Benzyl isothiocyanate (BITC), present in cruciferous vegetables, is an efficient substrate of human glutathione S-transferase P1-1 (hGST P1-1). BITC also acts as an affinity label of hGST P1-1 in the absence of glutathione, yielding an enzyme inactive toward BITC as substrate. As monitored by using BITC as substrate, the dependence of $k$ of inactivation ($K_i$) of hGST P1-1 on [BITC] is hyperbolic, with $K_i = 66 \pm 7 \mu M$. The enzyme incorporates 2 mol of BITC/mol of enzyme subunit upon complete inactivation. S-Methylglutathione and 8-anilino-1-naphthalene sulfonate (ANS) each yield partial protection against inactivation and decrease reagent incorporation, whereas S-(N-benzylthiocarbamoyl)glutathione or S-methylglutathione + ANS protects completely. Mapping of proteolytic digests of modified enzyme by using mass spectrometry reveals that Tyr$^{103}$ and Cys$^{47}$ are modified equally. S-Methylglutathione reduces modification of Cys$^{47}$, indicating this residue is at/near the glutathione binding region, whereas ANS decreases modification of Tyr$^{103}$, suggesting this residue is at/near the BITC substrate site, which is also near the binding site of ANS. The Y103F and Y103S mutant enzymes were generated, expressed, and purified. Both mutants handle substrate 1-chloro-2,4-dinitrobenzene normally; however, Y103S exhibits a 30-fold increase in $K_m$ for BITC and binds ANS poorly, whereas Y103F has a normal $K_m$ for BITC and $K_a$ for ANS. These results indicate that an aromatic residue at position 103 is essential for the binding of BITC and ANS. This study provides evidence for the existence of a novel xenobiotic substrate site in hGST P1-1, which can be occupied by benzyl isothiocyanate and is distinct from that of monobromobimane and 1-chloro-2,4-dinitrobenzene.

**Glutathione S-transferases (GSTs)**, EC 2.5.1.18), are a family of multifunctional enzymes that participate in the biotransformation of xenobiotics by catalyzing the addition of the tripeptide GSH to various electrophilic compounds (1, 2). Other GST functions include detoxification of lipid and DNA hydroperoxides by their intrinsic peroxidase activity (2), isomerization of certain steroids, and intracellular transport of various hydrophobic nonsubstrate ligands such as bile acids, bilirubin, and a number of drugs (1).

GSTs are distributed in a wide range of organisms from mammals to *Escherichia coli* (3). The catalytic diversity of mammalian cytosolic GSTs, which can exist as homo- or heterodimers, arises in part from the existence of at least eight distinct classes (named Alpha, Kappa, Mu, Omega, Pi, Sigma, Theta, and Zeta) (1, 4). The glutathione-binding site (G-site) and the catalytic mechanism of these enzymes have been the targets of many investigations involving chemical modification (5–8), site-directed mutagenesis (9–14), and x-ray crystallographic analysis (15–21). On the contrary, the electrophilic substrate-binding site (H-site) of GSTs is more complex and is class-specific. Crystallographic studies have shown that the H-site is quite dissimilar among different classes of GSTs (15–21). For example, in GST M1-1, the H-site is a hydrophobic cavity, whereas in GST P1-1 (15), the H-site is half-hydrophobic and half-hydrophilic with functionally important water molecules (16). Only a few amino acid residues have been identified in the H-site of GSTs, and relatively little is known about the key determinants of xenobiotic substrate specificity.

Among the various GSTs, the Pi class (GST P1-1) has attracted attention because it is overexpressed in a variety of malignancies, including lung, colon, ovary, esophagus, and stomach cancers (22–25). Furthermore, a number of studies have shown a correlation between overexpression of GST P1-1 and the development of resistance toward various anti-cancer drugs, such as Adriamycin, cisplatinum, melphalan, and etoposide in resistant tumor cells (22–25). The task of defining the xenobiotic substrate specificity of Pi class GST becomes a critical component in the rational design of highly potent GST Pi-selective inhibitors that may increase the effectiveness of commonly used anti-cancer agents. Previously, we showed there is a site for monobromobimane in the xenobiotic region of GST Pi distinct from that occupied by 1-chloro-2,4-dinitrobenzene, supporting the concept that this isozyme accomplishes its ability to react with a diversity of substrates in part by harboring distinct xenobiotic substrate sites (26).

Benzyl isothiocyanate (BITC), found abundantly in cruciferous vegetables (e.g. cabbage, cauliflower, and broccoli), contains a central carbon that can undergo facile addition reactions with $N$-, $O$-, or S-based nucleophiles, including the sulphydryl group of glutathione (27–29). It has been shown that BITC is a substrate of GSTs, with the Pi class being the most efficient catalyst (28). Consequently, locating its binding site in GST Pi should further contribute to the understanding of substrate specificity in relation to other GSTs. In this paper, we report the results of the affinity labeling of human GST P1-1 using BITC. Covalent modification of hGST P1-1 by BITC causes differential loss of activity toward two substrates, suggesting the existence of a novel binding site for xenobiotic substrates in the Pi isozyme distinct from that of monobromo-

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† To whom correspondence should be addressed. Tel.: 302-831-2973; Fax: 302-831-6335; E-mail: rfcollman@chem.udel.edu.
‡ The abbreviations used are: GST, glutathione S-transferase; hGST P1-1, human Pi class glutathione S-transferase; BITC, benzyl isothiocyanate; CDNB, 1-chloro-2,4-dinitrobenzene; mBr, monobromobimane; ANS, 8-anilino-1-naphthalene sulfonate; HPLC, high performance liquid chromatography; BSP, bromosulphphthalein; DTT, dithiothreitol; PDB, Protein Data Bank.

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*This paper is available on line at http://www.jbc.org*
bimane and 1-chloro-2,4-dinitrobenzene. A preliminary version of this work has been presented (30).

