Probing Subunit Interactions in Alpha Class Rat Liver Glutathione S-Transferase with the Photoaffinity Label Glutathionyl S-[4-(succinimidyl)benzophenone]*

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Glutathionyl S-[4-(succinimidyl)benzophenone] (GS-Succ-BP), an analogue of the product of glutathione and electrophilic substrate, acts as a photoaffinity label of dimeric rat liver glutathione S-transferase (GST), isoenzyme 1-1. A time-dependent loss of enzyme activity is observed upon irradiation of the enzyme with long wavelength UV light in the presence of the reagent. The initial rate of inactivation exhibits nonlinear dependence on the concentration of the reagent, characterized by an apparent dissociation constant of the enzyme-reagent complex (Kd) of 99 ± 2 μM and kmax of 0.082 ± 0.005 min⁻¹. Protection against this inactivation is provided by the electrophilic substrate (ethacrynic acid), electrophilic substrate analogue (dinitrophenol), and product analogues (S-hexylglutathione and p-nitrobenzylglutathione) but not by steroids (Δ5-androstene-3,17-dione and 17β-estradiol-3,17-disulfate). These results suggest that GS-Succ-BP binds and reacts with the enzyme within the xenobiotic substrate binding site, and this reaction site is distinct from the substrate and non-substrate steroid binding sites of the enzyme. About 1 mol of reagent is incorporated into 1 mol of enzyme dimer when the enzyme is completely inactivated. Met208 is the only amino acid target of the reagent, and modification of this residue in one enzyme subunit of the GST 1-1 dimer completely abolishes the enzyme activity of both subunits. In order to evaluate the role of subunit interactions in the Alpha class glutathione S-transferases, inactive GS-Succ-BP-modified GST 1-1 was mixed with unlabeled, active GST 2-2. The enzyme subunits were dissociated in dilute trifluoroacetic acid and then renatured at pH 7.8 and separated by chromatofocusing into GST 1-1, 1-2, and 2-2. The specific activities of the heterodimer toward several substrates indicate that the loss of catalytic activity in the unmodified subunit of the modified GST 1-1 is the indirect result of the interaction between the two enzyme subunits and that this subunit interaction is absent in the heterodimer GST 1-2.

Glutathione S-transferases (GST)† (EC 2.5.1.18) are a family of detoxification enzymes that catalyze the conjugation reaction of glutathione with a variety of endogenous electrophiles and xenobiotics (1–4). The enzyme catalyzes the reaction by lowering the pKₐ of the sulfhydryl group of the enzyme-bound glutathione (5, 6). The conjugation products are more water-soluble, usually less toxic, and can be degraded or transported outside of the cell. Furthermore, GSTs have been implicated in the development of resistance of various tumor cell lines to anti-cancer drugs. A higher level of expression of Alpha class GSTs was observed when Chinese hamster ovary cells were exposed to the nitrogen mustard alkylating agent chlorambucil (7), and cell lines exhibiting overexpression of these GSTs had increased resistance to the anti-cancer drugs chlorambucil and melphalan (8, 9); for recent reviews, see Hayes and Pulford (10) and Van der Aar et al. (11).

The mammalian cytosolic GSTs can be grouped into at least seven classes (Alpha, Mu, Pi, Theta, Kappa, Sigma, and Zeta) (1, 12, 13), and they exist as either homo- or heterodimers. Crystal structures have been reported for the representatives of most of these classes (14–20). All structures share similar topology, with each enzyme subunit having a glutathione binding site and an electrophilic substrate binding site. In addition, a nonsubstrate steroid binding site for Alpha class glutathione S-transferase, isoenzyme 1-1, was located in the cleft formed between the two enzyme subunits (21, 22). The two subunits in the structures of isoenzymes complexed with various products or product analogues show no significant structural difference between them, suggesting that the two active sites in the enzyme dimer act independently (1). Results from steady state kinetics using 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene as electrophilic substrates for rat Alpha class enzyme GST 1-1 and μ class enzymes GST 3–3 and GST 3–4 are consistent with two noncooperative active sites (23). However, the large bulky aflatoxin-glutathione conjugate has been shown recently to bind to mouse Alpha class 2-2 enzyme with a stoichiometry of 1 mol/mol of enzyme dimer, and binding of this ligand completely abolished the catalytic activity of both enzyme subunits (24).

In addition, binding studies of glutathione to the human Pi class enzyme show that binding displays positive cooperativity above 35 °C, whereas negative cooperativity occurs below 25 °C (25). These results suggest that the two active sites might not be independent. In this paper, we use the bulky product analogue glutathionyl S-[4-(succinimidyl)benzophenone] (GS-Succ-BP) as a photoaffinity label to probe the interactions between the two enzyme subunits.

GS-Succ-BP was used previously in this laboratory as a high pressure liquid chromatography.
Photoaffinity Labeling: Glutathione S-Transferase

photoaffinity label for the electrophilic substrate site of the rat liver Mu class GST 4-4 (26). In that study, Met-112 was identified as the reaction site. The benzophenone moiety of GS-Succ-BP binds in the cleft between the subunits, and modification of Met-112 of one enzyme subunit not only prevents the binding and the modification of the corresponding methionine residue on the other subunit but also inhibits the enzyme activities of both enzyme subunits. Comparison between the sequences of rat subunits 4 and 1 reveals only 29% identical plus similar amino acid residues. The sequence differences are undoubtedly responsible for the marked distinctions in substrate specificities between the 4-4 and 1-1 enzymes. In this paper, we show that GS-Succ-BP reacts with the Met-208 of the rat liver alpha class GST 1-1. This residue, unlike Met-112 of GST 4-4, is located away from the enzyme dimer interface. Modification of Met-208 of one enzyme subunit completely abolishes the catalytic activities of the enzyme dimer. Examination of the catalytic activities of the heterodimer GST 1-2, which was generated from the modified GST 1-1 and GST 2-2, suggests that two enzyme active sites of the dimer are coordinately regulated. The product was confirmed by TLC, HPLC, and MALDI TOF mass spectrometry.

