Affinity Labeling of Cys[111] of Glutathione S-Transferase, Isoenzyme 1-1, by *S*-(4-Bromo-2,3-dioxobutyl)glutathione†

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ABSTRACT: Incubation of *S*-(4-bromo-2,3-dioxobutyl)glutathione (S-BDB-G), a reactive analogue of glutathione, with the 1-1 isoenzyme of rat liver glutathione S-transferase at pH 6.5 and 25 °C results in a time-dependent inactivation of the enzyme. k_{obs} exhibits a nonlinear dependence on S-BDB-G from 50 to 1200 μM, with a k_{max} of 0.111 min^{-1} and K_I = 185 μM. The addition of 5 mM *S*-hexylglutathione, a competitive inhibitor with respect to glutathione, gives almost complete protection against inactivation by S-BDB-G. About 1.2 mol of [^{3}H]S-BDB-G/mol of enzyme subunit is incorporated when the enzyme is 85% inactivated, whereas 0.33 mol of reagent/mol of subunit is incorporated in the presence of *S*-hexylglutathione when the enzyme has lost only 17% of its original activity. Modified enzyme, prepared by incubating glutathione S-transferase with [^{3}H]S-BDB-G in the absence or in the presence of *S*-hexylglutathione, was reduced with sodium borohydride, reacted with *N*-ethylmaleimide, and digested with α-chymotrypsin. Analysis of the chymotryptic digests, fractionated by reverse-phase high-performance liquid chromatography, revealed Cys[111] as the amino acid whose reaction with S-BDB-G correlates with enzyme inactivation. It is concluded that Cys[111] lies within or near the hydrophobic substrate binding site of glutathione S-transferase, isoenzyme 1-1.

The glutathione S-transferases (EC 2.5.1.18) are a group of isoenzymes primarily involved in the detoxification of both endogenous compounds and xenobiotics. They catalyze reactions in which the thiol of glutathione undergoes nucleophilic addition to electrophilic substrates (Armstrong, 1987; Chasseaud, 1979; Jakoby, 1977; Listowsky et al., 1988; Mannervik, 1985; Pickett & Lu, 1989). The isoenzymes, found predominantly in the cell cytosol, can exist as either homo- or heterodimers in which each subunit contains a glutathione binding site and a second, hydrophobic binding site which tolerates structural variation in the electrophilic substrate (Mannervik & Danielson, 1988; Jakobson et al., 1979).

On the basis of isoelectric point, substrate specificity, immunological reactivity, and inhibition properties, as well as primary sequence similarity, the cytosolic glutathione S-transferases can be divided into at least four distinct classes: α, μ, π (Mannervik et al., 1985), and θ (Meyer et al., 1991). Isoenzyme subunits within the same gene class have sequence homologies which are quite high (75% or greater), whereas between classes, the sequence homology is in the range of 25–45%.

Rat liver isoenzyme 1-1, used in this study, belongs to the α class, which includes subunit types 1, 2, 8, and 10 (Mannervik et al., 1985; Armstrong, 1991). Although the primary sequence of the 1-1 isoenzyme is known (Pickett et al., 1984; Lai et al., 1984), the active site has not been determined within the primary structure.

In previous work in this laboratory with the μ-class isoenzyme 4-4 using the affinity label *S*-(4-bromo-2,3-dioxobutyl)-glutathione, (S-BDB-G),[1] Tyr[115] was identified as an amino acid of significance in the active site (Katusz & Colman, 1991). The 1-1 and 4-4 isoenzymes share only 19% identity and 10% similarity when their primary sequences are compared using the ALIGN program (PG Gene, IntelliGenetics). As shown in Table I, Tyr[115] is not conserved in glutathione S-transferase, isoenzyme 1-1. These differences between isoenzyme 1-1 and isoenzyme 4-4 suggest that these two classes of isoenzymes may have distinctive active-site structures. The purpose of this investigation is to compare examples of the α- and μ-class isoenzymes in terms of their reaction with the affinity label S-BDB-G.

EXPERIMENTAL PROCEDURES

Materials. *S*-(4-Bromo-2,3-dioxobutyl)glutathione was synthesized by the condensation of glutathione with recrystallized 1,4-dibromobutanedione according to the method of Katusz and Colman (1991). The bromide content of S-BDB-G measured after hydrolysis by 0.2 M NaOH was 1.03 mol/mol of reagent. Upon incubation in 0.1 M potassium phosphate buffer, pH 6.5, at 25 °C, a time-dependent increase in free bromide was observed to give approximately the same value as that obtained from base hydrolysis. The half-life for release of bromide from S-BDB-G at pH 6.5 was determined to be 48 min (Katusz & Colman, 1991). In the preparation of the radioactive reagent [*glycine*-2-^{3}H]glutathione was used and was purchased from Du Pont–New England Nuclear Research Products. The concentration was measured using ϵ_{324nm} = 4415 M^{-1} cm^{-1} and the reagent was stored in water (pH 3.0) at −80 °C.

Frozen Sprague-Dawley rat livers were purchased from Pel Freez Biologicals. Glutathione, the *S*-alkyl derivatives of glutathione, dithiothreitol, *N*-ethylmaleimide, *S*-hexylglutathione–Sepharose, bovine pancreatic α-chymotrypsin, protease from *Bacillus thermoproteolyticus rokko* (thermolysin), and 2,4-dinitrophenol were obtained from Sigma Chemical Co. 1-Chloro-2,4-dinitrobenzene and hexafluoroacetone trihydrate (98%) were from Aldrich Chemical Co. Whatman Biosystems supplied the DEAE-cellulose. Hydroxylapatite (Bio-Gel HT) was purchased from Bio-Rad Laboratories and PBE 118 and Pharmalyte were from Pharmacia. Coomassie Blue dye reagent was provided by Pierce Chemical Co., urea

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[1] Abbreviations: S-BDB-G, *S*-(4-bromo-2,3-dioxobutyl)glutathione; HPLC, high-performance liquid chromatography.