**EXPERIMENTAL PROCEDURES**

**Materials**—BITC, Sephadex G-50, 2,4-dinitrophenoephthalein, glutathione, 1-chloro-2,4-dinitrobenzene (CDNB), S-hexylglutathione, S-methylglutathione, S-b-nitrobenzylglutathione, bromosulfonylphthalein, 8-anilino-1-naphthalene sulfonate (ANS), N-ethylmaleimide, and ammonium bicarbonate were all obtained from Sigma. Monobromobimane (mBBR) was obtained from Molecular Probes. Trifluoroacetic acid was purchased from Aldrich. Oligonucleotides for mutagenesis and primers for DNA sequencing were purchased from BioSynthesis, Inc. Dye reagent concentrate was purchased from Bio-Rad. All other chemicals were of reagent grade.

**Plasmids and Mutagenesis**—The full-length cDNA for human glutathione S-transferase P1-1 was encoded in a pUC120 plasmid, as described by Manoharan et al. (31), and was a gift from W. E. Fahl (University of Wisconsin, Madison). Site-directed mutagenesis was performed using the Stratagene QuikChange kit. The following oligonucleotides and their complements were used to incorporate the mutations (position of the mutation underlined): Y103F, 5'-CTC CGC TGC AAA TTC ATC TGC TCC ACC ACC Y103SS, 5'-CTC ATC TGC AAA TTC ATC TGC TCC ACC ACC. Mutations were confirmed by DNA sequencing. Other plasmids were purchased from the University of Delaware Center for Agricultural Biotechnology using an ABI Prism model 377 DNA sequencer (PE Biosystems).

**Protein Purification**—GST P1-1 was expressed in E. coli JM109. Cells were grown at 37 °C, and when the A600 reached 0.4, the cells were induced with 1 mM isopropyl thio-

**Measurement of GST Activity**—Cells were grown at 37 °C, and when the A600 reached 0.4, the cells were induced with 1 mM isopropyl thio-

**Other Substrates Utilized to Assay for Enzymatic Activity Were BITC and mBBR**—For BITC, enzymatic activity was measured in a total volume of 1.0 ml using a Hewlett-Packard 8450 spectrophotom-

**Enzyme Assays**—Enzymatic activity toward CDNB was measured in a total volume of 1.0 ml using a Hewlett-Packard 8450 spectrophotom-

**Synthesis of S-(N-Benzylthiocarbamoyl)glutathione**—S-(N-Benzylthiocarbamoyl)glutathione was prepared by reaction of BITC with an equivalent concentration of glutathione. In a typical reaction: GST P1-1 was expressed in E. coli JM109. Cells were grown at 37 °C, and when the A600 reached 0.4, the cells were induced with 1 mM isopropyl thio-

**Measurement of Incorporation of BITC into hGST P1-1**—BITC and its amino acid conjugates display an absorption peak at 245 nm, which is not present in the spectrum of the precursor glutathione. The extinction coefficient was determined from a range of concentrations (4–400 μM) at a con-

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**Reaction of hGST P1-1 with BITC**—hGST P1-1 (0.3 mg/ml) was incubated in 90 mM phosphate buffer (pH 6.5) at 25 °C with various concentrations of BITC by the addition of appropriate stock solutions of BITC in acetonitrile. The concentration of BITC was determined from the absorbance at 245 nm using ε245 = 10,780 M⁻¹ cm⁻¹. The volume of acetonitrile was maintained at 10% of the total volume of the reaction mixture. In control experiments, the enzyme was incubated under the same conditions including 10% acetonitrile but without BITC. In every case, aliquots of the reaction mixture were removed at specified times and assayed for enzymatic activities using either CDNB or BITC as substrate. For both assays, aliquots of the reaction mixture were diluted 10-fold with 0.1 M potassium phosphate buffer (pH 6.5) at 25 °C and were assayed by the addition of 20 μl to the cuvette. The rate constants for reaction of the enzyme with mBBR were calculated from semi-logarithmic plots of E(t)/E₀ versus time, in accordance with the pseudo-first-order kinetic equation: ln(E'/E₀) = -k₁t, where E₀ is the activity of the enzyme at time 0; E represents the activity at a given time (t); and k₁ is the observed pseudo first-order rate constant.

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reaction conditions. Excess reagents were removed by gel filtration columns, and the protein concentration was determined by the Bio-Rad method, as described above. The amount of reagent incorporated was determined from the absorbance at 245 nm using \( e_{245\ nm} = 10,850 \frac{M^{-1} \ cm^{-1}}{M} \).

**Preparation of Modified hGST P1-1 for Kinetic Studies**—hGST P1-1 (0.3 mg/ml) was incubated for 40 min with 100 \( \mu \)M BITC in the presence of 5 mM S-methylglutathione. Excess reagents were removed by gel filtration, and the protein concentration was determined by the Bio-Rad dye-binding method. The prepared modified enzyme was frozen quickly and stored at \(-80^\circ\text{C}\).

**Measurement of ANS Binding to hGST P1-1**—Equilibrium binding studies with ANS were determined by measuring the enhancement of ANS fluorescence when the ligand (0–900 \( \mu \)M) binds to native and modified wild-type hGST P1-1, Y103F, and Y103S mutant enzymes (16 \( \mu \)M), as described by Bico et al. (36). Excitation was at 390 nm and emission at 460 nm. Fluorescence measurements were made in 0.1 M phosphate buffer (pH 6.5) at 25 \( ^\circ\text{C}\).

**Trypsin Digestion of Modified hGST P1-1**—hGST P1-1 (0.3 mg/ml) was incubated for 40 min with 100 \( \mu \)M BITC with or without the addition of ligands under standard reaction conditions. Excess reagent was removed by gel filtration as described above. Solubilized (to give 6 \( \mu \)M as the final concentration) and N-ethylmaleimide (final concentration, 10 mM) was added to the enzyme and incubated for 30 min at 25 \( ^\circ\text{C}\). The enzyme solution was then dialyzed overnight against 90 mM ammonium bicarbonate (pH 7.8) (4 liters).