Enzymatic Assays—Enzyme activity was routinely measured by monitoring the formation of the conjugate between 2.5 mM glutathione and 1.0 mM CDNB at 340 nm (ΔA340 nm 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. (31), using a Gilford model 240 spectrophotometer. The enzyme-catalyzed formation of the conjugate between 600 μM glutathione and 100 μM mBBr was measured in 0.1 M potassium phosphate buffer, pH 6.5, at 25 °C using the method of Hubert and Yakubu (32) with a Perkin-Elmer MFF-3 fluorescence spectrophotometer (excitation at 305 nm and emission at 480 nm). The enzyme-catalyzed isomerization reaction of 250 μM Δ3-androstene-3,17-dione (Δ3-AD) to Δ3-androstene-3,17-dione was determined spectrophotometrically from the change in ΔA242 nm (ΔA242 nm 16.3 mM-1 cm-1) (33) in 0.1 M potassium phosphate buffer, pH 6.5, at 25 °C in the presence of 0.1 mM glutathione and 0.1 mM dithiothreitol. Enzyme activity toward ethacrynic acid (EA) was measured by monitoring the formation of the conjugate between 400 μM EA and 625 μM glutathione in 0.1 M potassium phosphate buffer, pH 6.5, at 25 °C (ΔA395 nm 5 mM-1 cm-1) (34). Corrections for the nonenzymatic reactions were made for all assays.

Reaction of GS-Succ-BP with Glutathione S-Transferase, Isoenzyme 1-1—Glutathione S-transferase (0.4 mg/ml) was incubated in the dark with various concentrations of GS-Succ-BP (20–400 μM) in 0.1 M potassium phosphate buffer, pH 6.5, containing 15 mM 2,4-dinitrophenol and 10 mM dithiothreitol. Enzyme activity toward ethacrynic acid (625 μM) was determined using the CDNB assay. When various enzyme ligands were tested for their ability to protect against inactivation, they were preincubated with 10 μM glutathione for 10 min prior to the addition of GS-Succ-BP. To determine the decomposition rate of the GS-Succ-BP in the presence of 150 μM of reagent in the absence of the enzyme was irradiated. At various time points, aliquots (20 μl) of the solution were diluted in ethanol, and the UV-Vis spectra (240–400 nm) were measured. When the effects of the two stereoisomers of GS-Succ-BP on the inactivation of the enzyme were studied, equal molar concentrations of the stereoisomers at 150 μM was used in the reaction mixture as the reagent.

Determination of the Non-covalent Binding of the Enzyme and the Reagent—To determine the noncovalent binding of the enzyme and the reagent, GST 1-1 (0.4 mg/ml) was incubated with 150 μM of [3H]GS-Succ-BP (total of 500 μCi) at 0 °C in the dark for 20 min. Excess reagent was removed using the gel centrifugation method of Penefsky (35). The solution mixture was applied to one 5-ml Sephadex G-50 column equilibrated with 0.1 M potassium phosphate buffer, pH 6.5. The amount of the reagent binding to the enzyme was determined from the radioactivity measured using a Packard Tri-Carb liquid scintillation counter, model 1500. The protein concentration in the eluate was determined by the Bio-Rad protein assay, based on the Bradford method (30), using the GST 1-1 as the standard.

Enzymatic activities were assayed by HPLC on a Vydac C4 reverse-phase column (0.46 × 25 cm). The column was equilibrated with 0.1% trifluoroacetic acid containing 30% acetonitrile. Over a 50-min period, a linear gradient was established to 0.1% trifluoroacetic acid containing 60% acetonitrile. The GST 1 subunit exhibits a peak centered at 49% acetonitrile, while the GST 2 subunit peak is centered at 43% acetonitrile. The enzyme activities were measured by a simplified procedure using only affinity column chromatography on S-hexylglutathione-Sepharose followed by chromatofocusing on PBE 118 resin (25 cm) with a constant concentration of acetonitrile.

Photoaffinity labeling of Glutathione S-transferase—Glutathione S-transferase at 0.4 mg/ml was incubated with 150 μM [3H]GS-Succ-BP and irradiated with long wavelength UV light under the same conditions as described above. In some experiments, as indicated, unphotolyzed reagent in the solution was replenished by the addition of fresh reagent (150 μM) after 60 min of irradiation. At various times, an aliquot of the reaction mixture (500 μl) was diluted (500 fold) with 0.1 M potassium phosphate buffer, pH 6.5, at 0 °C and emission at 395 nm. The enzyme-catalyzed isomerization reaction of 250 μM Δ3-androstene-3,17-dione (Δ3-AD) to Δ3-androstene-3,17-dione was determined spectrophotometrically from the change in ΔA242 nm (ΔA242 nm 16.3 mM-1 cm-1) (33) in 0.1 M potassium phosphate buffer, pH 6.5, at 25 °C in the presence of 0.1 mM glutathione and 0.1 mM dithiothreitol. Enzyme activity toward ethacrynic acid (EA) was determined using the CDNB assay. When various enzyme ligands were tested for their ability to protect against inactivation, they were preincubated with 10 μM glutathione for 10 min prior to the addition of GS-Succ-BP. To determine the decomposition rate of the GS-Succ-BP under the same conditions, a solution of 150 μM of reagent in the absence of the enzyme was irradiated. At various time points, aliquots (20 μl) of the solution were diluted in ethanol, and UV-Vis spectra (240–400 nm) were measured. When the effects of the two stereoisomers of GS-Succ-BP on the inactivation of the enzyme were studied, equal molar concentrations of the stereoisomers at 150 μM was used in the reaction mixture as the reagent.

Determination of the Non-covalent Binding of the Enzyme and the Reagent—To determine the noncovalent binding of the enzyme and the reagent, GST 1-1 (0.4 mg/ml) was incubated with 150 μM of [3H]GS-Succ-BP (total of 500 μCi) at 0 °C in the dark for 20 min. Excess reagent was removed using the gel centrifugation method of Penefsky (35). The solution mixture was applied to one 5-ml Sephadex G-50 column equilibrated with 0.1 M potassium phosphate buffer, pH 6.5. The amount of the reagent binding to the enzyme was determined from the radioactivity measured using a Packard Tri-Carb liquid scintillation counter, model 1500. The protein concentration in the eluate was determined by the Bio-Rad protein assay, based on the Bradford method (30), using the GST 1-1 as the standard.
pH 2.0. Under these acidic conditions, the enzyme-reactant complex is denatured, so that only reagent covalently bound to the enzyme co-elutes from the column with the enzyme. The protein concentration in the eluate was determined by the Bio-Rad protein assay as described above.