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Table I: Comparison of Amino Acid Sequences in Selected Regions of Isoenzymes of Glutathione S-Transferase

subunit	class	source	amino acid sequence
1	α	Rat Liver	D^{100}-L-T-E-M-I-M-Q-L-V-I^{110}-C^{111}-P-P-D-Q-K-E^{117}
3	μ	Rat Liver	D^{105}-N-R-M-Q-L-I-M-L-C-Y^{115}-N^{116}-P-D-F-E-K-Q^{122}
4	μ	Rat Liver	D^{105}-T-R-L-Q-L-A-M-V-C-Y^{115}-S^{116}-P-D-F-E-R-K^{122}
7	π	Rat Liver	D^{98} -L-R-C-K-Y-G-T-L-I-Y^{108}-T^{109}---N-Y-E-N-G^{114}
4	μ	Human Muscle	D^{106}-S-R-M-Q-L-A-K-L-C-Y^{116}-D^{117}-P-D-F-E-K-L^{123}

was from Schwarz/Mann Biotech, and Liquiscint was from National Diagnostics. All other chemicals used were reagent grade.

Enzyme Preparation and Assay. The 1-1 isoenzyme of glutathione S-transferase was purified from the livers of Sprague-Dawley rats essentially by the methods of Cobb et al. (1983) and Jensson et al. (1985). After successive column chromatography on DEAE-cellulose, *S*-hexylglutathione–Sepharose, and hydroxylapatite, the α-isoenzymes (1-1, 1-2, and 2-2) were separated by chromatofocusing using PBE 118. The eluting buffer, Pharmalyte, was removed from the fractionated 1-1 isoenzyme by further chromatography on *S*-hexylglutathione–Sepharose. In a typical preparation using 25 rat livers (approximately 160 g frozen weight), 10–20 mg of isoenzyme 1-1 was obtained. The enzyme concentration was measured using a M_r of 25 500 per subunit (Mannervik & Danielson, 1988) and ϵ_{270nm} = 23 000 M^{-1} cm^{-1} determined from the ratio of A_{270}/A_{214} obtained from the absorption spectrum using a Cary 219 spectrophotometer. Ostlund Farrants et al. (1987) reported $\epsilon_{214nm} = 21 \times 10^4$ M^{-1} cm^{-1}; ϵ_{270nm} was chosen to correlate with the ϵ_{270nm} data of Graminski et al. (1989a,b) used previously in this laboratory to determine the concentration of isoenzymes 3-3 and 4-4. Using the same experimental approach, ϵ_{280nm} was determined to be 21 500 M^{-1} cm^{-1}. The purity of the final preparation was evaluated by high-performance liquid chromatography using a C_4 reverse-phase column (Vydac 214TP) equilibrated with 0.075% trifluoroacetic acid in 30% acetonitrile, followed by a linear gradient to 0.075% trifluoroacetic acid in 60% acetonitrile according to the method of Benson et al. (1989). The major peaks, representing the two major microheterogeneous species of the 1-1 isoenzymes (Lai et al., 1984), elute at 45% and 46% acetonitrile, respectively, and comprise 90–98% of the ultraviolet-absorbing material. Since the N-terminal amino acid of isoenzyme 1-1 is reported to be blocked (Beale et al., 1982; Frey et al., 1983), the identity of the isoenzyme was verified by gas-phase sequence analysis of chymotryptic peptides of the 1-1 isoenzyme.

According to the method of Habig et al. (1974), the enzymatic activity of isoenzyme 1-1 was measured on a Gilford 240 spectrophotometer by monitoring the formation of the conjugate of 1-chloro-2,4-dinitrobenzene (1 mM) and glutathione (2.5 mM) at 340 nm (ϵ = 9.6 mM^{-1} cm^{-1}) in 0.1 M potassium phosphate, pH 6.5, at 25 °C. All measurements were corrected for the spontaneous nonenzymatic rate of reaction between glutathione and 1-chloro-2,4-dinitrobenzene. As compared to a specific activity of 50 μmol min^{-1} mg^{-1} reported by Mannervik and Danielson (1988), a typical purified sample of glutathione S-transferase, isoenzyme 1-1, gave a value of 59 μmol min^{-1} mg^{-1}.

Reaction of S-BDB-G with Glutathione S-Transferase. Glutathione S-transferase, isoenzyme 1-1 (0.4 mg/mL), in 0.1 M potassium phosphate, pH 6.5, was incubated with various concentrations of S-BDB-G at 25 °C by the addition of appropriate volumes of a 3.6 mM stock solution, pH 3.0. Various volumes of acetic acid (0.14 M, pH 3.0) were added to compensate for the different volumes of S-BDB-G in solution, thereby keeping the total volume of the pH 3.0 solution added to a 1.0 mL reaction mixture at 0.19 mL. The acidity of the S-BDB-G was offset by adding 2 M potassium phosphate, pH 7.0, to the reaction mixture to give a final phosphate concentration of 0.44 M. The final pH of the reaction mixture was always 6.5. When protecting ligands were added, they were preincubated with enzyme for 10 min before the reagent was added. Control samples were incubated under the same conditions with acetic acid solution instead of S-BDB-G. At various times, 20-μL aliquots of the reaction mixture were withdrawn, diluted 40-fold with 0.1 M potassium phosphate, pH 6.5, at 0 °C, and assayed for residual activity. The rate of reaction of isoenzyme 1-1 with S-BDB-G was determined by analyzing the first 18 min on a semilogarithmic plot of E/E_0 as a function of time, where E_0 represents the initial activity of the enzyme and E represents the activity at a given time. Unreacted reagent was removed from the reaction mixture by the gel centrifugation procedure of Penefsky (1979) in certain experiments. Each 0.5-mL reactin mixture was applied to a 5-mL column of Sephadex G-50–80 equilibrated with 0.1 M potassium phosphate, pH 6.5. Based on the method of Bradford (1976), the protein concentration in the effluent was determined by the Coomassie Blue protein assay using a Bio-Rad 2550 RIA reader (600-nm filter). Purified glutathione S-transferase, isoenzyme 1-1, was used to establish the standard protein concentration curve for these measurements.