The enzyme solution was lyophilized and then resolubilized in 250 \( \mu \)l of 8 \( \mu \)M urea in 50 mM ammonium bicarbonate (pH 7.8). This solution was incubated at 37 \( ^\circ\text{C}\) for 2 h to denature the protein. Ammonium bicarbonate (750 \( \mu \)l, 50 mM (pH 7.8)) was then added to the solution to dilute the urea to 5 \( \mu \)M. Trypsin (5\% w/w) was added, and the enzyme sample was incubated for 2 h at 37 \( ^\circ\text{C}\). A second aliquot of the trypsin solution was added, and incubation was continued for another 2 h at 37 \( ^\circ\text{C}\).

**HPLC Separation of Modified Peptides**—The trypsic digest was injected onto a Varian 5000 LC HPLC (Varian, Walnut Creek, CA) equipped with a Phenomenex C18 reverse-phase column (0.46 \( \times \) 25 cm). At a flow rate of 1 ml/min, the peptides were separated by a 300-min gradient to 80\% solvent B, and finally a 20-min gradient to 100\% solvent B. The eluate was monitored at 220 and 245 nm with 1-ml fractions collected. Peaks I and II from the trypsin digest were pooled separately, lyophilized, and redissolved in 1 ml of 20 mM ammonium bicarbonate (pH 7.8). A second aliquot of trypsin (5\% w/w) was added, and the enzyme sample was incubated for 2 h at 37 \( ^\circ\text{C}\). A second aliquot of the trypsin solution was added, and incubation was continued for another 2 h at 37 \( ^\circ\text{C}\).

**Analysis of Isolated Peptides**—The purified labeled peptides from the HPLC were hydrolyzed, and the peptides were hydrolyzed and redissolved in 0.1\% trifluoroacetic acid/H\( _2\)O. Peptide molecular weights were determined using a ThermoFinnigan model LCQ mass spectrometer equipped with an electrospray attachment.

**Molecular Modeling**—Modeling was conducted using the program Insight II from Biosym Technologies on a Silicon Graphics work station. The molecular models of BITC and bromosulfophthalein were built and energy-minimized using the Builder module of the Insight II program. The approach of the BITC molecule to Tyr\(^{202}\) was modeled by positioning BITC at a site close to Tyr\(^{203}\) and the bromosulfophthalein site of human GST Pi (PDB codes 19GS (47) and 18GS (48)) by sequentially rotating and translating it along the \( x, y, \) and \( z \) axes. The intermolecular energy in terms of both van der Waals’ and electrostatic interactions as well as the interatomic distance between the oxygen atom of Tyr\(^{203}\) and the reactive central carbon atom of BITC were continuously monitored for conformations, with reasonable distances and potential energies constituting possible productive interactions for chemical modification of the tyrosyl hydroxyl group. The molecular model of the enzyme modified by BITC at Tyr\(^{203}\) was built and energy-minimized using the Builder and Homology modules of the Insight II program. Another possible configuration for the molecule was constructed by rotating BITC so that the reactive central carbon atom would face the thiol group of glutathione. The BITC was also docked at a site close to Cys\(^{47}\) of hGST P1-1. The manually docked complex as well as BITC-modified enzyme models were submitted to the Discover\(^{\circ}\) program from Biosym for extensive energy minimization using steepest descent and conjugate gradient methods to relieve residual van der Waals’ overlaps and optimize the structures.

**RESULTS**

**BITC as a Substrate for Human GST P1-1**—The formation of the glutathione conjugate with BITC is catalyzed by hGST P1-1, as determined by Kolm et al. (28). As part of this study, kinetic parameters for the enzymatic reaction have been confirmed, and the catalysis follows normal Michaelis-Menten kinetics. In hGST P1-1, the apparent \( K_m \) values for BITC and CDNB were 68 ± 5 and 650 ± 39 \( \mu \)M, respectively, and the apparent \( V_{max} \) values were 70 ± 4 and 65 ± 6 \( \mu \)mol/min/mg, respectively. Thus, the enzyme has a higher apparent affinity for BITC than for CDNB, the more traditional substrate for GST, but the two substrates have similar \( V_{max} \) values.

Because benzyl isothiocyanate is similar in structure to phenyl isothiocyanate, which is used in N-terminal polypeptide sequencing, the reaction of BITC with the tripeptide glutathione may be considered to involve either the free N-terminal group or the thiolate group. To address this question, hGST P1-1 was added to 0.1 M phosphate buffer at pH 6.5 containing 400 \( \mu \)M BITC and 450 \( \mu \)M S-methylglutathione, a glutathione derivative which has its thiolate group blocked. No formation of product was detected spectrophotometrically at 274 nm. We conclude that BITC must react with the thiolate of GSH under these conditions.

Previously, we found that S-(hydroxyethyl)bisulfonate functions as a competitive inhibitor of monobromobisulfonate (\( K_I = 56 \mu \)M) (26). We have now found that S- (hydroxyethyl)bisulfonate does not function as an inhibitor when BITC is the substrate, indicating BITC and mBBr occupy distinct substrate sites of hGST P1-1. This conclusion is based on the observation that addition of 50 or 100 \( \mu \)M of S- (hydroxyethyl)bisulfonate does not change either the \( K_m \) or \( V_{max} \) value for BITC. The nonsubstrate ligand ANS has been shown to function as a noncompetitive inhibitor with respect to other substrates (36), but its ability to inhibit BITC has not previously been tested. The addition of various concentrations of ANS does not appreciably change the \( K_m \) value for BITC but decreases \( V_{max} \); therefore, we conclude that ANS functions as a noncompetitive inhibitor with respect to BITC yielding a \( K_I \) of 6.1 ± 0.1 \( \mu \)M.