Reagent incorporation was also determined by mass spectrometry as described previously (26). Modified enzyme at various irradiation times was prepared as described above. The modified enzyme was separated from the excess reagent and unwanted phosphate present in the reaction mixture by HPLC with a Vydac C 18 column using solvent system I: 0.1% trifluoroacetic acid in H 2O as solvent A and 0.075% trifluoroacetic acid in acetonitrile as solvent B. After equilibration at ambient temperature for 30 min, a linear gradient was run to 60% solvent B at 40 min to recover the modified protein. The peak with modified enzyme was lyophilized and redissolved in 0.1% trifluoroacetic acid/H 2O solution. This enzyme solution was mixed with an equal volume of saturated sinapinic acid solution (70% H 2O, 30% acetonitrile), and 1 µl of this mixture (about 30 pmol of protein) was applied to the sample plate and air-dried. The mass spectra of the modified enzymes were determined by MALDI TOF mass spectrometry using a Voyager DE BioSpectrometry workstattion of PerSeptive Biosystems. Data were collected using the delayed extraction 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. The final spectrum for modified enzyme at various time points is the average of 256 mass spectra of these experiments. In these experiments, cytochrome C (M r 12,360) and lactate dehydrogenase from porcine muscle (M r 36,487) were used as external protein mass standards.

Proteolysis of GS-Succ-BP-modified Glutathione S-Transferase—Glutathione S-transferase (0.4 mg/ml) was incubated with 150 µM [3H]GS-Succ-BP and irradiated with long wavelength UV light (250 µW/well) on ice for 60 min. Fresh radioactive reagent (final concentration of 150 µM) was added, and the solution was further irradiated for an additional 50 min. Each 500-µl aliquot of the reaction mixture was applied to a 5-ml Sephadex G-50 column equilibrated with 0.1% trifluoroacetic acid/H 2O. In each case, the peptide mass standards. Incorporation of the reagent into the reconstituted GST 1-2 was determined by Bio-Rad using a 1:1 mixture of GST 1-1 and GST 2-2 as standard. Incorporation of the reagent into the enzyme was determined by radioactivity.

Circular Dichroism of Native and Modified Glutathione S-Transferase 1—Modified GST 1-1 was prepared as described above. The resulting modified enzyme solution was dialyzed first against 11 of 0.1 M phosphate buffer at 4 °C for 48 h with three buffer changes, followed by dialysis against 1 liter of 0.02 M phosphate buffer at 4 °C for 16 h. The protein concentration was determined using the Bio-Rad method, and the incorporation of the reagent into the enzyme was determined by radioactivity.

CD spectra of modified enzyme (0.15 mg/ml) and control enzyme (0.15 mg/ml), in 0.02 M phosphate buffer, were obtained using a Jasco model J-710 spectropolarimeter from 200 to 250 nm with a 0.1-cm path length cylindrical quartz cell. An average of five measurements were recorded and the spectra for each enzyme were subtracted.

Formation of Heterodimer of Glutathione S-Transferase 1-2—Modified GST 1-1 was prepared using 150 µM [3H]GS-Succ-BP as described above. A mixture of control GST 1-1 (0.8 mg) and control GST 2-2 (0.8 mg) or a mixture of modified GST 1-1 (0.32 mg) and control GST 2-2 (1.28 mg), at a final total protein concentration of 0.2 mg/ml, was dialyzed against 6 liters of 30% acetonitrile in H 2O containing 0.1% trifluoroacetic acid, pH 2.0, at 4 °C for 24 h to allow enzyme dimers to denature and dissociate. The resulting solutions were then dialyzed against 10 mM Tris buffer, pH 7.8, at 4 °C for 24 h to allow refolding and reassociation to dimers. Different isoenzymes (GST 1-1, 1-2, and 2-2) were separated by chromatofocusing on PBE 118 resin in the pH range of 10.8–7 (36). The protein concentration of the reconstituted GST 1-2 was determined by Bio-Rad using a 1:1 mixture of GST 1-1 and GST 2-2 as standard. Incorporation of the reagent into the reconstituted GST 1-2, from modified GST 1-1 and control GST 2-2, was determined by radioactivity measurement. The catalytic activities of the reconstituted GST 1-2 were determined using CDBN, EA, and Δ3-AD as electrophilic substrate.

Molecular Modeling—Molecular modeling was conducted using the Insight II program package from Molecular Simulation Inc. on Indigo workstations from Silicon Graphics. The model of rat GST 1-1 was constructed as described previously (26) based on the known crystal structure of the human GST 1-1 apoenzyme (1GSD) deposited in the Protein Data Bank (PDB). A 78% identity plus 12% similarity exists between the amino acid sequences of these rat and human isoenzymes. The Homology module was used to substitute the amino acids of the rat GST 1-1 isoenzyme for those in human GST 1-1. 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 1-1 isoenzyme.

The structure of the GS-Succ-BP was constructed using the Builder module. The docking of the GS-Succ-BP was conducted using the Docking module, which monitors both van der Waals and electrostatic inter-
actions between the reagent and the enzyme. After docking, the entire
enzyme-reagent complex was again submitted to the Discover 3 module
for global energy minimization.

RESULTS

Inactivation of Rat Liver Glutathione S-Transferase 1-1 by
GS-Succ-BP—GST 1-1 is inactivated by irradiation with long
wavelength UV light (365 nm) in the presence of GS-Succ-BP
(150 μM). Fig. 1 illustrates the decrease in the enzyme activity as
a function of time of irradiation. As controls, incubation of
enzyme with the reagent in the dark or irradiation of the
enzyme under the same conditions but without the reagent
caused no significant loss of enzyme activity. These results
show that loss of enzyme activity is due to the reaction of
enzyme and photoactivated reagent. Beyond 20 min, the rate of
loss of activity decreases. The curvature is probably due to the
photodecomposition of the reagent. Therefore, the rate constant
was determined from the first 20 min, as shown in the inset
of Fig. 1. The initial rate of inactivation under these
conditions was determined to be 0.053 ± 0.003 min⁻¹.
The residual enzyme activity of the inactivated enzyme after 60 min
of irradiation shows no significant difference when using 5Α-AD as the
electrophilic substrate instead of CDNB.