Determination of K_m for Glutathione and K_I for Hydrolyzed S-BDB-G. Using a constant concentration of 1-chloro-2,4-dinitrobenzene (1 mM), the K_m was evaluated by varying the concentration of glutathione (50–2000 μM) under standard assay conditions. Hydrolyzed reagent was prepared by diluting S-BDB-G with 2 M potassium phosphate buffer, pH 7.0, in a 1:1 ratio to attain a final pH of 6.5. The reagent was kept at 25 °C for 5 h, equivalent to 6.25 half-lives for the compound (Katusz & Colman, 1991). The K_I for hydrolyzed S-BDB-G was measured by determining the K_m for glutathione in the presence of three concentrations of hydrolyzed reagent (45, 93, and 149 μM) as described above.

Measurement of Incorporation of S-BDB-G into Glutathione S-Transferase. Under the conditions described above, glutathione S-transferase, isoenzyme 1-1 (0.4 mg/mL), was incubated with 200 μM [^{3}H]S-BDB-G in the absence or in the presence of 5 mM *S*-hexylglutathione. A 0.5-mL aliquot of the reaction mixture was withdrawn at various times and separated from excess reagent by the column centrifugation method previously described. For incorporation measurements, two consecutive Sephadex G-50–80 columns (5 mL) were equilibrated with 0.1 M potassium phosphate, pH 6.5. Following column centrifugation, the protein concentration

was measured using the Coomassie Blue dye reagent method described above. The amount of reagent was determined from the radioactivity measured in a Packard Tri-carb liquid scintillation counter, Model 1500, after aliquots were mixed with 5 mL of Liquiscint. The number of moles of radioactive reagent per mole of enzyme subunit was then calculated.

Preparation of α-Chymotryptic Digest of Modified Glutathione S-Transferase. Glutathione S-transferase, isoenzyme 1-1 (1.7 mg), was reacted, under standard reaction conditions, with 200 μM [^{3}H]S-BDB-G. After 60 min, to render any free compound unreactive and to reduce the keto groups of S-BDB-G covalently linked to the protein, the modified enzyme was reduced by one addition of 300 mM $NaBH_4$ (dissolved in 0.01 M NaOH), giving a final concentration of 3 mM $NaBH_4$. Fifteen minutes after the addition, the free sodium borohydride and any remaining unreacted reagent were removed by gel filtration, in 500-μL aliquots, through individual Sephadex G-50–80 columns equilibrated with 0.1 M potassium phosphate, pH 6.5. To ensure reduction of all residual keto groups of the reagent, the above procedure of reduction and removal of excess $NaBH_4$ was repeated. The effluents from all the spin columns were pooled and treated, in the presence of 9 M urea, with 10 mM *N*-ethylmaleimide to block any unreacted cysteines. After 30 min at 25 °C, the solution was dialyzed, at 4 °C, against 6 L of 50 mM ammonium bicarbonate, pH 8.0, with one change for a total of 18 h.

After dialysis, the solution of modified enzyme was lyophilized. To solubilize the enzyme, 400 μL of 10 M urea in 50 mM ammonium bicarbonate was added to the recovered 0.9 mg of modified enzyme for 2 h. To give a final urea concentration of 2 M, 1600 μL of ammonium bicarbonate was added to the solubilized enzyme. The modified glutathione S-transferase was digested at 37 °C with two additions of α-chymotrypsin (20% w/w) at 1-h intervals. Lyophilization was used to stop the reaction, and the digest was stored at −20 °C.

Separation of Modified Peptides by HPLC. The chymotryptic peptides were separated by HPLC on a Varian 5000 LC equipped with a Vydac C_{18} column (1 × 25 cm) and a UV-100 detector. The initial solvent system used was 0.1% trifluoroacetic acid (solvent A) and acetonitrile containing 0.075% trifluoroacetic acid (solvent B). After elution with solvent A for 10 min, a linear gradient was run to 5% solvent B at 30 min followed by successive linear gradients in solvent B to 15% at 160 min, 25% at 240 min, 50% at 340 min, and 100% at a total of 360 min (chromatography system 1). The flow rate was 1 mL/min. The effluent was monitored continuously for absorbance at 220 nm, and 1-mL fractions were collected. The amount of radioactivity was determined using aliquots from the fractions.

When further purification of peptides was required, samples were separated by HPLC on a Vydac C_4 column with a variation of the linear gradient of the trifluoroacetic acid solvent system. With chromatography system 2, after elution with solvent A for 10 min, a linear gradient was run to 30% solvent B at an incremental change of 0.074% acetonitrile/min.

In certain experiments, to verify the amino acid sequence, peptides purified by chromatography system 1 were lyophilized, dissolved in 1 mL of 50 mM ammonium bicarbonate, and treated twice with 5% (w/w) thermolysin for a total of 2 h. Redigested peptides were then lyophilized and repurified using chromatography system 2.

Analysis of Isolated Peptides. The amino acid sequences of peptides were determined on an Applied Biosystems gas-phase protein (peptide) sequencer, Model 470, equipped with a phenylthiohydantoin analyzer, Model 120, and a Model 900A computer. Typically, 20–1500 pmol of peptide, dissolved in 25 μL of hexafluoroacetone trihydrate (98%), was analyzed.