**Inactivation of Human GST P1-1 by BITC**—The effect of incubating homogeneous hGST P1-1 (0.3 mg/ml) with 100 \( \mu \)M BITC at pH 7.0 and 25 \( ^\circ\text{C}\) in the absence of glutathione was studied. The concentration of BITC was determined from the absorbance at 245 nm using \( e_{245\ nm} = 10,780 \frac{M^{-1} \ cm^{-1}}{M} \). The reaction was followed by assaying aliquots for their residual activity using either CDNB or BITC as substrates. This results in a time-dependent inactivation of the enzyme as assayed using BITC as substrate (\( k_{obs} = 0.067 \pm 0.007 \) min\(^{-1}\)), with a lesser effect on the use of CDNB as substrate for the enzyme (\( k_{obs} = 0.032 \pm 0.005 \) min\(^{-1}\) (Fig. 1). The control enzyme, incubated under the same conditions but in the absence of the reagent, showed no change in activity during the same period. As monitored by using BITC as substrate, the dependence of the \( k \) value of inactivation of hGST P1-1 on [BITC] is hyperbolic, with a \( K_I \) of 66 ± 7 \( \mu \)M and \( k_{max} = 0.118 \pm 0.007 \) min\(^{-1}\) (Fig. 2). This type of curve is typical for an affinity label, suggesting that an enzyme-reagent complex is formed reversibly prior to the irreversible modification of the enzyme (37). In contrast, when monitored using CDNB as substrate, the \( k \) value for inactivation exhibits a linear dependence on [BITC] with a second-order rate constant of 308 ± 11 min\(^{-1} \) M\(^{-1}\) (Fig. 2). These results suggest there are at least two different target sites for BITC.

**Effect of Substrate Analogs on the Rate of Inactivation of hGST P1-1 by BITC**—The ability of various substrate analogs to protect against inactivation of the enzyme by 100 \( \mu \)M BITC was examined to evaluate the site(s) at which BITC reacts.
GST Pi Has Three Distinguishable Xenobiotic Substrate Sites

Because the rate of inactivation of the enzyme was different when assayed using either substrate BITC or CDNB, protection against inactivation was monitored separately with either substrate product. Subsequently, the modified enzymes were isolated, and the incorporation of BITC was measured from its characteristic absorbance at 245 nm as described under "Experimental Procedures." The incorporation of BITC into hGST P1-1 was measured as a function of time (0–40 min) in the absence of added ligands, and the reagent incorporation was plotted as a function of residual activity at the same time (Fig. 3). Extrapolation to totally inactive enzyme yields about 2 mol of reagent/mol of subunit. These results suggest that inactivation results from modification of at most two amino acid residues.

The effect of the addition of ligands on the incorporation of BITC into hGST P1-1 was investigated. Incubation of hGST P1-1 with 100 μM BITC for 40 min affords a modified enzyme that is 8% active and contains 1.72 mol of reagent/mol of subunit. The addition to the reaction mixture of either S-methylglutathione or ANS yields enzyme that retains a little more activity (15 and 22%, respectively) and exhibits small decreases in incorporation to no less than 1.23 mol of reagent/mol of subunit. In contrast, when both S-methylglutathione and ANS are present in the reaction mixture, a dramatic decrease in incorporation is observed (0.31 mol of reagent/mol of subunit), and 92% enzymatic activity is retained. These results support the proposal that the BITC reaction occurs at two locations. Reaction at either one of these reaction sites is sufficient to cause complete inactivation of the enzyme.

Properties of BITC-modified hGST P1-1—The catalytic properties of the modified enzyme were investigated by using CDNB, BITC, and mBBr as substrates. As reported earlier, reaction of BITC occurs at two different sites. S-Methylglutathione offers partial protection against inactivation, suggesting that one of the reaction sites may be at or near the glutathione binding region and the other at a different site in the reaction mixture. The xenobiotic substrate analogs of CDNB and mBBr do not protect against inactivation by BITC, indicating that the affinity label BITC does not react at these substrate sites (Table I, lines 5 and 6). Conversely, the nonsubstrate hydrophobic ligands, such as 8-anilino-1-naphthalene sulfonate or bromosulfophthalein, provide partial protection against inactivation when used alone (Table I, lines 7 and 8) and complete protection when combined with S-methylglutathione (Table I, line 11). The best protection is provided by the combination of S-methylglutathione and ANS (Table I, line 11) and the substrate product 3-(N-benzylthiocarbamoyl)glutathione (Table I, line 13), suggesting that the second reaction occurs at or near the nonsubstrate hydrophobic site that is not far from the glutathione substrate site.

Incorporation of BITC by hGST P1-1—hGST P1-1 was incubated with 100 μM BITC in the absence or presence of added protectants. Subsequently, the modified enzymes were isolated, and the incorporation of BITC was measured from its characteristic absorbance at 245 nm as described under "Experimental Procedures." The incorporation of BITC into hGST P1-1 was measured as a function of time (0–40 min) in the absence of added ligands, and the reagent incorporation was plotted as a function of residual activity at the same time (Fig. 3). Extrapolation to totally inactive enzyme yields about 2 mol of reagent/mol of subunit. These results suggest that inactivation results from modification of at most two amino acid residues.
enzyme. Therefore, to study catalytic properties of modified enzyme, hGST P1-1 was incubated with 100 µM BITC at a single time (90 min) in the presence of S-methylglutathione to protect the glutathione-binding site, while leaving the other target site vulnerable to reaction. Table II compares the percent inactivation as monitored by various substrates. The residual activity is different with respect to all substrates. The enzyme retains almost full activity with respect to CDNB as substrate but is nearly completely inactive with respect to BITC. In contrast, the enzyme retains 40% residual activity with respect to mBBr. These results suggest that the sites occupied by BITC, CDNB, and mBBr are distinct.

It seemed possible that BITC modification of one site of the enzyme could indirectly alter the mBBr and CDNB sites. Thus, the kinetic constants for mBBr and CDNB were determined for modified hGST P1-1. As shown in Table II, the modification results in 60% inactivation when monitored by mBBr as a substrate; however, the observed per cent inactivation as monitored by various substrates. The residual activity is different with respect to all substrates. The enzyme retains almost full activity with respect to CDNB as substrate but is nearly completely inactive with respect to BITC. In contrast, the enzyme retains 40% residual activity with respect to mBBr. These results suggest that the sites occupied by BITC, CDNB, and mBBr are distinct.

In previous studies, we found that monobromobimane does not occupy the same site as the hydrophobic compound ANS (26). However, ANS provides partial protection against BITC inhibition of S-methylglutathione as measured by different xenobiotic substrates. Modified enzyme was prepared by incubating hGST P1-1 with 100 µM BITC in the presence of 5 mM S-methylglutathione for 90 min. The modified enzyme was subjected to gel filtration to remove excess reagents, and kinetic constants for BITC, CDNB, and mBBr were determined as described under “Experimental Procedures.”