Concentration Dependence of the Initial Rate of Inactivation

\[ k_{\text{obs}} \]—Solutions of GST 1-1 and various concentrations of GS-
Succ-BP (20–400 μM) were irradiated, and the initial rates of the
inactivation were determined. The apparent rate constant
\[ k_{\text{obs}} \] exhibits a nonlinear dependence on the GS-Succ-BP
concentration (Fig. 2). This saturation curve is typical of an
affinity label, suggesting that a reversible enzyme-reagent complex
is formed prior to the irreversible modification of the enzyme.
The saturation curve can be described by the equation
\[ k_{\text{obs}} = k_{\text{max}} / (1 + K_R / [\text{GS-Succ-BP}]), \]
where \( K_R \) is the apparent dissociation constant of the enzyme-reagent complex and \( k_{\text{max}} \) is the
maximum rate of inactivation at saturating concentrations of
the reagent under these conditions. A least squares curve fit of
the observed data yields \( K_R = 99 ± 2 \mu M \) and \( k_{\text{max}} = 0.082 ± 0.005 \text{ min}^{-1} \).

The UV-visible spectrum of GS-Succ-BP is shown in Fig. 3
(solid line). The reagent exhibits an ultraviolet absorption peak
at 260 nm, with an extinction coefficient of 17,800 M⁻¹ cm⁻¹ at
this wavelength. When a solution of the reagent in 0.1 M po-
tassium phosphate buffer, pH 6.5, containing 15% \( \text{N,N}^\prime\)-dim-
ethylformamide, was irradiated with long wavelength UV at
0 °C, this peak decreased. The change in the spectrum reached a
limit as shown by the dashed line in Fig. 3. The decomposition
rate of this compound \( (k_{\text{decomp}}) \) under these conditions was
calculated, using the extinction coefficient at 260 nm, from the
slope of \( \ln(E/E_0) \) versus time, in which, \( E_0 \) and \( E_t \)
are, respectively, the extinction coefficients of the reagent at
time 0, time \( t \), and 5095 M⁻¹ cm⁻¹, the limiting extinction
coefficient. Under these conditions, the \( k_{\text{decomp}} \) of the reagent
was determined as 0.173 min⁻¹, which corresponds to a half-
life of 4 min.

The determined \( k_{\text{max}} \) for enzyme inactivation is slow com-
pared with the decomposition rate of the reagent under the
same conditions, raising the issue of how to account for the
continued inactivation. We postulated that the continued slow
inactivation is due to the tight binding of the reagent to the
enzyme and the slow off-rate of the reagent from the enzyme-
reagent complex. To investigate that issue, the dissociation
constant \( (K_D) \) of the GS-Succ-BP-enzyme complex was deter-
mined by testing GS-Succ-BP as a competitive inhibitor with
respect to glutathione in the enzyme-catalyzed conjugation of
glutathione and CDNB. GS-Succ-BP causes an increase in the
apparent \( K_m \) for glutathione, leading to a determination of 2.53
μM as the inhibition constant, \( K_D \). As we have shown above,
the inactivation of the enzyme by the reagent is a two-step process:
first, enzyme and reagent form a reversible enzyme-reagent
complex; second, enzyme and bound reagent react irreversibly
to form a reagent-modified enzyme.

\[
E + R \rightleftharpoons ER \xrightarrow{k_1} E - R' \\
\text{REACTION 1}
\]

Since \( K_R = (k_{-1} + k_{\text{max}}) / k_1 \) and \( K_D = k_{-1} / k_1 \), \( k_{-1} \) and \( k_1 \) were
estimated to be \(0.0022 \text{ min}^{-1}\) and \(850 \text{ s}^{-1} \text{ min}^{-1}\), respectively. The \(k_{-1}\), which represents the off-rate of the reagent from the enzyme-reagent complex, indicates that this rate is slow, with a half-life of approximately 315 min for the reagent-enzyme complex under these conditions.

To measure the off-rate directly, a solution of enzyme and radioactive GS-Succ-BP was incubated at 0 °C in the dark for 30 min. Excess reagent not bound to the enzyme was removed by gel filtration using a Sephadex G-50 spin column equilibrated with 0.1 M potassium phosphate solution, pH 6.5. Under these conditions, the GS-Succ-BP does not react covalently, and the enzyme is not inactivated. A solution was left in the dark at 0 °C for 0, 4, and 8 h to allow some release of the GS-Succ-BP before applying the enzyme solution to a second Sephadex G-50 spin column. The amount of the radioactive reagent bound to the enzyme (in this case, due to the noncovalent interaction) was measured as 0.47, 0.43, and 0.41 mol/mol of enzyme subunit after 0, 4, and 8 h incubation, respectively, of the isolated enzyme-reagent complex, thus confirming that the off-rate of the reagent is slow.

A solution of enzyme-reagent complex (with apparent incorporation of 0.47), without excess reagent, was irradiated with long wavelength UV light at 0 °C to further test whether the enzyme can still be inactivated in the absence of the excess reagent. The initial rate of inactivation under these conditions was determined to be 0.037 \text{ min}^{-1}, which is comparable with the inactivation rate in the presence of excess reagent.

Effect of Substrate Analogue on the Inactivation of Glutathione S-Transferase by GS-Succ-BP—Various substrate analogues were included in the reaction mixture to test their protection abilities against inactivation of the enzyme by 150 \(\mu\)M of GS-Succ-BP. The results, given in Table I, are expressed as the ratio of the initial inactivation rate constant measured in the presence of a particular ligand \((k_{-1})\) to the initial inactivation rate constant measured in the absence of ligands \((k_{-1})\). The short-chain glutathione analogue S-methylglutathione affords little protection against inactivation (Table I, lines 6 and 7), suggesting that the target site of GS-Succ-BP is probably outside of the glutathione binding site of the enzyme. The long-chain glutathione analogues S-hexylglutathione (line 4) and \(p\)-nitrobenzylglutathione (line 5), as well as the CDNB analogue dinitrobenzene (line 3) afford complete protection against inactivation, suggesting that GS-Succ-BP occupies and reacts with the substrate binding site of the enzyme.

GST 1-1 is known to bind steroids, both at a substrate-binding site and at a nonsubstrate steroid binding site (22). The steroids \(\Delta^5\)-androstene-3,17-dione and \(17\beta\)-estradiol-3,17-disulfate were included in the reaction mixture to test whether they can decrease the rate of inactivation by GS-Succ-BP. The steroid \(\Delta^5\)-androstene-3,17-dione functions as a competitive inhibitor of this enzyme, with respect to CDNB, with a binding constant of 20 \(\mu\)M (22). This steroid (500 \(\mu\)M), at 25 times its binding constant, affords no protection against inactivation (line 8). The steroid \(17\beta\)-estradiol-3,17-disulfate, at a concentration of 250 times higher than its binding constant (500 \(\mu\)M, \(K_I = 2.1 \mu\)M), affords little protection against inactivation (line 11). These results suggest that the GS-Succ-BP binds and reacts with the enzyme outside the steroid binding sites.