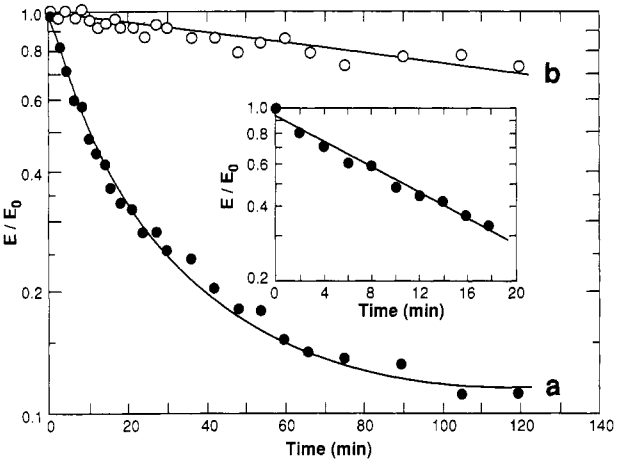

FIGURE 1: Inactivation of glutathione S-transferase, isoenzyme 1-1, by S-BDB-G in the (a) absence (●) and (b) presence (○) of substrate analogue as a function of time. Rat liver glutathione S-transferase, isoenzyme 1-1 (0.4 mg/mL), was incubated with 200 μM S-BDB-G at 25 °C at pH 6.5 in the absence and presence of 5 mM *S*-hexylglutathione. Residual activity, E/E_0, was measured as described under Experimental Procedures. (Inset) k_{obs} for the reaction in the absence of *S*-hexylglutathione was determined from the slope in ln (E/E_0) vs time for the first 18 min of the reaction. When the reagent concentration was 200 μM, k_{obs} = 0.059 min^{-1}.

RESULTS

Inactivation of Glutathione S-Transferase, Isoenzyme 1-1, with S-BDB-G. A time-dependent inactivation of glutathione S-transferase, as shown in Figure 1, line a, resulted when the enzyme was incubated with 200 μM S-BDB-G at pH 6.5 and 25 °C. The activity was constant over this time period when the control enzyme was incubated under the same conditions but in the absence of reagent. S-BDB-G is known to decompose with loss of bromide with a half-life of 48 min under these conditions (Katusz & Colman, 1991). When enzyme was added to reagent (200 μM) which had been preincubated under the standard reaction conditions for 5 h (6.25 half-lives), no loss of enzymatic activity was observed. This result indicates that inactivation by S-BDB-G (as in Figure 1, line a) requires the methylene bromide. Deviation from first-order kinetics of inactivation by S-BDB-G was observed beyond 20 min, as shown in Figure 1, line a. This deviation from linearity can be explained by the decomposition of the reagent and the effect of hydrolyzed reagent acting as a competitive inhibitor with respect to glutathione (Katusz & Colman, 1991). Using a K_m for glutathione of 304 μM under the conditions of the assay, the average K_I for the hydrolyzed reagent with the 1-1 isoenzyme was measured as 13 μM. For comparison, the K_I for *S*-butylglutathione, the alkyl derivative of glutathione with the same number of carbon atoms as the reagent, was determined to have an average value of 65 μM. After removal of hydrolyzed reagent, a second addition of 200 μM S-BDB-G (data not shown) led to complete inactivation of enzyme.

Concentration Dependence of the Reaction Rate of Glutathione S-Transferase with S-BDB-G. To determine the dependence of the rate of inactivation on the reagent concentration, glutathione S-transferase, isoenzyme 1-1, was incubated with various concentrations of S-BDB-G (50–1200 μM). As seen in Figure 2, k_{obs} exhibits a nonlinear dependence on the S-BDB-G concentration. This result suggests the

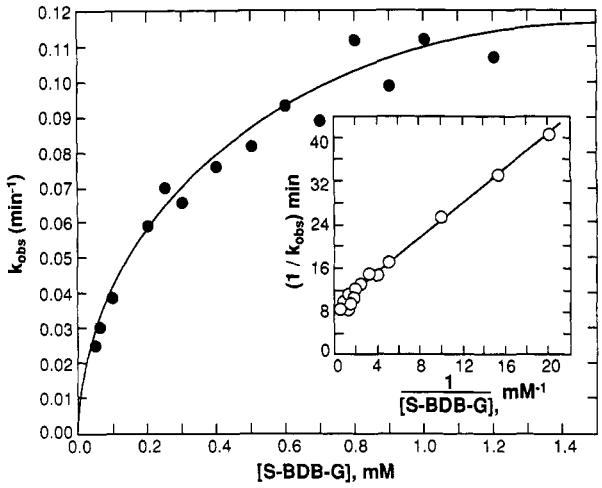

FIGURE 2: Dependence of the pseudo-first-order rate constant for inactivation of glutathione S-transferase on the concentration of S-BDB-G. Glutathione S-transferase, isoenzyme 1-1, was incubated with various concentrations of S-BDB-G under the conditions described for Figure 1. k_{obs} exhibits a nonlinear dependence on S-BDB-G concentration. (Inset) The double-reciprocal plot of $1/k_{obs}$ vs $1/[S\text{-BDB-G}]$ gives $k_{max} = 0.111$ min^{-1} and $K_I = 185$ μM.

Table II: Effect of Substrate Analogues on Rate Constant for Inactivation by 200 μM S-BDB-G

ligand added to reaction mixture	$k_{obs} \times 10^3$ (min^{-1})
none	59.0
S-hexylglutathione (5 mM)	2.2
S-pentylglutathione (5 mM)	8.0
S-butylglutathione (5 mM)	10.6
S-propylglutathione (5 mM)	23.0
S-ethylglutathione (5 mM)	30.9
S-methylglutathione (5 mM)	41.6
2,4-dinitrophenol (5 mM)	22.0
2,4-dinitrophenol (5 mM) + *S*-methylglutathione (5 mM)	11.2

initial formation of a reversible enzyme–reagent complex prior to irreversible modification, which is characteristic of an affinity label. The observed rate constant k_{obs} at a particular concentration of reagent (R) is described by

$$1/k_{obs} = 1/k_{max} + (K_I/k_{max})(1/R) \quad (1)$$

where $K_I = (k_{-1} + k_{max})/k_1$ and represents the concentration of reagent giving half the maximal inactivation rate (Huang & Colman, 1984). The double-reciprocal plot shown in the inset of Figure 2 was used to calculate $k_{max} = 0.111$ min^{-1} and $K_I = 185$ μM.