**Table I**

| Ligands added | k<sub>i</sub>/k<sub>−i</sub> | CDNB |
|---------------|-----------------|------|
| None          | 1.00            | 1.00 |
| 2. S-Methylglutathione (5 mM) | 0.75 | 0.12 |
| 3. S-Hexylglutathione (5 mM) | 0.51 | 0.00 |
| 4. S-(p-Nitrobenzyl)glutathione (5 mM) | 0.18 | 0.00 |
| 5. Dinitrophenol (10 mM) | 1.03 | 1.98 |
| 6. S-(Hydroxyethyl)bimane (0.33 mM) | 1.00 | 0.93 |
| 7. S-Anilino-1-naphthalene sulfonate (0.06 mM) | 0.52 | 0.73 |
| 8. Bromosulfophthalein (0.1 mM) | 0.29 | 0.93 |
| 9. S-(Hydroxyethyl)bimane (0.33 mM) + S-methylglutathione (5 mM) | 0.21 | 0.33 |
| 10. Dinitrophenol (10 mM) + S-methylglutathione (5 mM) | 0.14 | 0.06 |
| 11. S-Anilino-1-naphthalene sulfonate (0.06 mM) + S-methylglutathione (5 mM) | 0.04 | 0.12 |
| 12. Glutathionyl-bimane (0.33 mM) | 0.33 | 0.12 |
| 13. S-(N-Benzylthiocarbamoyl)glutathione (0.1 mM) | 0.06 | 0.05 |

**Table II**

| Varied substrates | Inactivation | K<sub>eff</sub> µM |
|-------------------|--------------|-----------------|
| BITC              | 97           | ND              |
| CDNB              | 11           | 4550 ± 97       |
| mBBr              | 60           | 34 ± 4          |

*Kinetic constants for BITC of modified enzyme were not determined because the enzyme was almost inactive.

**Fig. 3.** Incorporation of BITC into hGST P1-1 as a function of the percentage of maximum inactivation. Extrapolation to complete inactivation gives a maximum incorporation of about 2 mol of reagent/mol of enzyme subunit.
Both peaks were subjected to electrospray mass spectrometry to obtain evidence of chemical modification. Peak I yielded peptides of 1534.9 and 1684.7 atomic mass units. The 1534.9 atomic mass units is equivalent to the unmodified form of the peptide YISLIYTNYEAGK, corresponding to residues 103–115, whereas the 1684.7 atomic mass units corresponds to the predicted mass of the same peptide modified by BITC. In peak II, peptides of 1079.9 and 1229.6 atomic mass units were detected. The 1079.9 atomic mass units is comparable with the predicted mass of the unmodified form of the peptide ASCLYGQLPK corresponding to residues 45–54, whereas 1229.6 atomic mass units are identical to the predicted mass of the same peptide modified by BITC.

Both modified peptides corresponding to peaks I and II have many potential amino acid targets for BITC modification. Thus, they were treated with other proteases to identify further the modified amino acids. Peptide peak I was redigested with chymotrypsin, whereas peptide peak II was redigested with thermolysin, and both were subjected to the same HPLC solvent system as before. Peak Ia, which absorbed at 245 nm, eluted at 6% acetonitrile, earlier than peak I. Peak IIa, which also absorbed at 245 nm, eluted at 6% acetonitrile, earlier than peak II. Table III shows the results obtained when these two peaks were subjected to mass spectrometric analysis. Masses containing BITC in peak Ia correspond to Tyr (332.1 atomic mass units), Y103YISLIY108 (940.5 atomic mass units). These data indicate that the modified peptide resulting from peak Ia is Y103YISLIY108. Other peptides seen in peak Ia were most likely the result of fragmentation during the mass spectrometry analysis. Although 940.5 atomic mass units does not distinguish between reaction of BITC with Tyr103 or Tyr108, peptides I and Ia were both resistant to sequencing by Edman degradation, indicating that Tyr103 had been derivatized previously by BITC, thereby preventing reaction with phenylisothiocyanate. Furthermore, Tyr103 of GST Pi had been subjected previously to mutagenesis (38). Ahn et al. (38) studied Y103F and Y103S (16/H9262/M), showing little change in the kinetic parameters for CDNB as substrate. In peak IIa, masses containing BITC correspond to Cys47 (271.3 atomic mass units) and ASC47 (429.6 atomic mass units). From these data, the modified peptide from peak Ia is ASC47YISLIY108. Other peptides seen in peak Ia were most likely the result of fragmentation during the mass spectrometry analysis. Although 940.5 atomic mass units does not distinguish between reaction of BITC with Tyr103 or Tyr108, peptides I and Ia were both resistant to sequencing by Edman degradation, indicating that Tyr103 had been derivatized previously by BITC, thereby preventing reaction with phenylisothiocyanate. Furthermore, Tyr103 of GST Pi had been subjected previously to mutagenesis (38). Ahn et al. (38) studied Y103F and Y103S (16/H9262/M), showing little change in the kinetic parameters for CDNB as substrate. In peak IIa, masses containing BITC correspond to Cys47 (271.3 atomic mass units) and ASC47 (429.6 atomic mass units). From these data, the modified peptide from peak Ia is ASC47YISLIY108. Other peptides seen in peak Ia were most likely the result of fragmentation during the mass spectrometry analysis. These data indicate that the modified peptide resulting from peak Ia is ASC47YISLIY108. Other peptides seen in peak Ia were most likely the result of fragmentation during the mass spectrometry analysis. This information, in conjunction with the masses of the intact peptides and the primary sequence of the enzyme, clearly defines Tyr103 and Cys47 as the targets of BITC, and their modifications are responsible for the loss of enzymatic activity.

Effect of Protectants on Labeled Peptide Peaks—Table IV compares the amount of incorporation of BITC into the two labeled peaks at a single time (40 min) in the absence and presence of protectants. The enzyme was modified with 100 μM BITC at pH 6.5 and 25 °C for 40 min and subsequently digested with trypsin. The digest was fractionated on a C18 column, as described under “Experimental Procedures.” A, A220 nm; B, A245 nm shows profiles of digest of the modified enzyme prepared in the presence of protectants.

