S-(4-Bromo-2,3-dioxobutyl)glutathione: A New Affinity Label for the 4–4 Isoenzyme of Rat Liver Glutathione S-Transferase†

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ABSTRACT: *S*-(4-Bromo-2,3-dioxobutyl)glutathione (S-BDB-G), a reactive analogue of glutathione, has been synthesized and characterized by UV spectroscopy and thin-layer chromatography, as well as by bromide and primary amine analysis. Incubation of S-BDB-G (200 μM) with the 4–4 isoenzyme of rat liver glutathione S-transferase at pH 6.5 and 25 °C results in a time-dependent inactivation of the enzyme. The k_{obs} exhibits a nonlinear dependence on S-BDB-G concentration from 50 to 1000 μM, with a k_{max} of 0.078 min^{-1} and K_I = 66 μM. The addition of 5 mM *S*-hexylglutathione, a competitive inhibitor with respect to glutathione, completely protects against inactivation by S-BDB-G. About 1.3 mol of [^{3}H]S-BDB-G/mol of enzyme subunit is incorporated concomitant with 100% inactivation, whereas only 0.48 mol of reagent/mol of subunit is incorporated in the presence of *S*-hexylglutathione when activity is fully retained. 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 $NaBH_4$, carboxymethylated, and digested with trypsin. The tryptic digest was fractionated by reverse-phase high-performance liquid chromatography. Two radioactive peptides were identified: Lys^{82}-His-Asn-Leu-X-Gly-Glu-Thr-Glu-Glu-Glu-Arg^{93}, in which X is modified Cys^{86}, and Leu^{109}-Gln-Leu-Ala-Met-CmCys-Y-Ser-Pro-Asp-Phe-Glu-Arg^{121}, in which Y is modified Tyr^{115}. Only the Lys^{82}-Arg^{93} peptide was modified in the presence of *S*-hexylglutathione when the enzyme retained full activity. Cys^{86} is therefore regarded as a nonessential labeled residue, while Tyr^{115} is considered to be in the region of the active site. These results suggest that S-BDB-G functions as an affinity label at or near the active site of glutathione S-transferase and that modification of one site per enzyme subunit causes inactivation. It is proposed that the new compound, *S*-(4-bromo-2,3-dioxobutyl)glutathione, may have general applicability as an affinity label of other enzymes with glutathione binding sites.

The glutathione S-transferases (EC 2.5.1.18), a group of isoenzymes primarily involved in the detoxification of both endogenous compounds and xenobiotics, 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 exist as either homo- or heterodimers in which each subunit contains a glutathione binding site and a second, hydrophobic binding site which is tolerant of structural variation of an electrophilic substrate (Mannervik & Danielson, 1988; Jakobson et al., 1979).

The glutathione S-transferases have been found predominantly in the cell cytosol in a number of species and in various organs of mammals (Jakoby, 1977; Mannervik & Danielson, 1988). On the basis of isoelectric point, substrate specificity, immunological reactivity, and inhibition properties as well as primary sequence similarity, the isoenzymes indicate the existence of three distinct gene classes: α, μ, and π. Rat liver isoenzyme 4–4, used in this study, belongs to the μ gene class which encodes subunit types 3, 4, 6, 9, and 11 (Mannervik & Danielson, 1988; Armstrong, 1991).

The primary structure of the 4–4 isoenzyme has been determined by both amino acid and cDNA sequencing (Alin et al., 1986; Ding et al., 1986). However, the active site has not been clearly identified within the primary sequence. Affinity labeling is a powerful approach to limiting chemical modification to a specific site of an enzyme. In this technique, a reagent is designed that is structurally similar to the natural substrate but also features a functional group capable of covalent reaction with many different amino acids.

In this paper, the synthesis and characterization of a new glutathione analogue, *S*-(4-bromo-2,3-dioxobutyl)glutathione (S-BDB-G),[1] is described. The reagent, S-BDB-G, retains the glutathione backbone but contains, in addition, a bromodioxobutyl group which can react with the nucleophilic side chains of several amino acids including cysteine, tyrosine, histidine, aspartic acid, glutamic acid, lysine, and arginine (Colman, 1989). This paper presents evidence that S-BDB-G reacts in a specific and limited manner at or near the active site of rat liver glutathione S-transferase, isoenzyme 4–4. A preliminary version of this work has been presented (Katusz & Colman, 1990).

Experimental Procedures

Materials. Frozen Sprague-Dawley rat livers were purchased from Pel Freez Biologicals. Glutathione, the *S*-alkyl derivatives of glutathione, *S*-hexylglutathione–Sepharose, *N*-tosyl-L-phenylalanine chloromethyl ketone treated trypsin, 2,4-dinitrophenol, and 2,4,6-trinitrobenzenesulfonic acid were all obtained from Sigma Chemical Co. 1-Chloro-2,4-dinitrobenzene was supplied by Aldrich Chemical Co. 1,4-Dibromobutanedione was also purchased from Aldrich Chemical Co. and was recrystallized from petroleum ether before use. Whatman Biosystems supplied the DEAE-cellulose, and Analtech, Inc. provided the MN300F cellulose TLC

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

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plates with fluorescent indicator. Coomassie Blue Dye Reagent was obtained from Pierce Chemical Co. Hydroxylapatite (Bio-Gel HT) was supplied by Bio-Rad Laboratories, urea was from Schwarz/Mann Biotech, and Liquiscint was from National Diagnostics. [*glycine*-2-^{3}H]Glutathione was purchased from Du Pont New England Nuclear Research Products. All other chemicals used were reagent grade.

Synthesis of S-(4-Bromo-2,3-dioxobutyl)glutathione. S-BDB-G was prepared by incubation of glutathione with a much higher concentration of 1,4-dibromobutanedione. In a typical reaction, 1.76 mmol of 1,4-dibromobutanedione were dissolved in 6 mL of acetone and added to 0.058 mmol of glutathione previously dissolved in 1 mL of water (pH 3.0). The solution was allowed to stand at room temperature for 10 min. At 4 °C, the reaction mixture was extracted with 15 mL of chloroform three times to remove excess 1,4-dibromobutanedione, and the water layer (approximately 1 mL) was applied to two 20 cm × 20 cm 250-μm cellulose TLC plates with fluorescent indicator (0.029 mmol of product per plate). Development at 4 °C in *n*-butanol/acetic acid/water (4:1:1.6) yielded purified *S*-(4-bromo-2,3-dioxobutyl)glutathione. Following extraction from the cellulose (three times with 2 mL of water each time), the product (pH 3.0) was frozen rapidly and stored at −80 °C. In the preparation of radioactive reagent, 125 μCi of [*glycine*-2-^{3}H]glutathione was diluted with 0.058 mmol of nonlabeled glutathione, and the mixture was adjusted to pH 3.0 with one drop of 17.6 N acetic acid. Synthesis was then conducted as described above.

Characterization of S-(4-Bromo-2,3-dioxobutyl)glutathione. The content of primary amine present in a solution of S-BDB-G was determined by reaction with 2,4,6-trinitrobenzenesulfonic acid (Mokrasch, 1967). In the determination, reduced glutathione was used to generate the standard curve. The bromide content was determined as described by Colman et al. (1984).