Effect of Substrate Analogues on the Inactivation Rate of Glutathione S-Transferase by S-BDB-G. Table II shows the effect of substrate analogues on the reaction rate of 200 μM S-BDB-G with glutathione S-transferase, isoenzyme 1-1. The concentration of the analogues (5 mM) was chosen to be 25 times the highest K_I reported for any of the *S*-alkyl-substituted glutathione, *S*-methylglutathione (Graminski et al., 1989a). The effect of *S*-hexylglutathione is illustrated in Figure 1, line b. Protection against enzyme inactivation is dependent upon the length of the hydrophobic side chain of the glutathione analogue. The rate constant decreases as the length of the side chain increases (Table II). *S*-Methylglutathione and 2,4-dinitrophenol together yield greater protection against S-BDB-G than either substrate analogue does alone (Table II). These data are consistent with the conclusion that the reaction of S-BDB-G occurs in the region of the active site of the enzyme, probably in the area normally occupied by the electrophilic substrate.

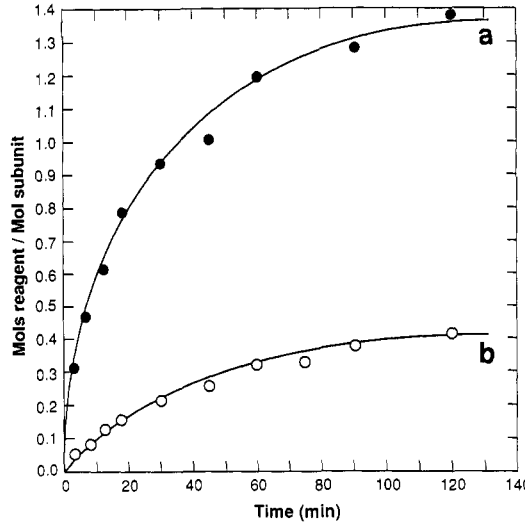

FIGURE 3: Incorporation of S-BDB-G per mole of subunit of glutathione S-transferase in the (a) absence (●) and (b) presence (○) of substrate analogue as a function of time. Glutathione S-transferase, isoenzyme 1-1 (0.4 mg/mL), was incubated with 200 μM S-BDB-G in the absence or presence of 5 mM *S*-hexylglutathione. Incorporation was determined at indicated time points as described under Experimental Procedures.

Incorporation of S-BDB-G by Glutathione S-Transferase. In the absence or presence of 5 mM substrate analogue, *S*-hexylglutathione, glutathione S-transferase, isoenzyme 1-1, was incubated with 200 μM as described under Experimental Procedures. Figure 3 (line a) shows the time-dependent incorporation of [^{3}H]S-BDB-G into the 1-1 isoenzyme of glutathione S-transferase. At 60 min, when the enzyme was, on the average, 85% inactivated, the incorporation measured was 1.2 mol of reagent/mol of enzyme subunit. These results indicate that the extent of reaction of S-BDB-G with the enzyme is limited. On a graph of residual enzyme activity vs incorporation, extrapolation to 0% residual enzyme activity gives an incorporation of 1.3 mol of reagent/mol of subunit (data not shown). In the presence of the protecting substrate analogue, *S*-hexylglutathione (Figure 3, line b), incorporation at 60 min was 0.33 mol of reagent/mol of subunit when the enzyme was 17% inactivated (Figure 1, line b). These data suggest the occurrence of reaction at one amino acid which is important for enzymatic activity and which is substantially protected by the substrate analogue.

Characterization of α-Chymotryptic Peptides from Modified Glutathione S-Transferase. The modified enzyme, prepared by reaction with 200 μM [^{3}H]S-BDB-G for 60 min, was treated with $NaBH_4$ and *N*-ethylmaleimide and then digested with α-chymotrypsin as described under Experimental Procedures. As shown in Figure 4A, the digest was fractionated using chromatography system 1. The major radioactive peptide regions are designated I and II.

Glutathione S-transferase, incubated with 200 μM [^{3}H]S-BDB-G for 60 min in the presence of protectant (*S*-hexylglutathione) to produce enzyme which retains most of its activity, was also digested with chymotrypsin. The digest, separated using chromatography system 1, is shown in Figure 4B. Although both peaks I and II are present in the chromatogram, they are greatly reduced in magnitude, consistent with the observation that marked (but not total) protection was provided by the *S*-hexylglutathione. These results suggest that the labeled peptide(s) found in peaks I and II is responsible for loss of 85% of the original activity after reaction in the absence of protectant.

Peak I, when rechromatographed using chromatography system 2, yields two peaks (Ia and Ib) with similar amino