FIG. 4. Effect of modification or mutations of hGST P1-1 on its binding of ANS. A, the equilibrium binding of ANS to native (○) and BITC-modified (●) hGST P1-1 (16 μM) was determined by measuring the enhanced fluorescence of enzyme-bound ANS at 480 nm (excitation at 390 nm) relative to free ANS (Δ); the fluorescence is measured on a high sensitivity scale. B, in addition to native (○) and modified (●) wild-type hGST P1-1, enhanced fluorescence at a lower sensitivity scale (1/3 of the scale shown in A) is shown for Y103F (□) and Y103S (○) (16 μM). Inset in B shows fluorescent readings of enzyme-bound ANS at lower [ANS].

FIG. 5. HPLC separation of tryptic digests of protein resulting from the 40-min modification of hGST P1-1 by BITC. The enzyme was modified with 100 μM BITC at pH 6.5 and 25 °C for 40 min and subsequently digested with trypsin. The digest was fractionated on a C18 column, as described under “Experimental Procedures.” A, A220 nm; B, A245 nm shows profiles of digest of the modified enzyme prepared in the presence of protectants.
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presence of ligands. Approximately equal amounts of BITC are found in peaks I and II in the absence of protecting ligands. Compared with the absence of ligands, a markedly lower incorporation was found in peptide II when S-methylglutathione was present in the reaction mixture (Table IV, line 2), indicating this peptide is within the glutathione binding region. Comparison of lines 1 and 3 in Table IV reveals the major decrease of BITC incorporation is in peptide I when the enzyme is protected by ANS. This observation suggests that peptide I is primarily within the ANS binding region, and this site appears protected by ANS. This observation suggests that peptide I is incorporated into both peptides I and II, indicating the glutathione binding region and the BITC site are not far apart.

Kinetic Properties of Mutant Enzymes—Because the affinity labeling study points to Tyr103 as the residue responsible for inactivation toward BITC as substrate, mutant enzymes were constructed with substitutions at position 103 to provide insights into the role of this residue in catalysis. Nishihira et al. (39), Park et al. (14), and Ricci et al. (40) have previously studied mutant enzymes with replacements for Cys47; therefore, this residue was not studied further.

Because Tyr103 has both an aromatic and a hydroxyl group, two mutants were constructed as follows: Y103F, to retain the aromatic moiety while eliminating the hydroxy group, and Y103S, to retain the hydroxy group while eliminating the aromatic component. These mutant enzymes were expressed at 25 °C in E. coli strain JM105 and then isolated and purified to homogeneity using S-hexylglutathione-agarse affinity chromatography.

Four different substrates, including BITC, were studied. It was expected that significant differences would be observed for substrate BITC in the mutants as compared with the wild-type enzyme if Tyr103 is involved exclusively in the BITC substrate site; furthermore, it was anticipated that the kinetic parameters for the other substrates would be similar for mutant and wild-type enzymes. The actual data are shown in Table V. For wild-type hGST P1-1, the Vmax for mBBr is ∼1/3 that for all the other substrates, whereas the Km for CDNB is about 10 times that of the other substrates (Table V). For both mutants, the Km and Vmax values of CDNB and GSH (with CDNB as substrate) do not change appreciably. In contrast, for Y103S, the Km for BITC is ∼30 times greater than the wild-type enzyme, but the Vmax does not change. Y103F retains the same kinetic characteristics as wild-type enzyme for BITC as a substrate. These results demonstrate that Tyr103 contributes to the affinity of the enzyme for BITC possibly through Pi-Pi stacking interactions between the rings of Tyr103 and BITC. In the case of mBBr, there was an unexpected decrease in Vmax for both mutants with an unchanged Km value, suggesting that mBBr and BITC may share the same site; however, this is unlikely because we have shown that S-(hydroxyethyl)bimane, an mBBr analog, failed to protect against inactivation by affinity label BITC and did not inhibit the reaction of BITC as a substrate. We propose that BITC binds at a discrete site of hGST P1-1 (see “Discussion” for further consideration of this issue).

The identification of Tyr103 as a major target of the affinity label BITC was strengthened by incubating Y103F with 100 μM BITC in the presence of S-methylglutathione. The mutant enzyme was not inactivated by BITC, indicating that the phenoxy

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### Table III

| Peaks Ia and IIA | Measured mass (atomic mass units) | Corresponding sequence |
|-----------------|----------------------------------|------------------------|
| Peak Ia         | 150.1                            | X = BITC               |
|                 | 182.7                            | Y                      |
|                 | 332.1                            | X-Y                   |
|                 | 790.7                            | 103YISLI408            |
|                 | 940.5                            | 102X-YISLI408          |
|                 | 608.2                            | 104I4ISLI408          |
| Peak IIA        | 149.9                            | X = BITC               |
|                 | 121.0                            | 47C                   |
|                 | 271.3                            | 47X-C                 |
|                 | 194.6                            | 45AS45                |
|                 | 279.4                            | 45AS45                |
|                 | 429.6                            | 45AS-X-C47            |

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### Table IV

| Effect of protectants on the magnitude of the labeled peptide peaks |
|------------------------------------------------------------------|
| hGST P1-1 (0.3 mg/ml) was incubated for 40 min with 100 μM BITC at pH 6.5 and 25 °C in the presence or absence of protectants and later subjected to proteolytic digestion and fractionation by HPLC. |
| Additions to the reaction mixture | Inactivation | Incorporation of BITC | BITC incorporated into labeled peptide peaks |
|---------------------------------|-------------|----------------------|-------------------------------------------|
|                                 | % | mol BITC | substrate of GST | I | II |
| 1. None                         | 92 | 1.72 | 0.97 | 0.74 |
| 2. S-Methylglutathione          | 85 | 1.36 | 1.24 | 0.11 |
| 5. S-Anilino-1-naphthalene sulphonate | 78 | 1.23 | 0.09 | 1.14 |
| 6. S-Anilino-1-naphthalene sulphonate + S-methylglutathione | 8 | 0.31 | 0.29 | 0.02 |
| 5. S-(N-Benzylthiocarbamoyl)glutathione | 5 | 0.21 | 0.13 | 0.08 |