Inactivation of Glutathione S-Transferase by Two Isomers of GS-Succ-BP—To test the effects of the two stereoisomers of the reagent on enzyme inactivation, a solution of enzyme and each isolated isomer (150 \(\mu\)M) was irradiated under the same conditions as described above. The initial rate of inactivation was determined, respectively, to be 0.046 ± 0.003 \text{ min}^{-1} and 0.062 ± 0.004 \text{ min}^{-1}, for isomer I (eluting earlier from HPLC) and isomer II (eluting later from HPLC). These results suggest that both isomers inactivate the enzyme at a similar rate. In the rest of this study, a mixture of reagent isomers was used.

Incorporation of GS-Succ-BP into Glutathione S-Transferase 1-1—A solution of glutathione S-transferase (0.4 mg/ml) and \([3\text{H}]\)GS-Succ-BP (150 \(\mu\)M) was irradiated for different periods of time with long wavelength UV light at 0 °C. Incorporation of the radioactive reagent into the enzyme occurs concomitant with the loss of enzyme activity. Fig. 4 shows a plot of percentage of residual activity versus incorporation. The reagent incorporation extrapolates to 0.51 mol of GS-Succ-BP incorporated per mol of enzyme subunit (or 1 mol of reagent per mol of enzyme dimer) when the enzyme is completely inactivated.

To further evaluate whether, upon complete inactivation, only half of the enzyme subunits are modified, the mass of the enzyme subunits was determined using MALDI TOF mass spectrometry. The MALDI mass spectra were obtained for modified enzyme at various time points during the inactivation. As shown in Fig. 5, the control enzyme (time 0) exhibits a major peak at \(m/z\) 25,471 and a minor peak at \(m/z\) 25,680, which corresponds to a photochemically generated adduct of the enzyme with the matrix (sinapinic acid) (38). As the reaction progresses, a new higher mass peak appears (time 10, 20, and 150 min) corresponding to the modified enzyme. At an irradiation time of 150 min, when the enzyme is about 90% inactivated, there are clearly two major peaks, with a substantial amount of material remaining in the peak corresponding to the

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**TABLE I**

Effects of enzyme ligands on the photoinactivation of glutathione S-transferase by 150 \(\mu\)M GS-Succ-BP

| Ligand added | \(k_{-1}/k_{-1}\) |
|--------------|-----------------|
| 1. None      | 1.00 ± 0.13     |
| 2. Ethacrynic Acid (5 mM) | 0.33 ± 0.04 |
| 3. Dinitrophenol (5 mM) | 0             |
| 4. S-Hexylglutathione (5 mM) | 0             |
| 5. \(p\)-Nitrobenzylglutathione (5 mM) | 0             |
| 6. S-Methylglutathione (5 mM) | 0.78 ± 0.14    |
| 7. S-Methylglutathione (10 mM) | 0.78 ± 0.10    |
| 8. \(\Delta^5\)-Androstene-3,17-dione (500 \(\mu\)M) | 0.99 ± 0.14    |
| 9. \(17\beta\)-Estradiol-3,17-sulfate (100 \(\mu\)M) | 0.81 ± 0.17    |
| 10. \(17\beta\)-Estradiol-3,17-sulfate (200 \(\mu\)M) | 0.56 ± 0.08    |
| 11. \(17\beta\)-Estradiol-3,17-sulfate (500 \(\mu\)M) | 0.50 ± 0.10    |

\(a\) \(k_{-1}/k_{-1}\) was determined by the ratio of initial inactivation rate with added ligand to that observed in the absence of the ligand. The S.D. was determined using SigmaPlot.
unmodified enzyme. The difference in mass between these two major peaks is 581, which is close to the mass of the reagent (M_r = 585). The area corresponding to the peaks of modified and unmodified subunits, respectively, was estimated, and the incorporation was approximated from the ratio of modified subunit to total subunits. The resulting reagent incorporation versus residual activity (Fig. 4, filled circles) is consistent with the more precise incorporation results determined from radioactivity measurements. Further addition of the fresh reagent into the reaction mixture followed by further irradiation does not cause the reagent incorporation into the enzyme to exceed 0.5 mol/mol subunit.

**Figure 4.** Percentage of 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 0.51 ± 0.01 mol of reagent/mol of enzyme subunit.

**Isolation and Characterization of Tryptic Peptides from GS-Succ-BP-modified Glutathione S-Transferase**—Modified enzyme with 12% residual activity was prepared and digested with trypsin. The digest was fractionated by HPLC using a reverse-phase column (C18) and solvent system II at pH 2.0 as described under “Experimental Procedures” (Fig. 6). Three pools were made (A, B, and C), and each was further fractionated at pH 6.0 using solvent system III (Fig. 7). The radioactive peaks were subjected to gas phase amino acid sequencing, with the results shown in Table II. Peaks I, II, IV, V, and VI contain the same pure peptide, as indicated by the amino acid sequences. The sequence Lys-Pro-Ala-X-Asp-Ala-Lys corresponds to residues 205–211 in the known amino acid sequence (39, 40). No phenylthiohydantoin derivative was detected in cycle 4. Since the known amino acid sequence contains a Met at this position, we conclude that Met-208 is the target of GS-Succ-BP, and its modification is responsible for the loss of enzyme activity. The peptide of peak III results from the amino acid sequence difference at positions 205 and 206 between the two isoforms of the 1-1 isoenzyme (39–41); Met-208 is also the sequence difference at positions 205 and 206 in the known amino acid sequence (39, 40).

**Figure 5.** MALDI TOF mass spectra of unmodified and modified enzymes. Enzyme was modified by GS-Succ-BP for 0, 10, 20, and 150 min as described under “Experimental Procedures.” The mass spectrum of unmodified enzyme (T = 0 min) gives a major peak centered at m/z = 25,471 and a minor peak centered at m/z = 25,680. At T = 10, 20, and 150 min, the mass spectrum of modified enzyme shows an additional major peak whose mass is about 581 mass units higher than that of the first major peak, which corresponds to the unmodified enzyme subunit.