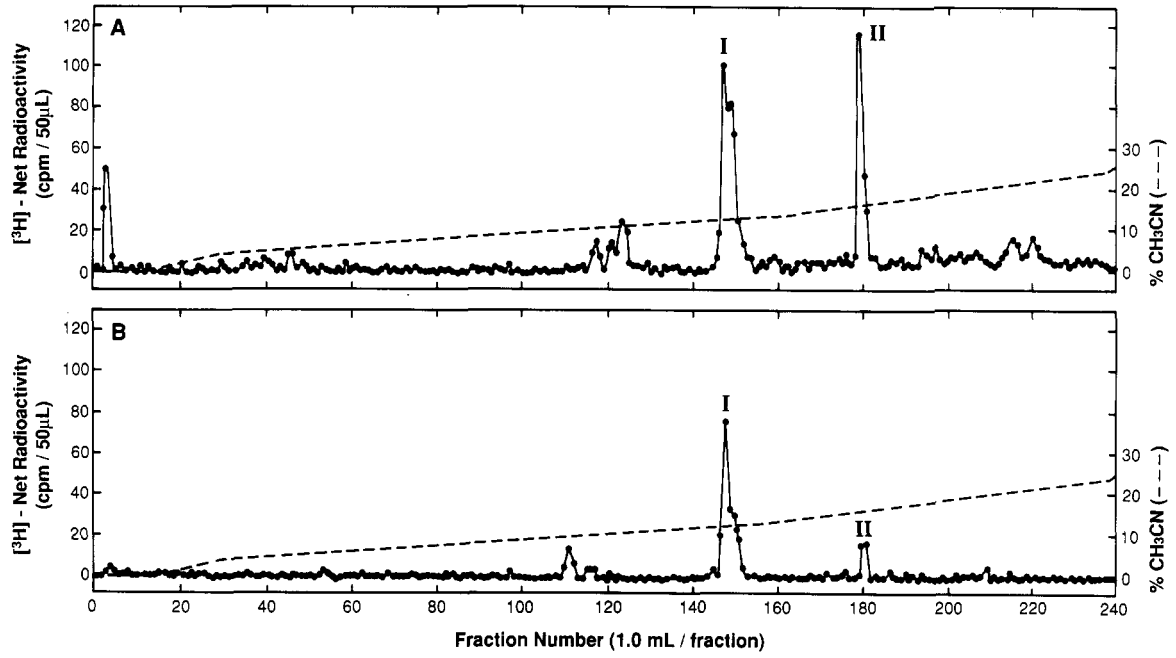

FIGURE 4: Fractionation of peptides by HPLC. Peptides derived from the chymotryptic digest of modified glutathione S-transferase, isoenzyme 1-1, were separated by chromatography system 1 as described under Experimental Procedures. (A) Distribution of radioactivity in the digest of modified enzyme prepared in the absence of substrate analogue. (B) Distribution of radioactivity in the digest of enzyme modified in the presence of 5 mM *S*-hexylglutathione. No significant amount of radioactivity was detected in regions of the chromatogram not shown.

Table III: Representative Sequences of Modified Peptides Present for both Active and Inactive Enzyme[a]

cycle no.	peak Ia amino acid (pmol)	peak Ib amino acid (pmol)	peak II[b] amino acid (pmol)
1	Val (247)	Val (352)	Gln (61)[c]
2	Ile (194)	Ile (308)	Leu (229)
3	X	X	Val (55)
4	Pro (175)	Pro (216)	Ile (50)
5	Pro (159)	Pro (217)	X
6	Asp (62)	Asp (109)	Pro (45)
7	Gln (114)	Gln (187)	Pro (43)
8	Lys (124)	Arg (46)	Asp (26)
9	Glu (80)	Glu (123)	Gln (38)
10	Ala (148)	Ala (212)	Lys (25)
11	Lys (124)	Lys (114)	Glu (21)
12	Thr (26)	Thr (32)	Ala (17)
13	Ala (58)	Ala (72)	Lys (11)
14	Leu (17)	Leu (17)	Thr (4)
mol of reagent/ mol of peptide[d]	0.70	1.01	0.52

[a] The final purification of peaks Ia and Ib was achieved by chromatography system 2. [b] This sequence resulted from the thermolysin digestion of peak II followed by purification using chromatography system 2.[c] The low recovery of this peptide on the sequencer may be due to the fact that Gln has, in part, converted to pyroglutamic acid (Blomback, 1967), a cyclic form which does not undergo Edman degradation. [d] Moles of reagent was calculated on the basis of radioactivity applied to the sequencer; moles of peptide was the average of picomoles of PTH-amino acids from the first 4 cycles of the sequencer, excluding X.

sequences, as shown in Table III. The sequence Val-Ile-X-Pro-Pro-Asp-Gln-Lys(Arg)-Glu-Ala-Lys-Thr-Ala-Leu corresponds to residues 109–122 in the known amino acid sequence (Pickett et al., 1984; Lai et al., 1984). An X at a given cycle indicates that no phenylthiohydantoin derivative was detected in this cycle. Since the known amino acid sequence contains a cysteine at this position, the peptide is most likely modified at the cysteine (Cys^{111}). Furthermore, in this peptide preparation cysteine residues not labeled by S-BDB-G are blocked by *N*-ethylmaleimide. In gas-phase sequence analysis, NEM-Cys can be detected as a doublet migrating on the HPLC column between the PTH derivatives of Pro and Met (Smyth & Colman, 1991). No doublet was apparent in cycle 5 of the sequence analysis of any of the peptides isolated. The alternate amino acids (Lys and Arg) detected in position 116 can be attributed to the microheterogeneity of the 1-1 isoenzyme in this region (Lai et al., 1984).

When digested with thermolysin and repurified using chromatography system 2, peak II[2] gave the radioactive peptide indicated by the amino acid sequence given in Table III. The sequence Gln-Leu-Val-Ile-X-Pro-Pro-Asp-Gln-Lys-Glu-Ala-Lys-Thr contains the same Cys^{111} labeled by S-BDB-G in peak I. Peaks I and II appear to be derived from different cleavages by α-chymotrypsin. These results suggest that Cys^{111} is the only major target of S-BDB-G and is the amino acid responsible for enzyme inactivation when isoenzyme 1-1 is labeled by the reagent.