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### Table V

| Kinetic parameters for the glutathione conjugation to different substrates by wild-type and mutant GST P1-1 |
|--------------------------------------------------|
| Substrate | WT  | Vmax | Km | Y103F | Vmax | Km | Y103S | Vmax |
|-----------|-----|------|----|-------|------|----|-------|------|
| CDNB      | 650 ± 39 | 65 ± 6 | 780 ± 20 | 60 ± 6 | 598 ± 80 | 81 ± 4 |
| GSH       | 51 ± 1  | 59 ± 2 | 51 ± 2 | 58 ± 2 | 57 ± 4  | 84 ± 1 |
| BITC      | 68 ± 5  | 70 ± 4 | 58 ± 3 | 61 ± 5 | 2171 ± 4 | 93 ± 9 |
| mBBr      | 42 ± 2  | 21 ± 3 | 32 ± 1 | 4 ± 0 | 42 ± 2  | 9 ± 1 |

*GSH kinetics were measured using CDNB as the electrophilic substrate.*
group of Tyr^{103} is the nucleophilic group that reacts with BITC under these conditions.

The ability of mutant enzymes to bind ANS was also determined. Fig. 4B compares the ability of wild-type and mutant enzymes to bind ANS at concentrations ranging from 3 to 900 μM (concentrations considerably higher than those shown in Fig. 4A). Results from the wild-type unmodified enzyme indicate there are at least two sites for ANS (K_D = 6.4 ± 0.4 μM, shown in Fig. 4B, inset, and K_D = 153 ± 20 μM shown in Fig. 4B). As shown in Fig. 4A, modification of the enzyme by BITC eliminated the high affinity site for ANS; however, the BITC-modified enzyme still binds ANS at higher concentrations (K_D = 148 ± 18 μM). The binding of ANS to Y103F (K_D = 3.3 ± 0.3 μM and K_D = 151 ± 9 μM) is similar to that of wild-type enzyme, indicating this mutation does not affect the ANS affinity. In contrast, mutation of Tyr^{103} to Ser greatly weakens the high affinity site for ANS (see Fig. 4B, inset), whereas the low affinity binding of ANS is not appreciably changed (K_D = 169 ± 18 μM). The effect of these substitutions suggests that Tyr^{103} in hGST P1-1 contributes to the tight binding of ANS. A major determinant in the binding of ANS is the aromatic ring of Tyr^{103}, rather than its hydroxyl group.

**DISCUSSION**

Most of the substrates of GSTs that have been studied in detail are synthetic compounds not present in nature (28); however, benzyl isothiocyanate (BITC), a substrate for human GST P1-1, is abundant in edible plants (28). In addition, recent studies (27–29) suggest BITC offers chemoprotection against tumor formation by inducing the synthesis of GSTs. Thus, locating the binding site of BITC in hGST P1-1 not only provides structural insights into the several functions of the enzyme but contributes to an understanding of the metabolism of benzyl isothiocyanate in humans. BITC is indeed an efficient substrate for hGST P1-1 with a lower apparent K_m value than for the more common xenobiotic substrate CDNB. In addition, when added to the enzyme in the absence of glutathione, BITC acts as an affinity label that reacts covalently with the enzyme.

The results of this study of hGST P1-1 demonstrate that there are two target sites for BITC, Tyr^{103} and Cys^{47}. 8-Anilino-1-naphthalene sulfonate decreases incorporation into Tyr^{103} of subunit B (PDB code 19GS), whereas S-methylglutathione decreases the reaction of BITC with Cys^{47}.

Previously, we demonstrated through affinity labeling with monobromobimane that Cys^{47} is at or near the glutathione-
binding site (26). In this study, BITC also targets Cys47 and the addition of S-methylglutathione decreases the reaction of BITC with Cys47, further supporting the location of this residue at or near the glutathione-binding site. We did not conduct mutagenesis at position 47 because others have provided insights into the role of this residue in the enzyme. For example, Nishihira et al. (39) showed that reaction of Cys47 with the bulky reagent 7-fluoro-4-sulfamoyl-1,2,3-benzodiazole inactivates hGST Pi by steric hindrance of the glutathione-binding site. Park et al. (14) demonstrated that the binding affinity between hGST P1-1 and glutathione decreases 5-fold when Cys47 is mutated to Ser. In addition, Cacurri et al. (41) reported that the human GST Pi is inactivated by 1-chloro-2,4-dinitrobenzene upon modification of Cys47, with protection being provided by S-methylglutathione; and the replacement of Cys47 with Ala or Ser decreases the affinity for glutathione. These results all support the location of Cys47 at or near the glutathione-binding site.

This is the first time that Tyr103 has been implicated in any function of GST Pi. We demonstrated here that labeling of Tyr103 in hGST P1-1 results in loss of activity toward BITC as a substrate. Most interestingly, ANS, which is a hydrophobic nonsubstrate ligand, protects against inactivation as monitored by BITC as a substrate and decreases incorporation into Tyr103. Examination of the kinetic characteristics of Y103F and Y103S enzymes allows evaluation of the role of Tyr103 in catalysis. The $K_m$ value for BITC is dramatically increased in Y103S, suggesting that Tyr103 is critical for binding of BITC but is not required for catalysis. Such binding may be governed by Pi-Pi stacking interactions because Y103F exhibits the same affinity for BITC as wild type, whereas elimination of the aromatic group (Y103S) decreases the BITC affinity markedly. The decrease in the $V_{max}$ value for mBBr of both mutants was unexpected. This result could be interpreted to indicate that mBBr and BITC share the same site; however, the failure of S-(hydroxyethyl)bimane, a monobromobimane analog, to protect against inactivation from BITC indicates this is unlikely. Additionally, S-(hydroxyethyl)bimane does not function as a competitive inhibitor of BITC, as it does with mBBr. Also, the mutations at Tyr103 had different effects on the kinetic parameters for the mBBr and BITC reactions; the $V_{max}$ value in the mBBr reaction is strikingly decreased when either phenylalanine or serine replaces the original tyrosine. If BITC and mBBr shared the same site, then the two substrates would be affected equally by both mutations. Therefore, we propose that BITC binds at a discrete site of hGST P1-1 and that Tyr103 makes different contributions toward the reactions of the electrophilic substrates bound at these two sites.