**Figure 6.** Fractionation by HPLC of trypsin digest of [3H]GS-Succ-BP-modified glutathione S-transferase. Peptides from the trypsin digest of the 150 μM GS-Succ-BP-modified enzyme were isolated by HPLC using solvent system II, as described under “Experimental Procedures.” Top graph. A_220 nm profile of the digest of modified enzyme. Bottom graph, distribution of radioactivity in the digest shown in top graph. Pool A includes fraction 53 and 54; pool B includes fraction 55 and 56; and pool C includes fractions 57–59. 

**Characterization of GS-Succ-BP-modified Glutathione S-Transferase**—To investigate whether modification of the enzyme causes overall change in the folding of the protein, the circular dichroism spectra were determined for both control and modified enzyme. No significant difference was found be-
tween the spectra of these enzymes (data not shown), suggesting that the loss of enzyme activity is not due to the unfolding of the enzyme.

Since only one subunit of the enzyme dimer is covalently modified by GS-Succ-BP, even when enzyme is completely inactivated, we sought to determine whether the unmodified subunit is still capable of binding substrates. mBBr has been shown previously to act as a substrate and an affinity label of the xenobiotic site of this enzyme, reacting covalently with both Cys-17 and Cys-111 (36). GS-Succ-BP-modified enzyme (15% residual activity) was treated with 4 mM of mBBr. The incorporation of mBBr into the modified enzyme after a 120-min incubation was determined to be 1.03 mol/mol of enzyme subunit compared with that of 1.84 mol/mol of enzyme subunit with control enzyme. These results suggest that about half (1.03 out of 1.84) of the reaction sites available to mBBr in the unmodified enzyme are still available in the GS-Succ-BP-modified enzyme. To investigate whether mBBr reacts equally with these two Cys residues, control and GS-Succ-BP-modified enzyme were treated with mBBr and digested with thermolysin, and the resulting peptides were fractionated by HPLC. Peaks corresponding to the mBBr-modified peptides resulting from the GS-Succ-BP-modified enzyme decrease when compared with those corresponding peaks resulting from control enzyme. The magnitude of the decrease in these peaks is about the same (data not shown), suggesting that both Cys are equally protected by modification of the enzyme with GS-Succ-BP. These results suggest that the unmodified subunit of GS-Succ-BP-modified enzyme dimer can still bind and react with mBBr at two sites (while the modified subunit does not react at all) to give a measured incorporation of 1 mol of mBBr per average subunit.

**Formation of Heterodimer of Glutathione S-Transferase 1-2**—Since enzyme modified at only one subunit of the dimeric enzyme is inactive when assayed using CDNB as substrate, it is clear that the subunit that does not contain radioactive GS-Succ-BP has no catalytic activity toward the substrates tested. We wondered whether the inactivation of the apparently unmodified subunit was due to a "silent" modification (such as oxidation) that might have occurred without reagent incorporation during the irradiation of the enzyme; alternatively, within the intact dimer, the interaction between the modified and unmodified subunits might have caused both to be inactive. These two explanations can be distinguished by testing the enzymatic activity of the nonlabeled enzyme subunit in the absence of its labeled counterpart.

To accomplish this goal, it is necessary to dissociate the two subunits of the enzyme dimer and to reassociate subunits later to yield dimer. The subunit composition of the reformed dimer should reflect the concentration of each subunit in the pool of monomers. In evaluating conditions for the dissociation and reassociation, we utilized the fact that rat liver GST 1 and GST 2 can form heterodimer (GST 1-2) in vivo and that GST 1-2 can be easily separated from GST 1-1 and GST 2-2 by chromatofocusing. Furthermore, the catalytic activity contributed by the GST 1 and GST 2 subunit in the GST 1-2 dimer can easily be distinguished by their differential catalytic activity toward several substrates, such as EA and Δ5-AD (2). GST 1 is much more active than GST 2 when Δ5-AD is used as a substrate (Table III, lines 1 and 2), while GST 2 is much more active when EA is used as substrate.

A solution of equal amounts of control GST 1-1 and GST 2-2 was dialyzed against 0.1% trifluoroacetic acid at pH 2.0 to dissociate the dimers, followed by dialysis against 10 mM Tris buffer at pH 7.8 to allow reassociation, as detailed under "Experimental Procedures." After dialysis, there was no sign of precipitation, suggesting that there is no loss of protein during these processes. The resulting dimers (GST 1-1, GST 2-2, and newly formed heterodimer GST 1-2) were separated by chromatofocusing as illustrated in Fig. 8A. The three protein peaks elute from the chromatofocusing column at pH 8.6, 8.5, and 8.4, respectively, corresponding to GST 1-1, GST 1-2, and GST 2-2.

The identity and composition of the peaks were confirmed by HPLC (43). The total amount of protein corresponding to all these peaks were determined, yielding a ratio close to 1:2:1 (GST 1-1:GST 1-2:GST 2-2), which is expected from the equal concentrations of the two types of subunit in the monomer pool. These results suggest that the binding affinity of GST 1 subunit to GST 2 subunit is about the same as its binding affinity to GST 1. The activities of these separated peaks are shown in Table III for the control enzyme. The specific activity of GST 1-2 toward all substrates tested (Table III, line 3) is about the average of those of GST 1-1 and GST 2-2, indicating that in the enzyme dimer, the two enzyme subunits probably function independently in catalysis. The specific activities of the rena-
tured GST 1-1, GST 2-2, and GST 1-2 show no significant difference from the specific activities of the corresponding control enzyme purified from rat liver (data not shown).

Heterodimer was also generated from GS-Succ-BP-modified GST 1-1 and unmodified GST 2-2 as described under "Experimental Procedures." Modified GST 1-1 with about 0.44 mol of [3H]GS-Succ-BP incorporated per mol of enzyme subunit was mixed with 4 times as much GST 2-2 in order to enhance the percentage of GST 1 subunit obtained in GST 1-2. The result from the chromatofocusing column is shown in Fig. 8B; the elution pH values of the peaks are the same as those of the control enzymes (Fig. 8A). The isoenzyme compositions of the three peaks were confirmed by HPLC; e.g., the chromatofocusing peak at pH 8.51 contains equal amounts of subunit 1 and 2 as determined by HPLC (43). In the purified GST 1-2 (peak eluting at pH 8.51 of Fig. 8B), the radioactive incorporation was measured as 0.21 mol of GS-Succ-BP/mol of enzyme subunit, indicating that about half of the GST 1-2 is composed of modified GST 1 and unmodified GST 2, while the other half is composed of unmodified GST 1 and unmodified GST 2 subunit; the modified and unmodified 1-2 enzymes differ by only one negative charge per dimer and are not separated by this col-

![Image](http://www.jbc.org/)
Measurement of the catalytic activity of this generated GST 1-2 can provide evidence as to whether the unmodified GST 1 subunit is active.