DISCUSSION

In its reaction with the 1-1 isoenzyme of glutathione S-transferase, *S*-(4-bromo-2,3-dioxobutyl)glutathione acts as an affinity label. The rate constant of the reaction exhibits a nonlinear dependence on reagent concentration, indicating the formation of a reversible enzyme–reagent complex prior to irreversible modification; at high concentrations of the reagent, the rate constant becomes independent of reagent concentration. Substrate analogues with hydrophobic side chains from one to six carbons provide protection against inactivation by S-BDB-G; however, *S*-hexylglutathione gives almost total protection, while the inactivation rate constant is about 20 times higher in the presence of *S*-methylglutathione than with *S*-hexylglutathione. These results correlate with investigations showing the effect of competitive inhibitors on glutathione S-transferase. Listowsky et al. (1988), using isoen-

[2] Peak II, when subjected to gas-phase sequencing directly after initial purification by chromatography system 1, included the sequence Gln^{107}–Thr^{120}, along with another peptide present in amounts much greater than the amount of the radioactive reagent. Treatment with thermolysin, followed by rechromatography, allowed the isolation of the pure radioactive peptide found in Table III with no other radioactive peaks apparent in the HPLC chromatogram.

zyme 4-4, determined the K_I for *S*-hexylglutathione as 35 μM, while Graminski et al. (1989a) reported the K_I for *S*-methylglutathione as 200 μM. Similarly, Askelof et al. (1975) showed that the extent of inhibition of isoenzymes 3-3 and 3-4 increased with the length of the alkyl side chains of S-substituted glutathione derivatives. A limited number of sites on the enzyme are covalently bound when [^{3}H]S-BDB-G reacts with the 1-1 isoenzyme: only 1.2 mol of reagent/mol of subunit is incorporated when the enzyme is 85% inactivated.

One peptide containing covalently linked S-BDB-G has been isolated from the proteolytic digest of modified enzyme. Residues 109–120 in the known amino acid sequence of rat liver glutathione S-transferase, isoenzyme 1-1 (Pickett et al., 1984; Lai et al., 1984) are contained in this peptide. The substrate analogue, *S*-hexylglutathione, decreases reaction at this peptide, suggesting that it is at or near the active site of the enzyme. On the basis of the absence of a recognizable phenylthiohydantoin derivative at its expected position in the amino acid sequence, Cys^{111} appears to be the residue modified by S-BDB-G in this peptide.

Previous work with the μ-class isoenzyme 4-4 (Katusz & Colman, 1991), identified Tyr^{115} as the amino acid covalently linked to S-BDB-G when the enzyme was 83% inactive. Although both the 1-1 and the 4-4 isoenzymes had limited incorporation of the reagent (1.1 and 1.2 mol of S-BDB-G/mol of subunit, respectively, when the enzymes were 83-85% inactivated), isoenzyme 1-1 exhibits a weaker affinity for S-BDB-G (K_I = 185 μM) than does the 4-4 isoenzyme (K_I = 66 μM), indicating that the binding pocket for substrate may be slightly different for the two classes of enzyme. Consistent with this suggestion are the results that *S*-hexylglutathione, although providing complete protection against inactivation by S-BDB-G to the 4-4 isoenzyme, gave substantial but not complete protection to the 1-1 isoenzyme. Comparison of the K_I values for hydrolyzed S-BDB-G (13 μM) and *S*-butylglutathione (65 μM) suggest that additional interactions due to the presence of the carbonyl groups in the reagent may be facilitating binding of S-BDB-G.

Alignment of the primary sequence of the 1-1 isoenzyme with that of the 4-4 isoenzyme using the CLUSTAL program (PC Gene, Intelligenetics), as shown in Table I, indicates that Cys^{111} (of 1-1) and Tyr^{115} (of 4-4) differ in one position. When isoenzyme 7-7, a π-class isoenzyme of glutathione S-transferase, is included in the analysis, Tyr^{108} aligns with Tyr^{115} of isoenzyme 4-4.

The three-dimensional structure of the π-class 7-7 pig lung isoenzyme is the only glutathione S-transferase structure published to date (Reinemer et al., 1991). In this structure, each subunit of the isoenzyme is folded into two domains. The competitive inhibitor glutathione sulfonate binds on domain I and is found in part of a cleft between intrasubunit domains. Although the investigators indicate uncertainty about the exact location of the electrophilic binding site, of the three possibilities proposed, Tyr^{106} is included as an amino acid whose side chain could be involved in forming the cleft which binds the hydrophobic substrate. In primary sequence alignments presented by these investigators, Tyr^{106} seems to be equivalent to Tyr^{108} of the rat glutathione S-transferase, isoenzyme 7-7. Additionally, in the figures with glutathione sulfonate present (Reinemer et al., 1991), Tyr^{106} appears sufficiently close to the bound inhibitor to be a residue capable of reaction with the methylene bromide if S-BDB-G were substituted for glutathione sulfonate.

These deductions are consistent with recent work on glutathione S-transferase 3-3, a μ-class isoenzyme whose Tyr^{115} is equivalent to Tyr^{115} of isoenzyme 4-4 in the primary sequence alignments (Table I). The phenolic oxygen of Tyr^{115}, in the three-dimensional structure, has been measured to be 7.5 Å from the sulfur atom of glutathione (Richard N. Armstrong, personal communication). This value is close to 5.2 Å, the distance between the sulfur and the carbon of -CH_2Br, or to 6.8 Å, the distance between the sulfur and the bromo group of -CH_2Br, determined by molecular modeling[3] when the reagent, S-BDB-G, is in an extended conformation. In both the π- and μ-class isoenzymes, the respective tyrosine residues (Tyr^{106} or Tyr^{115}) could be of importance in binding the electrophilic substrate through hydrophobic interactions.

The inclusion of Tyr^{115} (Tyr^{106} in the π-class isoenzyme) in the electrophilic substrate binding site of the isoenzymes and its importance in providing hydrophobic interactions to the non-glutathione substrate are consistent with the recent investigation of Penington and Rule (1992). In this study, all the tyrosines of the human class μ muscle glutathione S-transferase were changed to phenylalanines by site-directed mutagenesis and the substrate-binding site of the enzyme was mapped using nuclear magnetic resonance spectroscopy in the absence and presence of spin label. The mutation of Tyr^{116} (equivalent to Tyr^{115} in the rat liver μ isoenzyme and Tyr^{106} in the pig lung π isoenzyme, as seen in Table I) to phenylalanine caused no decrease in specific activity using 1-chloro-2,4-dinitrobenzene as substrate. This result leads to the conclusion that this tyrosine itself is not essential for function since it can readily be replaced by another hydrophobic amino acid. However, Penington and Rule also demonstrated that the spin label sl-GSH (a conjugate between glutathione and iodoacetamide proxyl) causes broadening of the resonance line of Tyr^{116}, indicating proximity between this residue and the paramagnetic portion of the enzyme-bound glutathione adduct.