Determination of Rate of Decomposition of S-(4-Bromo-2,3-dioxobutyl)glutathione. As shown in Figure 1, the reagent was observed to undergo a time-dependent change in λ_{max} from 324 to 318 nm with an isosbestic point at 351 nm when incubated in 0.1 M potassium phosphate buffer, pH 6.5, at 25 °C. At 370 nm, where the hydrolyzed reagent exhibits a substantially higher absorbance as compared to the unhydrolyzed reagent, the spectral change was monitored under these conditions for 300 min using a Cary 219 spectrophotometer. The rate of absorbance change was determined from a semilogarithmic plot of $[(A_\infty - A_t)/(A_\infty - A_0)]$ as a function of time, where A_∞ represents the absorbance at 300 min, A_t represents the absorbance at a given time, and A_0 represents the initial absorbance. The extent of bromide hydrolysis as a function of time was also measured as previously described (Colman et al., 1984). In this determination, 5 M triethanolamine acetate buffer (pH 7.0) was substituted for phosphate buffer to reduce interference in the measurement of bromide.

Enzyme Preparation and Assay. The 4–4 isoenzyme of glutathione S-transferase was purified from the livers of Sprague-Dawley rats essentially by the method of Chen et al. (1988) using column chromatography on DEAE-cellulose, *S*-hexylglutathione–Sepharose, and hydroxylapatite. In a typical preparation using 25 rat livers (160 g approximate frozen weight), 10–15 mg of isoenzyme 4–4 glutathione S-transferase was recovered. The enzyme concentration was determined by using ϵ_{270nm} = 36 700 M^{-1} cm^{-1} (Graminski et al., 1989) and a M_r of 26 500 per subunit (Mannervik et al., 1985). 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 peak, representing 90–98% of the ultraviolet-absorbing material, eluted at 40% acetonitrile. When subjected to gas-phase sequence analysis, the amino-terminal sequence was Pro-Met-Thr-Leu-Gly-Tyr-Trp-Asp-Ile-Arg-Gly-Leu-Ala-His-Ala-Ile-Arg-Leu-Phe, consistent with that known for the 4–4 isoenzyme (Mannervik, 1985; Alin et al., 1986; Ding et al., 1986). In contrast, the most closely related isoenzyme (3–3) has Ile at position 3, Asn at position 8, Val at position 9, Thr at position 13, Pro at position 15, and Leu at position 19 (Mannervik, 1985).

Enzymatic activity was measured on a Gilford 240 spectrophotometer by monitoring the formation of the conjugate of glutathione (2.5 mM) and 1-chloro-2,4-dinitrobenzene (1 mM) at 340 nm (ϵ = 9.6 mM^{-1} cm^{-1}) in 0.1 M potassium phosphate buffer, pH 6.5, at 25 °C according to the method described by Habig et al. (1974). All measurements were corrected for the spontaneous non-enzymatic rate of the reaction between glutathione and 1-chloro-2,4-dinitrobenzene. A typical purified sample of glutathione S-transferase, isoenzyme 4–4, gave an average specific activity of 21 μmol min^{-1} mg^{-1} as compared to that of 18 μmol min^{-1} mg^{-1} reported by Mannervik (1985).

Reaction of S-BDB-G with Glutathione S-Transferase. Glutathione S-transferase, isoenzyme 4–4 (0.41 mg/mL), in 0.1 M potassium phosphate buffer, pH 6.5, was incubated, at 25 °C, with various concentrations of S-BDB-G by the addition of appropriate volumes of a stock solution of 2.4 mM, pH 3.0. The total volume of the pH 3.0 solution added to a 1.0-mL reaction mixture was 0.19 mL; 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. To offset the acidity of the S-BDB-G, 2 M potassium phosphate, pH 7.0, was added to the reaction mixture to yield a final phosphate concentration of 0.44 M. The final pH of the reaction mixture was always 6.5. When protecting ligands were added, enzyme was preincubated with them for 10 min before the reagent was added. Control samples were incubated under the same conditions without S-BDB-G, but with acetic acid solution added instead. At various times, aliquots of the reaction mixture were withdrawn, diluted approximately 15-fold with 0.1 M potassium phosphate buffer, pH 6.5, at 0 °C, and assayed for residual enzymatic activity. The rate of reaction of isoenzyme 4–4 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 at time zero and E represents the activity at a given time. When unreacted reagent was removed from the reaction mixture, the gel centrifugation procedure of Penefsky (1979) was used. Each 0.5 mL reaction mixture was applied to a 5-mL column of Sephadex G-50-80 equilibrated with 0.1 M potassium phosphate, pH 6.5. The protein concentration in the filtrate was determined by using the Coomassie Blue protein assay based on the method of Bradford (1976), using a Bio-Rad 2550 RIA reader (600-nm filter). For these determinations, purified glutathione S-transferase, isoenzyme 4–4, was used to establish the standard protein concentration curve.

Determination of K_m for Glutathione and K_I for Hydrolyzed S-BDB-G. The K_m was determined by varying the concentration of glutathione (50–2500 μM) under the standard assay

conditions with a constant concentration of 1-chloro-2,4-dinitrobenzene (1 mM). 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 maintained at 25 °C for 5 h, equivalent to 6.25 half-lives for the compound (see Results). The K_I for hydrolyzed S-BDB-G was determined by measuring the K_m for glutathione as described above except for the presence of three different concentrations of hydrolyzed reagent (5.4, 9.0, and 20 μM).

Measurement of Incorporation of S-BDB-G into Glutathione S-Transferase. The enzyme (0.41 mg/mL) was incubated with 200 μM radioactive reagent, in the absence or in the presence of 5 mM *S*-hexylglutathione under the conditions described. At various times, a 0.5-mL aliquot of the reaction mixture was withdrawn 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 buffer: the first, with 0.1 M potassium phosphate buffer, pH 6.5; the second, with 0.1 M potassium phosphate buffer, pH 8.0. The protein concentration following column centrifugation was measured using the Coomassie Blue dye reagent method as previously described. The incorporation of S-BDB-G into the 4–4 isoenzyme of glutathione S-transferase was measured by determination of the number of moles of radioactive reagent per mole of enzyme subunit.

Preparation of Proteolytic Digest of Modified Glutathione S-Transferase. Glutathione S-transferase, isoenzyme 4–4 (1.7 mg), was reacted with 200 μM radioactive S-BDB-G for 60 min under standard reaction conditions. The mixture was divided into 500-μL aliquots, and each was applied to one Sephadex G-50-80 spin column equilibrated with 0.1 M potassium phosphate buffer, pH 6.5. The effluents from all the spin columns were pooled and treated with sodium borohydride to reduce any unreacted keto groups of the reagent which were covalently linked to the protein. The modified enzyme was reduced by two additions of 300 mM $NaBH_4$ (dissolved in 0.01 M NaOH) with 15 min between additions, giving a final concentration of 3 mM $NaBH_4$. Fifteen minutes after the second addition, the free sodium borohydride was removed by a Sephadex G-50-80 spin column equilibrated with 0.1 M potassium phosphate buffer, pH 8.0. The effluents from all the spin columns were again pooled and treated, in the presence of 8 M urea, with iodoacetic acid to yield a final concentration of 20 mM. After 30 min at 25 °C, the iodoacetic acid reaction was stopped by addition of mercaptoethanol to a concentration of 200 mM. The solution was dialyzed 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, 250 μL of 8 M urea in 50 mM ammonium bicarbonate was added. After 90 min, 750 μL of 50 mM ammonium bicarbonate was added to give a final urea concentration of 2 M. The modified glutathione S-transferase was digested at 37 °C with two additions of *N*-tosyl-L-phenylalanine chloromethyl ketone treated trypsin (20% w/w) at 1-h intervals. The reaction was stopped by lyophilization, and the digest was stored at −20 °C.