For each of four possible situations, the theoretical specific activities of the GST 1-2 toward different substrates were calculated, with the assumption that subunits in the enzyme dimer are acting independently; these theoretical specific activities are shown in Table III. The specific activities for case 1 (all GST 1 inactive, all GST 2 active) is one-half of those of GST 2-2; the specific activities for case 2 (half GST 1 active, all GST 2 active) are the sum of one-fourth of those of GST 1-1 and one-half of those of GST 2-2; the specific activities for case 3 (all GST 1 inactive, half GST 2 active) are one-fourth of those of GST 2-2; and the specific activities for case 4 (half GST 1 active, half GST 2 active) are the sum of one-fourth of those of GST 1-1 and one-fourth of those of GST 2-2.

The experimentally determined activities of the heterodimer GST 1-2, generated from modified GST 1-1 and unmodified GST 2-2 (Fig. 8B), were 21.6, 0.61, and 0.69 \( \mu \text{mol min}^{-1} \text{mg}^{-1} \), respectively, toward CDNB, EA, and \( \Delta^5\)-AD (Table III, last row). These values are very close to those shown for theoretical case 2, indicating that the unmodified GST 1 subunit must be fully active when it forms a dimer with unmodified GST 2 isoenzyme. In other words, loss of the enzymatic activities of the unmodified subunit of GST 1-1 dimer is due to its interaction with its modified counterpart. Interestingly, these results also show that GST 2 subunit, even when it forms a dimer with the GS-Succ-BP-modified GST 1 subunit, is still active, indicating that the interaction between the two subunits of isoenzyme 1, which causes the loss of enzymatic activities of the unmodified subunit, is absent in the heterodimer GST 1-2.

**DISCUSSION**

GS-Succ-BP functions as a photoaffinity label for rat liver GST 1-1. The initial rate of inactivation shows a nonlinear dependence on the concentration of the reagent, suggesting that a reversible enzyme-reactant complex forms prior to the irreversible modification of the enzyme. Protection against inactivation by the reagent is afforded by the long chain glutathione analogues (\( S \)-hexylglutathione or \( p \)-nitrobenzylglutathione) or the electrophilic substrate analogues (ethacrynic acid).

### TABLE II

Representative sequences of modified peptides isolated from the tryptic digest of \(^3\)H/GS-Succ-BP-labeled GST 1-1

| Cycle | Peak I | Peak II | Peak III | Peak IV | Peak V | Peak VI |
|-------|--------|---------|----------|---------|--------|---------|
| 1     | Lys (12) | Lys (25) | Lys (87) | Lys (7) | Lys (36) | Lys (99) |
| 2     | Pro (18) | Pro (23) | Leu (49) | Pro (40) | Pro (22) | Pro (96) |
| 3     | Ala (29) | Ala (37) | Pro (33) | Ala (52) | Ala (23) | Ala (152) |
| 4     | X       | X       | X        | X       | X       | X       |
| 5     | Asp (9) | Asp (15) | Asp (33) | Asp (18) | Asp (60) | Asp (48) |
| 6     | Ala (20) | Ala (21) | Ala (71) | Ala (24) | Ala (60) | Ala (56) |
| 7     | Lys (4) | Lys (6) | Lys (13) | Lys (2) | Lys (10) | Lys (13) |

- Mass measured (MH\(^+_1\))
  - 1345.0
  - ND
  - 1401.3
  - 1326.7
  - 1360.6
  - 1360.2

- Mass calculated from sequence (MH\(^+_1\))
  - 1344.8
  - 1344.8
  - 1386.8
  - 1344.8
  - 1344.8
  - 1344.8

- Mass difference
  - 0.2
  - ND
  - 14.5
  - -18.1
  - 15.8
  - 15.4

*ND, not determined.

### TABLE III

Enzymatic activity of renatured unmodified (control) and modified (experimental) glutathione S-transferase 1-2

| Type of enzyme sample | Subunits in dimeric enzyme | Specific activity |
|-----------------------|---------------------------|------------------|
|                       |                           | CDNB | EA | \( \Delta^5\)-AD |
| Control               | GST 1-1                   | 50.5 | 0.11 | 2.04 |
|                       | GST 2-2                   | 16.4 | 1.31 | 0.15 |
|                       | GST 1-2                   | 31.8 | 0.66 | 1.02 |
| Theoretical: GST 1-2 activity for modified GST 1 + unmodified GST 2 | Case 1: all 1 inactive, all 2 active | 8.2 | 0.66 | 0.075 |
|                       | Case 2: half 1 active, all 2 active | 20.8 | 0.68 | 0.59 |
|                       | Case 3: all 1 inactive, half 2 active | 4.1 | 0.33 | 0.037 |
|                       | Case 4: half 1 active, half 2 active | 16.7 | 0.36 | 0.55 |
| Experimental: modified GST 1 + unmodified GST 2 | GST 1-2 (generated) | 21.6 | 0.61 | 0.69 |

**FIG. 8.** Purification of generated homo- and heterodimers. Different combinations of enzyme dimers, resulting from the dissociation and reassociation of control GST 1-1 and control GST 2-2 at 1:1 ratio (A) or of modified GST 1-1 and control GST 2-2 at 1:4 ratio (B), were separated by chromatofocusing using a PBE 118 resin in the pH range of 10–8 as described under "Experimental Procedures." Glutathione S-transferase 1-1 elutes first at pH 8.6, followed by 1-2 at pH 8.5 and 2-2 at pH 8.3.
acid or dinitrobenzene), but not by the short chain glutathione analogue, S-methylglutathione. These results indicate that the reagent binds to the enzyme and reacts within its electrophilic substrate binding site. Steroids (Δ5-androstene-3,17-dione and 17β-estradiol-3,17-disulfate) afford little protection against inactivation, suggesting that the reagent binds and reacts outside of the steroid binding sites of the enzyme.