As shown in Table I, the α-class isoenzyme 1-1 has an Ile^{110} aligned with Tyr^{115} of the 4-4 isoenzyme, thereby conserving a hydrophobic residue in this position. Tyrosine, in addition to being hydrophobic in nature, is nucleophilic, which enables it to react with -CH_2Br of S-BDB-G. In terms of hydrophobicity, Ile^{110} in the 1-1 isoenzyme is an acceptable alternative to Tyr but it is unreactive with the reagent. Fortuitously, Cys^{111}, the amino acid labeled by S-BDB-G in isoenzyme 1-1, is adjacent to the isoleucine where it is accessible for covalent reaction with reagent but, as indicated by the available three-dimensional structures, may not be an essential part of the hydrophobic binding pocket.

When the primary sequence of the 1-1 isoenzyme is aligned with that of the α-class 2-2 isoenzyme (Telakowski-Hopkins et al., 1985), Cys^{111} of isoenzyme 1-1 aligns with Ile^{111} in the 2-2 isoenzyme. Results of the present study with the 1-1 isoenzyme predict that the presence of isoleucine rather than a nucleophilic amino acid in position 111 of the sequence would prevent loss of activity if S-BDB-G were incubated with the 2-2 isoenzyme. When this experiment was conducted and the 2-2 isoenzyme was incubated with 200 μM S-BDB-G under the conditions described for Figure 1, line a, little or no loss of enzymatic activity was observed. This result further supports the suggestion that Cys^{111} is important for the inactivation of isoenzyme 1-1 and demonstrates that S-BDB-G inactivates the isoenzymes of glutathione S-transferase selectively.

Carne et al. (1979) have reported that small sulfhydryl reagents such as iodoacetate and *N*-ethylmaleimide do not inactivate ligandin (a mixture of isoenzymes 1-1 and 1-2). However, when *p*-mercuribenzoate or *N*-(4-dimethylamino-

[3] Conducted using the program Chem 3D, Cambridge Scientific Corp.

3,5-dinitrophenoxy)maleimide was used, a maximum of 40% loss of enzymatic activity was observed, with little decrease in the affinity for glutathione. These results led the investigators to conclude that a thiol group may be associated with the hydrophobic binding site of ligandin.

Hoesch and Boyer (1989) used the photoaffinity label *S*-(*p*-azidophenacyl)-[^{3}H]glutathione to identify what they referred to as a portion of the active site for the α-class isoenzyme 1-1 and 2-2. Although for both isoenzymes the investigators indicate that the C-terminal region was labeled by *S*-(*p*-azidophenacyl)glutathione, they found only the region between amino acids 90 and 110 was modified in the 2-2 isoenzyme. On the basis of the hydropathy index, Hoesch and Boyer suggest that the C-terminus may not be the most hydrophobic region of the active site and that the region between amino acids 90 and 110 may contain the hydrophobic binding portion of the active site for both isoenzymes.

These previous investigations with chemical modification reagents are consistent with the results of the present study, which indicate that labeled Cys^{111}, located in a hydrophobic portion of the primary sequence, may be near the active site of the 1-1 isoenzyme. In addition, both the π and μ three-dimensional structures indicate that some of the residues close to the C-terminus of the pig 7-7 and rat 3-3 isoenzymes may be near the amino acid (Tyr^{106}, Tyr^{115}, or Cys^{111}) which either is, or is expected to be, modified by S-BDB-G.

In examining the primary sequence of the 1-1 isoenzyme, it has been observed that Cys^{111} (the residue labeled by S-BDB-G) is adjacent to the hydrophobic residue, Ile^{110}, which aligns with Tyr^{115} in the 4-4 isoenzyme. There is also a cysteine residue (Cys^{114}) adjacent to Tyr^{115} in the primary sequence of the 4-4 isoenzyme (and 3-3 isoenzyme), but it is not reactive with S-BDB-G. In addition, recent work using site-directed mutagenesis of the 3-3 isoenzyme in which Cys^{86}, Cys^{114}, and Cys^{173} were replaced by serine or alanine residues, as well as chemical modification studies with iodoacetamide, have led Hsieh et al. (1991) and Tam et al. (1992) to suggest that cysteines are not directly involved in the enzymatic mechanism but that Cys^{114} may be located in the hydrophobic substrate binding site. This conclusion raises the question of why Cys^{114} does not react with S-BDB-G in the 4-4 isoenzyme. Examination of the three-dimensional structures of both the 7-7 isoenzyme and the 3-3 isoenzyme (Richard N. Armstrong, personal communication) place both Tyr^{115} and Cys^{114} as part of an α-helix with Tyr^{115} (the last amino acid of the helix) facing the putative binding pocket and Cys^{114} pointing away from the pocket, making it less available for reaction with S-BDB-G.

S-(4-Bromo-2,3-dioxobutyl)glutathione has been used in this investigation to label Cys^{111} in rat liver glutathione S-transferase, isoenzyme 1-1. Although Cys^{111} may not itself be a contributor to the hydrophobic interactions which facilitate the binding of the non-glutathione substrates, the results of this study, especially those with the substrate analogue *S*-hexylglutathione, indicate that Cys^{111} is at or near the hydrophobic binding pocket of the enzyme.

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Registry No. S-BDB-G, 136954-46-6; Cys, 52-90-4; *S*-hexylglutathione, 24425-56-7; *S*-pentylglutathione, 24425-55-6; *S*-butylglutathione, 6803-16-3; *S*-propylglutathione, 24425-53-4; *S*-ethylglutathione, 24425-52-3; *S*-methylglutathione, 2922-56-7; 2,4-dinitrophenol, 51-28-5; glutathione *S*-transferase, 50812-37-8.