Separation of Modified Peptides by HPLC. The tryptic 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% tifluoroacetic 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% B at a total of 360 min (chromatography system I). The flow rate was 1 mL/min. The effluent was monitored continuously for absorbance at 220 nm, and 1-mL fractions were collected. Aliquots of fractions were mixed with 5 mL of Liquiscint and were monitored for radioactivity using a Packard Tri-Carb liquid scintillation counter, Model 1500.

When further purification of peptides was needed, samples were separated by HPLC on a Vydac C_4 column with variations of linear gradients of the trifluoroacetic acid solvent system. With chromatography system II, after elution with solvent A for 10 min, a linear gradient was run to 20% solvent B at an incremental change of 0.074% acetonitrile/min. With chromatography system III, after elution with solvent A for 10 min, a linear gradient was run to 5% solvent B at 30 min, followed by a linear gradient to 30% solvent B at 360 min.

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 was analyzed. The amount of CmCys was calculated using the PTH-Gln standard.

Results

Characterization of S-(4-Bromo-2,3-dioxobutyl)glutathione. The overall yield in the synthesis of S-BDB-G from glutathione was approximately 40%. Product purity was assessed by thin-layer chromatography on cellulose plates with a fluorescent indicator using *n*-butanol/acetic acid/water (5:1:1.6) as the solvent system. A single spot which was ninhydrin positive was observed with an R_f value of 0.41. For comparison, the precursors, glutathione and 1,4-dibromobutanedione, exhibited spots at R_f 0.33 and 1.00, respectively.

The product exhibits an ultraviolet absorption spectrum with a peak at 324 nm (Figure 1) which is not present in the spectrum of the precursor glutathione. The compound contains a free amino group as indicated by its reaction with ninhydrin and with 2,4,6-trinitrobenzenesulfonic acid. The extinction coefficient was measured as 4415 M^{-1} cm^{-1} in water at the λ_{max} of 324 nm on the basis of the S-BDB-G concentrations determined from the primary amine content.

The bromide content of S-BDB-G, measured as previously described (Colman et al., 1984), was 1.03 mol of hydrolyzable bromide/mol of reagent. The half-life of the reagent at pH 6.5 and 25 °C was measured as 48 min both by spectrophotometric methods (see Experimental Procedures) and by the rate of release of free bromide. These data are all consistent with the structure for *S*-(4-bromo-2,3-dioxobutyl)glutathione.

Inactivation of Glutathione S-Transferase, Isoenzyme 4–4, with S-BDB-G. Incubation of glutathione S-transferase with 200 μM S-BDB-G at pH 6.5 and 25 °C resulted in a time-dependent inactivation of the enzyme as shown in Figure 2. The control enzyme, incubated under the same conditions but in the absence of reagent, showed constant activity over this time period. Deviation from first-order kinetics was observed beyond 20 min, as shown in Figure 2; this deviation from linearity can be explained by the decomposition rate of the reagent ($t_{1/2}$ = 48 min) and the effect of hydrolyzed reagent as a competitive inhibitor with respect to glutathione. The K_I for the hydrolyzed reagent with the 4–4 isoenzyme was determined as 4 μM, as described under Experimental Procedures. For comparison, the K_I for *S*-butylglutathione, the alkyl derivative of glutathione with the same number of carbon atoms as the reagent, yielded an average value of 10 μM.

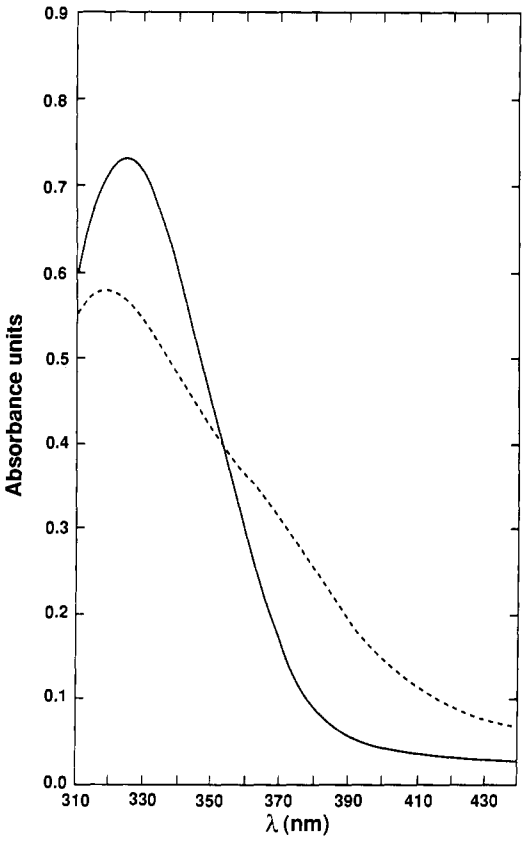

FIGURE 1: Absorption spectrum (—) of *S*-(4-bromo-2,3-dioxobutyl)glutathione in water (ϵ = 4415 M^{-1} cm^{-1} at λ_{max} 324 nm); absorption spectrum (---) of S-BDB-G after hydrolysis for 210 min in 0.1 M potassium phosphate buffer, pH 6.5, at 25 °C (λ_{max} = 318 nm).

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

ligands added to reaction mixture	$k_{obs} \times 10^3$ (min^{-1})
1 none	58.0
2 *S*-hexylglutathione (5 mM)	0
3 *S*-pentylglutathione (5 mM)	0
4 *S*-butylglutathione (5 mM)	0
5 *S*-propylglutathione (5 mM)	1.7
6 *S*-ethylglutathione (5 mM)	3.3
7 *S*-methylglutathione (5 mM)	10.4
8 2,4-dinitrophenol (5 mM)	20.2
9 *S*-methylglutathione (5 mM) + 2,4-dinitrophenol (5 mM)	3.4
10 hydrolyzed S-BDB-G (200 μM)	27.7

Under the conditions of the assay described under Experimental Procedures, the K_m for glutathione was 123 μM for the 4–4 isoenzyme. A second addition of 200 μM S-BDB-G after the removal of hydrolyzed reagent (data not shown) leads to nearly complete enzyme inactivation (4.5% remaining activity). It is postulated that as the hydrolyzed reagent is formed during the incubation of enzyme with S-BDB-G, it binds to the glutathione site of the enzyme and markedly decreases the subsequent inactivation rate.