GST Pi has been implicated in the intracellular transport and storage of a broad range of structurally diverse hydrophobic ligands (36). The hydrophobic nonsubstrate or “ligandin”-binding site has been proposed as distinct from the G- and H-sites (36). However, controversy still exists as to whether ligandin sites are separate or the same as those occupied by the xenobiotic substrates (42). We have demonstrated that either bromosulfophthalein or ANS prevents inactivation and that ANS decreases incorporation of BITC into Tyr103, raising the possibility that the BITC site overlaps a ligandin site of hGST P1-1. We therefore tested the ability of the BITC-modified enzyme to bind ANS, and we demonstrated that modification with BITC eliminates the high affinity binding site for ANS. The binding site for ANS may thus be near the xenobiotic substrate region. In addition, we tested the ability of Y103F and Y103S enzyme to bind ANS. The ANS binding of wild-type and Y103S enzyme is similar. In contrast, replacement of Tyr103 by serine weakens binding of ANS to the high affinity site, suggesting that Tyr103 is a major contributor to this site for ANS and a major component in binding is the aromatic ring of Tyr103, rather than its hydroxyl group.

For all of the enzymes (modified, wild-type, or mutant) ANS was bound at higher concentrations. This finding indicates the existence of more than one binding site for ANS. Because ANS is commonly used as a probe to assess the surface hydrophobicity of proteins (43), other sites in hGST P1-1 for ANS binding may be located at hydrophobic patches throughout the enzyme. Similarly, a high affinity and several low affinity sites have been reported recently (44) for a different nonsubstrate ligand, bromosulfophthalein, of the Alpha class GST A1-1. We here demonstrate that the high affinity binding site of ANS on hGST P1-1 is located near the BITC substrate region; however, the location of the low affinity site(s) remains unknown.

Examination of the crystal structure of hGST P1-1 allows us to better understand the experimental results. In Fig. 6A, glutathione is positioned in an orientation suitable for reaction with BITC at the active site. Because the aromatic ring of Tyr103 is important for binding BITC; the aromatic rings of both Tyr103 and BITC have been positioned appropriately for Pi-Pi stacking interactions, i.e. the rings should be $<5.6$ Å apart (45). The distance between the reactive carbon of BITC and the $\pm$SH of glutathione is $\pm2.4$ Å, and the distance between the two aromatics is $\pm4$ Å. Thus, the catalytic reaction of BITC and glutathione is feasible. The binding site for BSP, but not ANS, in hGST P1-1 is known from a crystal structure (46). Because BSP binds competitively with respect to ANS (36), the BSP-binding site was used to interpret results related to ANS. With BITC positioned to react with glutathione, as in Fig. 6A, it is clear that BITC and BSP bind at distinct sites, thereby accounting for the noncompetitive inhibition by ANS with respect to BITC as substrate.

An alternate mode of docking of BITC is shown in Fig. 6B, in which the reactive isothiocyanate group is oriented close to the $\pm$OH of Tyr103. The distance between the reactive carbon of BITC and the $\pm$OH of Tyr103 is $\pm2.3$ Å, whereas it is about 10.9 Å to the $\pm$SH of glutathione. This orientation can account for the protection provided by BSP and ANS against reaction at Tyr103. In Fig. 6C, an energy-minimized structure is shown...
that depicts the covalently modified Tyr\textsuperscript{103}. We propose that BITC approaches Tyr\textsuperscript{103} by the route indicated in Fig. 6B that passes through or close to the site occupied by BSP (Fig. 6B). However, after reaction occurs, the most stable conformation of the covalently modified Tyr\textsuperscript{103} places the BITC moiety in the BITC substrate site (Fig. 6C); the location of the modified Tyr\textsuperscript{103} thus accounts for the complete loss of activity with BITC as substrate but retention of some activity with either CDN or monobromobimane as substrate.

BITC also reacts covalently with Cys\textsuperscript{47}. In the structure shown in Fig. 6D, BITC has been docked into a site close to Cys\textsuperscript{47}; the distance between the reactive carbon of BITC and \textbf{–SH} of Cys\textsuperscript{47} is \textasciitilde1.8 Å. This structure shows that the aromatic ring of BITC overlaps the enzyme-bound glutathione; glutathione and BITC cannot be bound simultaneously, thereby explaining the protection provided by S-methylglutathione against reaction of BITC with Cys\textsuperscript{47}.

To visualize whether BITC can really occupy a different location from monobromobimane and the dinitrobenzyl group of CDN, Fig. 7 shows the structure of hGST P1-1 complexed with dinitrophenyl-glutathione and monobromobimane overlaid on the structure of the enzyme with BITC docked into the substrate site as in Fig. 6A. BITC, monobromobimane, and dinitrophenyl are clearly in three discrete locations in this model, indicating that the site for BITC, can be distinct from that of the other xenobiotic substrates.

Glutathione S-transferases function to protect an organism against potentially toxic compounds. Because an organism may encounter a wide variety of foreign chemicals through its lifetime, there is an advantage to having multiple classes of glutathione S-transferases with diverse substrate specificities. However, it is physiologically even more efficient for a given isozyme to evolve several xenobiotic substrate sites within the H-site to handle the wide array of foreign chemicals.

In summary, the results presented in this paper provide evidence for the existence of a novel binding site for xenobiotic substrates in hGST P1-1, which can be occupied by BITC, and support the existence of at least three discrete binding sites for electrophilic substrates within the xenobiotic substrate region of hGST P1-1. Benzyl isothiocyanate, in addition to serving as a substrate for hGST P1-1, acts as an affinity label. Modification of Tyr\textsuperscript{103} occurs from the xenobiotic substrate site occupied by BITC during its reaction with glutathione, whereas reaction with Cys\textsuperscript{47} takes place with BITC positioned at or near the glutathione-binding site.

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