Inactivation of the enzyme is proportional to the covalent incorporation of the reagent into the enzyme. When the enzyme is completely inactivated, an incorporation of about 0.5 mol of reagent per mol of enzyme subunit is observed. Met-208 was identified as the only amino acid being modified by GS-Succ-BP. The location of this residue is quite different from Met-112 of GST 4–4, which was the reaction site of GS-Succ-BP (26). (Met-112 is not conserved in GST 1-1.) Met-208 is located at the beginning of the C-terminal region of GST 1-1, which undergoes a structural change upon binding substrate analogues (14, 44, 46).

The docking of GS-Succ-BP into the enzyme was modeled, as described under "Experimental Procedures." The structure of the rat GST 1-1 from residue 1 to 208 was based on the x-ray coordinates of the human GST 1-1 apoenzyme (1GSD in the PDB). The glutathionyl moiety of the reagent was positioned in one of the enzyme's active sites so that it coincided with the glutathionyl S-benzylglutathione bound to the human GST A1-1 (1GUH in the PDB). Bonds between the sulfur atom of the glutathione and succinimidylbenzophenone, between the sulfur atom and the rest of the glutathione moiety, and between the nitrogen atom of the succinimidyl and benzophenone were rotated, while the intermolecular van der Waals potential and electrostatic potential between the apoenzyme and the reagent was monitored in order to position the carbonyl group of the benzophenone moiety of the reagent actually binds in the cleft between the two enzyme subunits. The results indicate that the large reagent (and corresponding large electrophilic substrates) binds distictively in different isoenzymes. The electrophilic substrate does not protrude into the steroid binding site of the enzyme in the Alpha class isoenzyme, as suggested by McHugh et al. (24).

The C terminus (starting from Met-208) of the human GST 1-1 is not observed in the crystal structure of the apoenzyme (presumably, it is mobile), while it forms a helical cap over the active site of the enzyme enclosing the electrophilic substrate in the structure of enzyme complexed with EA-glutathione or with S-benzylglutathione (14, 45). The C-terminal helix in the enzyme-S-benzylglutathione complex (1GUH) is shown in white in Fig. 9. Severe steric hindrance exists between the reagent modeled into the apoenzyme and residues of this helix, suggesting that in the enzyme-GS-Succ-BP complex, the position of this helix is not the same as that of enzyme S-benzylglutathione complex; probably, it is a little bit lower to accommodate the bulky moiety of our reagent.

The dissociation rate of GS-Succ-BP from the enzyme-reagent complex is extremely slow. This observation is probably due to the dynamics of the C-terminal structural transition between enzymes with and without the large product analogue. It has been shown that the binding of the product analogue and the enzyme involves a conformational change (isomerization) of the enzyme-reagent complex, which mainly involves the helix formation of the C terminus of the enzyme (46). For the large electrophilic substrates of this enzyme, the low catalytic activity is probably due to the slow rate of the product release from the enzyme.

In this study, when the enzyme is completely inactivated, only one of the enzyme subunits of the enzyme dimer is modified. This result is consistent with our observation that only 1 mol of reagent binds covalently with 1 mol of enzyme dimer in the dark. This finding is somewhat surprising, because each subunit of the enzyme dimer has an active site, and, in the crystal structure of the human GST 1-1 complexed with the EA-glutathione conjugate (1GSE in PDB) or in the crystal structure of GST 1-1 complexed with S-benzylglutathione (1GUH in PDB), it is clear that both active sites of the enzyme are occupied by the product (or product analogue). It has been reported that larger conjugation products (or product ana-
finding suggests that the two enzyme active sites in GST 1-1 are coordinated. On the contrary, results of activity measurements from the GST 1-2 generated from the modified GST 1-1 and control GST 2-2 indicate that modification of the GST 1 subunit of the heterodimer has no effect on the catalytic activity of the GST 2 subunit. Thus, the two active sites in the heterodimer GST 1-2 appear to be independent. These two isoenzymes share 68% sequence identity. It remains to be seen what interaction(s) between the two enzyme subunits cause the active sites to be coordinated in the case of homodimers (GST 1-1) but independent in the case of heterodimers (GST 1-2). Additional study is needed to ascertain whether our results from this pair of homo- and heterodimers are specific or can be generalized to all isoenzymes.

In recent years, equilibrium unfolding studies have been performed on Pi class (47–49), Schistosoma japonicum (50), Alpha class (51), and Sigma class (52) glutathione S-transferases. In these studies, urea or guanidinium chloride was added to the enzyme solution to cause denaturation and inactivation, and its dye binding ability, as well as UV, circular dichroism, and fluorescence spectral characteristics were monitored to study the unfolding pathway. GSTs from Pi class, Schistosoma japonicum and Alpha class unfold via a two-state pathway in which only folded dimers or unfolded monomers are detectable at equilibrium (47–51), while Sigma class GST unfolds via a partially active dimeric intermediate and an inactive monomeric intermediate (52). In most of these studies, the refolding of the urea or guanidinium chloride-denatured enzymes was accomplished by dilution (at least 10-fold) in buffer without the denaturant. The enzyme catalytic activity recovered varied from 40–55% in the case of Pi enzyme (48) to 95–98% for the Alpha enzyme (51). In this study, we were able not only to dissociate the enzyme dimer unequivocally but also to reassociate and recover the enzyme dimer without loss of the protein and the enzyme activities. This approach provides a method to directly study the interaction between enzyme subunits and to elucidate the residue(s) that determines the specificity during dimer formation.

In summary, GS-Succ-BP acts as a photoaffinity label for rat GST 1-1, modifying the Met-208 residue. Modification of one subunit of the enzyme dimer completely abolishes the enzyme catalytic activity. Analysis of the ligands that protect against photoinactivation and of models of the enzyme structure suggests that the reagent binds outside the steroid binding site of the enzyme, which is in the cleft between the subunits. Furthermore, reaction of GS-Succ-BP with one subunit causes a conformational change that prevents the binding of the reagent to the other subunit and inhibits the enzyme activity on the unmodified subunit. This interaction is absent in GST 1-2.

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Probing Subunit Interactions in Alpha Class Rat Liver Glutathione S-Transferase with the Photoaffinity Label Glutathionyl S-[4-(Succinimidyl)benzophenone]

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