Concentration Dependence of the Reaction Rate of Glutathione S-Transferase with S-BDB-G. Glutathione S-transferase, isoenzyme 4–4, was incubated with various concentrations of S-BDB-G (50–1000 μM) to determine the dependence of the rate of inactivation on the reagent concentration. As seen in Figure 3, k_{obs} exhibits a nonlinear dependence on the S-BDB-G concentration. This result suggests the initial formation of a reversible enzyme–reagent complex prior to irreversible modification which is characteristic of an

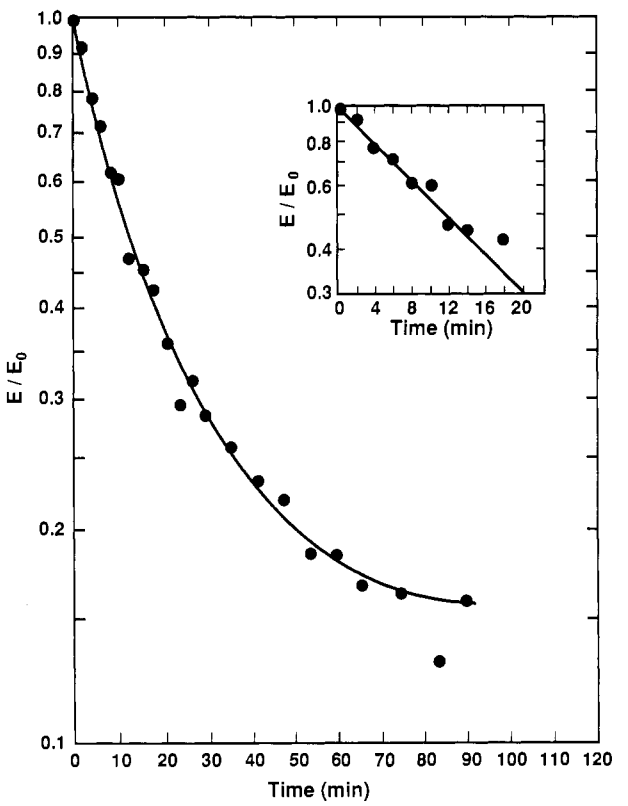

FIGURE 2: Inactivation of glutathione S-transferase, isoenzyme 4–4 by S-BDB-G. Rat liver glutathione S-transferase, isoenzyme 4–4 (0.41 mg/mL), was incubated with 200 μM S-BDB-G at 25 °C at pH 6.5. Residual activity, E/E_0, was measured as described under Experimental Procedures. Inset: The k_{obs} for the reaction was determined from the slope in ln (E/E_0) vs time for the first 18 min of the reaction. When reagent concentration was 200 μM, k_{obs} = 0.058 min^{-1}.

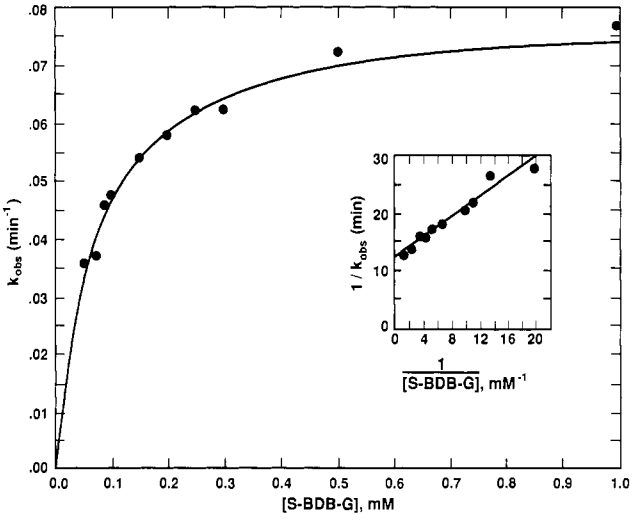

FIGURE 3: Dependence of the pseudo-first-order rate constant for inactivation of glutathione S-transferase on the concentration of S-BDB-G. Glutathione S-transferase, isoenzyme 4–4, was incubated with various concentrations of reagent under the conditions described in Figure 2. The k_{obs} exhibits a nonlinear dependence on S-BDB-G concentration. Inset: The double-reciprocal plot of $1/k_{obs}$ vs 1/[S-BDB-G] gives k_{max} = 0.078 min^{-1} and K_I = 66 μM.

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 of the maximal inactivation rate (Huang

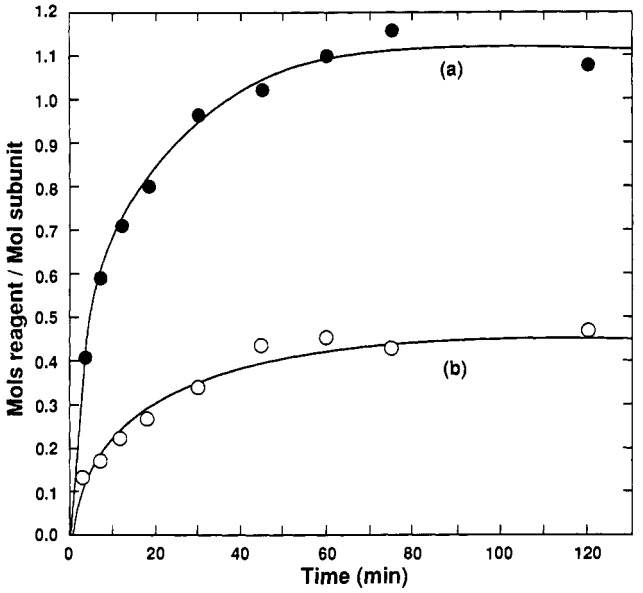

FIGURE 4: Incorporation of S-BDB-G per mole of subunit of glutathione S-transferase in the (a) absence (●) and (b) presence (○) of substrate analogue as a function of time. Glutathione S-transferase, isoenzyme 4–4 (0.41 mg/mL), was incubated with 200 μM S-BDB-G in the absence or presence of 5 mM *S*-hexylglutathione. Incorporation was determined at the indicated time points as described under Experimental Procedures.

& Colman, 1984). The double-reciprocal plot shown in the inset of Figure 3 was used to calculate k_{max} = 0.078 min^{-1} and K_I = 66 μM.

Effect of Substrate Analogues on the Inactivation Rate of Glutathione S-Transferase by S-BDB-G. The effect of substrate analogues on the reaction rate of 200 μM S-BDB-G with glutathione S-transferase, isoenzyme 4–4, was investigated with the results shown in Table I. 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., 1989). Glutathione analogues with hydrophobic side chains of at least four carbons give complete protection against inactivation by S-BDB-G (Table I, lines 2–4). Less protection is provided by analogues with shorter side-chain length (Table I, lines 5–7). *S*-Methylglutathione and 2,4-dinitrophenol together yield greater protection against S-BDB-G than either substrate analogue does alone (Table I, lines 7–9). Hydrolyzed reagent provides substantial protection against inactivation by S-BDB-G (Table I, line 10). These data are consistent with the conclusion that the reaction of S-BDB-G occurs in the vicinity of the active site of the enzyme, probably in the region normally occupied by the electrophilic substrate.

Incorporation of S-BDB-G by Glutathione S-Transferase. Glutathione S-transferase, isoenzyme 4–4, was incubated with 200 μM S-BDB-G, in the absence or presence of 5 mM substrate analogue, *S*-hexylglutathione, as described under Experimental Procedures. The time-dependent incorporation of the [^{3}H]S-BDB-G into the 4–4 isoenzyme of glutathione S-transferase, in the absence of the substrate analogue, is shown in Figure 4 (line a). The maximum incorporation measured was 1.1 mol of reagent/mol of enzyme subunit when the enzyme was, on the average, 83% inactivated. These results indicate that the extent of reaction of S-BDB-G with the enzyme is limited. In the presence of the protecting substrate analogue, *S*-hexylglutathione (Figure 4, line b), the maximum incorporation was 0.48 mol of reagent/mol of subunit, and the enzyme retained all its activity.

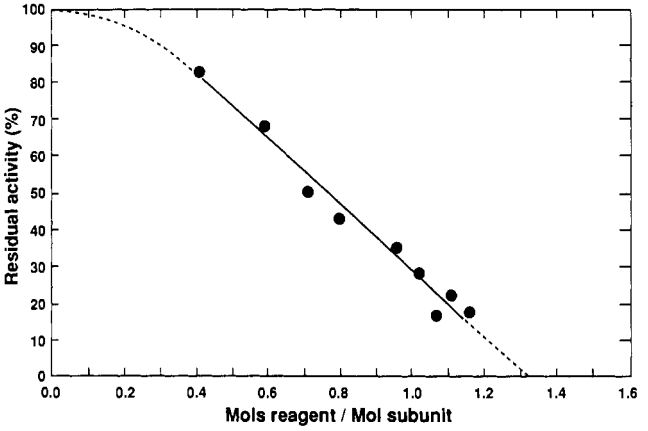

FIGURE 5: Residual activity as a function of incorporation for the inactivation of glutathione S-transferase by [^{3}H]S-BDB-G. The fraction of the maximum inactivation of glutathione S-transferase, isoenzyme 4–4, in the presence of 200 μM S-BDB-G (shown in Figure 2) is plotted vs incorporation (represented in Figure 4, line a). Extrapolation to 0% residual activity gives a maximum incorporation of 1.3 mol of reagent/mol of enzyme subunit.

Figure 5 shows the correlation between enzyme inactivation and incorporation of [^{3}H]S-BDB-G. The decrease in residual activity is directly related to the increase in reagent incorporation, suggesting that an amino acid at or near the active site of the enzyme is modified by the reagent. However, an incorporation of 0.41 mol of reagent/mol of subunit was measured at an early time in the reaction (t = 3 min) when the loss of enzyme activity was only 17%. Extrapolation to 0% residual enzyme activity gives an incorporation of 1.3 mol of reagent/mol of subunit. Both of these data suggest some additional reaction at an amino acid(s) not essential for enzymatic activity.

Isolation of Tryptic Peptides from Modified Glutathione S-Transferase. Glutathione S-transferase (1.7 mg/mL) was 78% inactivated by incubation for 60 min with 200 μM S-BDB-G at pH 6.5 and 25 °C. The modified enzyme was treated with $NaBH_4$, carboxymethylated, and then digested with trypsin as described under Experimental Procedures. The digest was fractionated using chromatography system I, as illustrated in Figure 6A,B. The four major radioactive peptide regions are designated Ia–d, II, III, and IV.

Glutathione S-transferase (1.7 mg/mL), incubated with 200 μM [^{3}H]S-BDB-G in the presence of 5 mM *S*-hexylglutathione to produce catalytically active modified enzyme, was digested with trypsin as described previously. The digest, fractionated by chromatography system I, is shown in Figure 6C. Only peaks Ia–d are present in the digests of both inactive (Figure 6B) and active (Figure 6C) enzymes, indicating that this labeled peptide is not necessary for enzymatic activity. Since peaks II, III, and IV are absent from the digest of active enzyme (Figure 6C), it appears that these peaks contain peptides important for enzymatic activity.

Characterization of Modified Peptides. Peaks Ia–d all contain the same peptide as indicated by the amino acid sequences given in Table II. The sequence Lys-His-Asn-Leu-X-Gly-Glu-Thr-Glu-Glu-Glu-Arg corresponds to residues 82–93 in the known amino acid sequence (Alin et al., 1986; Ding et al., 1986). The X at cycle 5 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^{86}). In the case of this peptide and all other S-BDB-G-modified peptides from glutathione S-transferase, isoenzyme 4–4, the radioactivity did not elute from the sequencer with

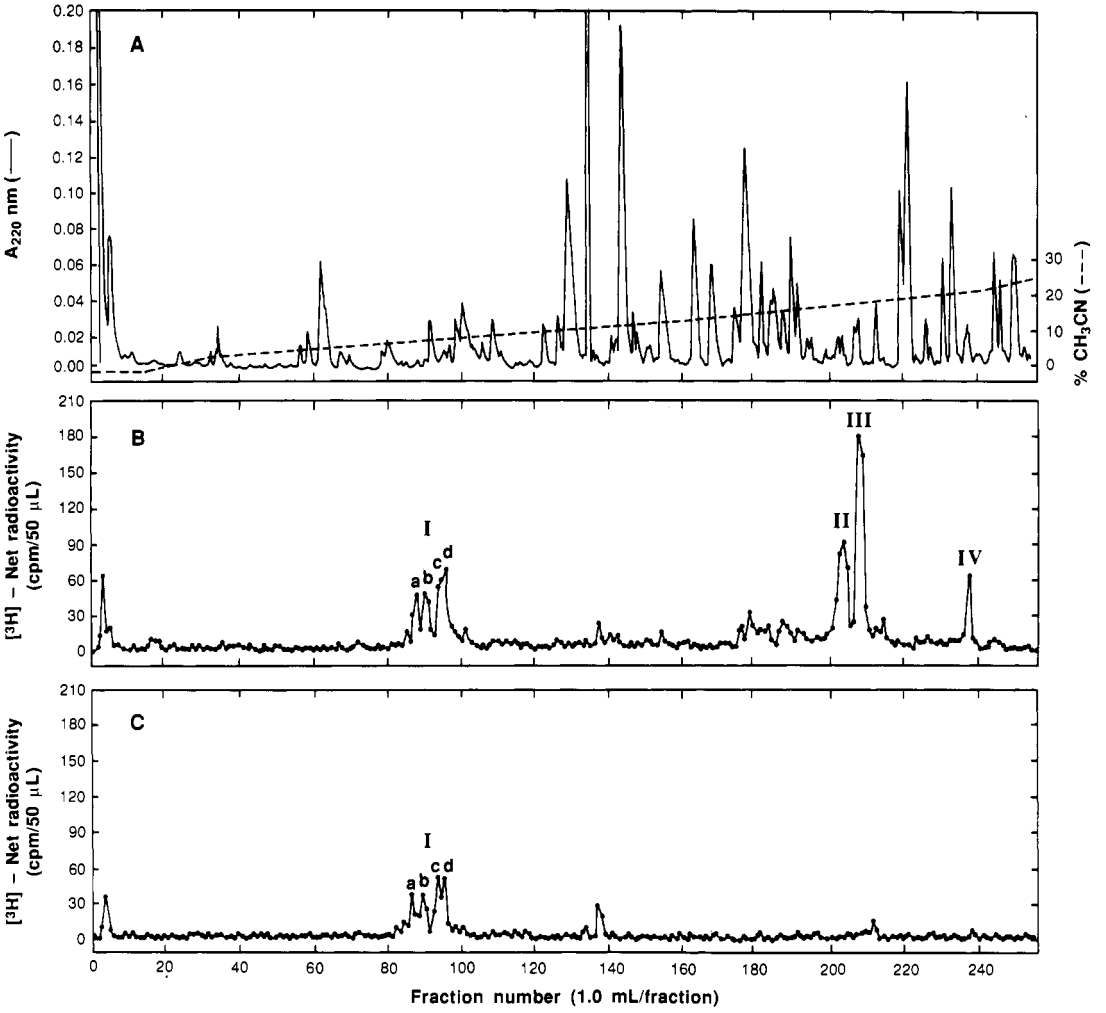

FIGURE 6: Fractionation of peptides by HPLC. Peptides resulting from the modification of glutathione S-transferase, isoenzyme 4–4, by 200 μM [^{3}H]S-BDB-G were separated by chromatography system I as described under Experimental Procedures. (A) A_{220} nm profile of the digest of modified enzyme prepared in the absence of *S*-hexylglutathione. (B) Distribution of radioactivity in the digest shown in (A). (C) Distribution of radioactivity in digest modified in the presence of 5 mM *S*-hexylglutathione. No significant amount of radioactivity was detected in regions of the chromatogram not shown.

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

	peak Ia			peak Ic		
cycle no.	amino acid (pmol)	amino acid (pmol)	peak Ib amino acid (pmol)	amino acid (pmol)	amino acid (pmol)	peak Id amino acid (pmol)
1	Lys (62)	His (17)	Lys (57)	Lys (35)	His (14)	Lys (86)
2	His (15)	Asn (45)	His (8)	His (50)	Asn (32)	His (29)
3	Asn (45)	Leu (20)	Asn (15)	Asn (55)	Leu (41)	Asn (74)
4	Leu (45)	X	Leu (80)	Leu (67)	X	Leu (77)
5	X	Gly (17)	X	X	Gly (5)	X
6	Gly (26)	Glu (15)	Gly (36)	Gly (13)	Glu (7)	Gly (46)
7	Glu (28)	Thr (7)	Glu (27)	Glu (12)	Thr (17)	Glu (64)
8	Thr (10)	Glu (40)	Thr (14)	Thr (20)	Glu (18)	Thr (18)
9	Glu (18)	Glu (18)	Glu (34)	Glu (12)	Glu (12)	Glu (56)
10	Glu (19)	Glu (19)	Glu (36)	Glu (13)	Glu (13)	Glu (61)
11	Glu (43)	Arg (20)	Glu (41)	Glu (33)	Arg[b]	Glu (54)
12	Arg (18)		Arg (3)	Arg (1)		Arg (5)

[a] The purification of peaks Ia–d was achieved by chromatography system I, and the amino acid sequences for these peptides were obtained as explained under Experimental Procedures. [b] Amount was too low to detect reliably.

any PTH-amino acid but remained bound to the filter.

Peaks Ia and Ic contain a mixture of two peptides which are the result of alternative cleavages by trypsin. Since, in the known amino acid sequence, an Arg is found in position 81, trypsin cleavage after either Arg^{81} or Lys^{82} yields a mixture of the amino acid sequence His-Asn-Leu-X-Gly-Glu-Thr-Glu-Glu-Glu-Arg and the sequence Lys^{82}–Arg^{93} noted above. Peaks Ib and Id contain only the peptide starting with Lys^{82} as a result of a single cleavage by trypsin.

Peaks Ia and Ib, when rechromatographed using chromatography system II, lose all their radioactivity. In contrast, peaks Ic and Id, when similarly rechromatographed, fractionate into two distinct peaks which retain their radioactivity (data not shown). These observations suggest that S-BDB-G can react to yield two types of products, one of which is stable in acid, while the second is not. The structure of S-BDB-G exhibits two types of reactive groups: the carbonyl moieties and the methylene bromide. The product formed between

Table III: Representative Sequences of Modified Peptides Present Only for Inactive Enzyme[a]

	peak IIa				peak IV	
cycle no.	amino acid (pmol)	amino acid (pmol)	peak IIb amino acid (pmol)	peak III amino acid (pmol)	amino acid (pmol)	amino acid (pmol)
1	Leu (76)	Lys (65)	Leu (85)	Leu (2290)	Leu (264)	X
2	Gln (82)	His (18)	Gln (70)	Gln (959)	Gln (218)	Leu (563)
3	Leu (52)	Asn (53)	Leu (74)	Leu (1270)	Leu (245)	Asp (224)
4	Ala (67)	Leu (56)	Ala (69)	Ala (1270)	Ala (369)	Ala (369)
5	Met (55)	X	Met (69)	Met (1750)	Met (189)	Phe (385)
6	Val (40)	Gly (32)	Val (51)	Val (972)	Val (122)	Pro (277)
7	X	Glu (33)	X	CmCys (487)	X	Asn (257)
8	X	Thr (34)	X	X	X	Leu (286)
9	Ser (44)	Glu (28)	Ser (57)	Ser (515)	Ser (124)	Lys (177)
10	Pro (26)	Glu (28)	Pro (36)	Pro (464)	Pro (72)	Asp (54)
11	Asp (22)	Glu (26)	Asp (36)	Asp (540)	Asp (54)	Phe (102)
12	Phe (24)	Arg (2)	Phe (30)	Phe (471)	Phe (104)	Val (83)
13	Glu (18)		Glu (27)	Glu (361)	Glu (45)	Ala (45)
14	Arg (4)		Arg (7)	Arg (578)	Arg (10)	Arg (10)
moles of reagent/ moles of peptide[b]	0.73		1.2	0.87	1.2	

[a] The purification of Peaks IIa and IIb was achieved by chromatography system III; the purification of Peaks III and IV by chromatography system I. The amino acid sequences for these peptides were obtained as explained under Experimental Procedures. These are representative sequences and were not taken from the same digests of modified enzyme. Therefore, the amounts of the peptides from the several enzyme samples do not reflect the relative magnitude of the peaks shown in Figure 6. [b] Moles of reagent was calculated on the basis of radioactivity that was applied to the sequencer; moles of peptide was the average of the picomoles of sample from the first five cycles of the sequencer.

S-BDB-G and the Lys^{82}–Arg^{93} peptide, which is acid labile, most likely results from reaction of Cys^{86} at a carbonyl of the reagent (Ozturk & Colman, 1991).[2] The acid-stable species apparently results from displacement by Cys^{86} of the bromide. The four peaks Ia–d differ, therefore, as a consequence of either differences in trypsin cleavage patterns or in the reaction product of the peptide and S-BDB-G.

Peak III (the largest radioactive peak, Figure 6B) contains the peptide indicated by the amino acid sequence given in Table III. The sequence Leu-Gln-Leu-Ala-Met-Val-CmCys-X-Ser-Pro-Asp-Phe-Glu-Arg corresponds to residues 108–121 in the known amino acid sequence. The X in cycle 8, where no phenylthiohydantoin derivative was detected, corresponds to a tyrosine in the known amino acid sequence. The ratio of moles of reagent per mole of peptide of peak III applied to the amino acid sequencer (Table III) is approximately 1 (0.87). These results suggest that this peptide is modified by S-BDB-G at only Tyr^{115}.

Peak II (Figure 6B), when rechromatographed using chromatography system III, yields two radioactive peaks designated IIa and IIb. Peak IIa contains two peptides, present in approximately equal amounts, as shown in Table III. One of these two amino acid sequences (Lys^{82}–Arg^{93}) corresponds to that of peak I which contains Cys^{86}; the second amino acid sequence (Leu^{108}–Arg^{121}) is the same as that of peak III which contains Tyr^{115} in the known amino acid sequence. Examination of the sequence of the two peptides in peak IIa shows: they are present in a 1:1 ratio; Cys^{114} is not carboxymethylated; and Tyr^{115} yields no recognizable phenylthiohydantoin derivative. Since the ratio of moles of reagent per mole of peptide applied to the amino acid sequencer for this run was approximately 1 (0.73), the data indicate that Tyr^{115} is the amino acid labeled by the reagent but suggests that a secondary oxidation of Cys^{86} and Cys^{114} occurs yielding a disulfide cross-link.

[2] An alternate possibility, that the labile product is an arginine derivative, is unlikely for the following reasons: the peptides found in peaks Ia and Ib end in arginine residues and trypsin would not be expected to hydrolyze a peptide bond following a modified arginine; and, no (carboxymethyl)cysteine is detected at the sequencing cycle corresponding to Cys^{86}, making it most probable that this cysteine is modified by S-BDB-G.

Peak IIb, as shown in Table III, has the same amino acid sequence as that of peak III but lacks the carboxymethylated cysteine. The ratio of moles of reagent per mole of peptide applied to the amino acid sequencer for this run was approximately 1 (1.2), suggesting that when the Tyr^{115} is modified by S-BDB-G, Cys^{114} is less accessible for carboxymethylation.

Peak IV (Figure 6B) contains two peptides, as shown in Table III. The sequence of one of the peptides corresponds to that of peak III which contains Tyr^{115}. The second peptide has the sequence X-Leu-Asp-Ala-Phe-Pro-Asn-Leu-Lys-Asp-Phe-Val-Ala-Arg which corresponds to residues 173–186 in the known amino acid sequence. The X in cycle 1, in which no phenylthiohydantoin derivative was detected, corresponds to Cys^{173} in the known amino acid sequence. Examination of the two peptides in peak IV (Table III) shows results similar to those for peak IIa: the two peptides are present in a 1:1 ratio; Cys^{114} is not carboxymethylated; and Tyr^{115} yields no detectable phenylthiohydantoin derivative. In this case, the ratio of moles of reagent per moles of peptide applied to the amino acid sequencer was also approximately 1 (1.2). As in the case of peak IIa, the data indicate that Tyr^{115} is the amino acid labeled by S-BDB-G but suggest that a secondary oxidation of Cys^{114} and Cys^{173} yields the disulfide cross-linked peptide of peak IV.

The amount of [^{3}H]S-BDB-G incorporated into peaks I–IV can be determined by multiplying the percentage of the total radioactive peptide peaks in Figure 6B by the amount of reagent incorporated into enzyme at 60 min (1.1 mol of reagent/mol of subunit). By this method of calculation, peaks Ia–d represent 0.4 mol of reagent/mol of subunit incorporated into the [^{3}H]S-BDB-G-labeled peptides at 60 min. Peak II contains 0.26 mol of reagent/mol of subunit, 0.07 mol of which, when calculated from the distribution of radioactivity between peaks IIa and IIb resolved using chromatography system III (data not shown), can be attributed to peak IIa. Peak III represents 0.37 mol of the radioactive reagent/mol of subunit, while peak IV contains 0.07 mol of reagent/mol of subunit. The radioactivity of peaks II, III, and IV together represent 0.70 mol of reagent/mol of subunit incorporated by the enzyme at 60 min. The amount of radioactive reagent incorporated exclusively into the cross-linked peptides in which

interaction between cysteinyl residues is indicated (peak IIa and peak IV) totals only 0.14 mol. These results suggest that it is the reaction of [^{3}H]S-BDB-G at Tyr^{115} and not the secondary reactions between cysteines which is responsible for the measured 78% inactivity of the enzyme at 60 min.

In addition, when the period of incubation between the enzyme and S-BDB-G was extended to 120 min (data not shown), peak III (which contains peptide 108–121 including CmCys) decreased while peak IIb (which contains peptide 108–121 but without CmCys) increased. At both 60 and 120 min, the inactivation of the enzyme remains at 78%. This observation supports the postulate that the absence of CmCys (indicative of the modification of Cys) is a secondary phenomenon not directly related to the inactivation of the enzyme.

Discussion

S-(4-Bromo-2,3-dioxobutyl)glutathione demonstrates the characteristics of an affinity label in its reaction with rat liver glutathione S-transferase, isoenzyme 4–4. It reacts covalently with a limited number of sites on the enzyme: only 1.1 mol of reagent/mol of subunit is incorporated when the enzyme is 83% inactivated. The rate constant of the reaction of S-BDB-G with glutathione S-transferase exhibits a nonlinear dependence on reagent concentration, indicating the formation of a reversible enzyme–reagent complex prior to irreversible modification; at higher concentrations of the reagent, the rate constant becomes independent of reagent concentration. Substrate analogues, especially glutathione analogues with hydrophobic side chains of at least four carbons, are effective in protecting against enzyme inactivation.

From the tryptic digest of modified enzyme, two peptides containing covalently linked S-BDB-G have been isolated. One of these peptides contains residues 108–121 in the known amino acid sequence of rat liver glutathione S-transferase, isoenzyme 4–4 (Alin et al., 1986; Ding et al., 1986). Reaction at this peptide with S-BDB-G is prevented by the presence of the substrate analogue *S*-hexylglutathione, indicating that it is at or near the active site of the enzyme. From the lack of a recognizable phenylthiohydantoin derivative in the amino acid sequence analysis, Tyr^{115} appears to be modified by S-BDB-G in this peptide. Although the labeled Tyr^{115} is found in several peptide forms (Figure 6, peaks II–IV), the extent of inactivation correlates only with the total amount of modified Tyr^{115}. The second peptide modified by S-BDB-G contains residues 82–93. From amino acid sequence analysis, the residue modified by S-BDB-G in this peptide is Cys^{86}. Since this radioactively labeled peptide is found both in the presence and in the absence of the substrate analogue *S*-hexylglutathione when the enzyme is respectively catalytically active or inactivated, it can be concluded that modification of Cys^{86} does not cause inactivation.

Previous studies (Van Ommen et al., 1988, 1989) on the chemical modification of the rat liver isoenzyme 4–4 by tetrachloro-1,4-benzoquinone and its glutathione conjugate led the investigators to conclude that inactivation was caused by reaction with cysteine residues in or close to the active site. However, these residues were not identified within the sequence of the enzyme. Furthermore, Hsieh et al. (1990) and Zhang and Armstrong [P. Zhang and R. N. Armstrong, quoted in Armstrong (1991)] showed that the substitution of serines for Cys^{86}, Cys^{114}, or Cys^{173} yields mutant glutathione S-transferases which are catalytically functional, thereby indicating that the three cysteines are not essential for enzymatic activity. The results from the present study with S-BDB-G showing that modification of Cys^{86} does not cause inactivation support the conclusions drawn from the site-specific mutagenesis experiments. In no chemical modification or site-directed mutagenesis studies with the 4–4 isoenzyme published to date has Tyr^{115} been reported to be important for enzymatic activity.

Possible roles for the important Tyr^{115} labeled by S-BDB-G must be considered. The active site of glutathione S-transferase contains two binding sites: one specific for glutathione and a second, hydrophobic site more tolerant of structural variations in electrophilic substrates (Mannervik & Danielson, 1988; Jakobson et al., 1979). Mechanistic studies of the glutathione S-transferases suggest that the thiolate ($E–GS^-$) is the predominant ionic state in the binary complex formed between glutathione and the electrophilic substrate (Graminski et al., 1989). The pK_a of the bound glutathione has been determined to be in the range 6.4–6.7, substantially below the pK_a for glutathione in aqueous solution (9.0) (Chen et al., 1988), indicating that one function of the enzyme may be to lower the pK_a of the bound thiol of glutathione. It has been suggested that this is accomplished by the existence of a positively charged electrostatic field in the active site of the enzyme (Armstrong, 1991) or by shielding of the glutathione thiolate from solvent when it is enzyme-bound (Armstrong, 1991; Huskey et al., 1991). Additional stabilization of the glutathione thiolate may be provided by hydrogen bonding to Tyr^{115}. However, the results of this study show that protection against enzyme inactivation by S-BDB-G increases with length of the hydrophobic side chain of glutathione analogues. This result suggests that the critical Tyr^{115} is located in the hydrophobic substrate binding portion of the enzyme's active site, rather than directly within the glutathione binding site. It seems most likely, then, that the major function of Tyr^{115} is to facilitate the binding of the non-glutathione substrate by hydrophobic interactions.

Tyrosine is conserved in position 115 of the amino acid sequence of the μ class of glutathione S-transferase isoenzymes. When representative sequences of the subunits for the α class (rat 1, 2) and π class (rat 7, human p) of isoenzymes of glutathione S-transferase are aligned by the Clustal program (PC Gene, IntelliGenetics), the only non-tyrosine residue found in position 115 of the amino acid sequence is isoleucine in the rat 1 subunit. These sequence comparisons support the idea that tyrosine's importance to the enzyme's function may be in its hydrophobic character which facilitates the binding of the non-glutathione substrates.

S-(4-Bromo-2,3-dioxobutyl)glutathione has been used in this study to modify a tyrosine residue at or near the active site of rat liver glutathione S-transferase, isoenzyme 4–4. As a glutathione analogue which also contains a reactive bromodioxobutyl group, S-BDB-G may be an effective affinity label for other enzymes which contain a glutathione binding site such as glyoxylase (Creighton & Pourmotabbed, 1988), γ-glutamylcysteine synthetase, glutathione synthetase and γ-glutamyltranspeptidase (Meister & Anderson, 1983).

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References

Alin, P., Mannervik, B., & Jörnvall, H. (1986) *Eur. J. Biochem. 156*, 343–350.

Armstrong, R. N. (1987) *CRC Crit. Rev. Biochem. 22*, 39–88.

Armstrong, R. N. (1991) *Chem. Res. Toxicol. 4*, 131–140.

Benson, A. M., Hunkeler, M. J., & York, J. L. (1989) *Biochem. J. 261*, 1023–1029.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248-254.
Chasseaud, L. F. (1979) *Adv. Cancer Res. 29*, 175-274.
Chen, W.-J., Graminski, G. F., & Armstrong, R. N. (1988) *Biochemistry 27*, 647-654.
Colman, R. F. (1989) in *Protein Structure and Function* (Creighton, T. E., Ed.) pp 77-99 IRL Press, New York.
Colman, R. F., Huang, Y.-C., King, M. M., & Erb, M. (1984) *Biochemistry 23*, 3281-3286.
Creighton, D. J., & Pourmotabbed, T. (1988) in *Molecular Structure and Energetics: Mechanistic Principles of Enzyme Activity* (Liebman, J. F., & Greenberg, A., Eds.) pp 353-386, VCH Publishers, New York.
Ding, G. J.-F., Ding, V. D.-H., Rodkey, J. A., Bennett, C. D., Lu, A. Y. H., & Pickett, C. B. (1986) *J. Biol. Chem. 261*, 7952-7957.
Graminski, G. G., Kubo, Y., & Armstrong, R. N. (1989) *Biochemistry 28*, 3562-3568.
Habig, W. H., Pabst, M. J., & Jakoby, W. B. (1974) *J. Biol. Chem. 249*, 7130-7139.
Hsieh, J.-D., Huang, S.-C., Chen, W.-L., Lai, Y.-C., & Tam, M. F. (1990) *The Protein Society Fourth Symposium*, August 1990, San Diego, CA.
Huang, Y.-C., & Colman, R. F. (1984) *J. Biol. Chem. 259*, 12481-12488.
Huskey, S.-E., Huskey, W. P., & Lu, A. Y. H. (1990) *J. Am. Chem. Soc. 113*, 2283-2290.
Jakobson, I., Warholm, M., & Mannervik, B. (1979) *J. Biol. Chem. 254*, 7085-7089.
Jakoby, W. B. (1977) *Adv. Enzymol. Relat. Areas Mol. Biol. 46*, 383-414.
Katusz, R. M., & Colman, R. F. (1990) *The Protein Society Fourth Symposium*, August 1990, San Diego, CA.
Listoswky, I., Abramovitz, M., Homma, H., & Niitsu, Y. (1988) *Drug Metab. Rev. 19*, 305-318.
Mannervik, B. (1985) *Adv. Enzymol. Relat. Areas Mol. Biol. 56*, 357-417.
Mannervik, B., & Danielson, U. H. (1988) *CRC Crit. Rev. Biochem. 23*, 283-337.
Meister, A., & Anderson, M. E. (1983) *Annu. Rev. Biochem. 52*, 711-760.
Mokrasch, L. C. (1967) *Anal. Biochem. 18*, 64-71.
Ozturk, D. H., & Colman, R. F. (1991) *Biochemistry 30*, 7126-7134.
Penefsky, H. S. (1979) *Methods Enzymol. 56*, 527-530.
Pickett, C. B., & Lu, A. Y. H. (1989) *Annu. Rev. Biochem. 58*, 743-764.
Van Ommen, B., den Besten, C., Rutten, A. L. M., Ploemen, J. H. T. M., Vos, R. M. E., Muller, F., & Van Bladeren, P. J. (1988) *J. Biol. Chem. 263*, 12939-12942.
Van Ommen, B., Ploemen, J. H. T. M., Ruven, H. J., Vos, R. M. E., Bogaards, J. J. P., Van Berkel, W. J. H., & Van Bladeren, P. J. (1989) *Eur. J. Biochem. 181*, 423-429.

Chromium(III) Bound to DNA Templates Promotes Increased Polymerase Processivity and Decreased Fidelity during Replication in Vitro†

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ABSTRACT: Carcinogenic chromium [Cr(VI)] compounds are reduced intracellularly to DNA- and protein-reactive chromium(III) species. However, the role of Cr(III) ions in chromium-induced genotoxicity remains unclear. We have investigated the effects of chromium(III) binding on DNA replication and polymerase processivity in vitro. Chromium ions bind slowly and in a dose-dependent manner to DNA. Micromolar concentrations of free chromium inhibit DNA replication, but if the unbound chromium is removed by gel filtration, the rate of DNA replication by polymerase I (Klenow fragment) on the chromium-bound template is increased greater than 6-fold relative to the control. This increase is paralleled by as much as a 4-fold increase in processivity and a 2-fold decrease in replication fidelity. These effects are optimum when very low concentrations of chromium ions are bound to the DNA [3-4 Cr(III) ions per 1000 nucleotide phosphates]. Increased concentrations of chromium lead to the production of DNA-DNA cross-links and inhibition of polymerase activity. These results suggest that low levels of DNA-bound chromium(III) ions may contribute to chromium mutagenesis and carcinogenesis by altering the kinetics and fidelity of DNA replication.

Chromate is one of the best documented human and animal carcinogens. Occupational exposure to chromium compounds has been widespread, and many studies of the biological effects of chromium have been conducted (Stern, 1982; Bianchi et al., 1983; De Floro & Wetterhahn, 1989). However, because of its complex intracellular metabolism, molecular mechanisms of chromium-induced genotoxicity are not thorougly understood.

Chromium exists in a number of oxidation states, of which only Cr(VI) and Cr(III) are environmentally stable (Goyer, 1986). While chromium(VI), as chromate, is biologically active, because it is taken up by cells, it is rapidly reduced through relatively unstable Cr(V) and Cr(IV) intermediates to kinetically stable Cr(III) species (Arslan et al., 1987). Because Cr(VI) is cellularly reduced, numerous studies have attempted to determine which intracellular form of chromium is the ultimate carcinogenic and/or mutagenic species (Lofroth, 1978; Macrae et al., 1979; Whiting et al., 1979). While the

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