Biochemical Pharmacology, Vol. 52, pp. 281–288, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00205-5

Structural Studies of a Human Pi Class Glutathione S-Transferase

PHOTOAFFINITY LABELING OF THE ACTIVE SITE AND TARGET SIZE ANALYSIS

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ABSTRACT. The glutathione S-transferases (GSTs; EC 2.5.1.18) are a family of dimeric proteins that catalyze reactions between glutathione (GSH) and various electrophiles. A partial cDNA for human GST π was obtained and the open reading frame completed. The completed cDNA was cloned, and GST π protein was expressed in bacteria. Cloned enzyme was purified and had the same kinetic constants, molecular mass, pI value, and N-terminal sequence as placental GST π except that some of the polypeptides had N-terminal methionines. A radiolabeled azido derivative of GSH, S-(*p*-azidophenacyl)-[^{3}H]glutathione, was used to photoaffinity-label the active site of the cloned enzyme. Labeled enzyme did not bind to a GSH-agarose affinity column. Labeling was prevented in the presence of S-hexylglutathione, and noncovalently-bound azido affinity label was a competitive inhibitor towards 1-chloro-2,4-dinitrobenzene and GSH. These results suggest that the azido label was binding at the active site of the enzyme. Photoaffinity-labeled enzyme was trypsinized, and two labeled peptides were purified and sequenced. One peptide corresponded to residues 183–188, whereas the other corresponded to residues 183–186. These residues appear to form part of the hydrophobic (H-site) binding region of human GST π that has not been shown previously. Cloned enzyme was subjected to radiation inactivation to assess the importance of subunit interactions in the maintenance of catalytic activity. The target size of enzymatic activity (23 kDa) was not significantly different from that of the protein monomer (24 kDa). Therefore, each subunit of human GST π appears to be capable of independent catalytic activity. BIOCHEM PHARMACOL 52;2: 281–288, 1996.

KEY WORDS. glutathione transferases; human; active site; affinity labels; protein structure; radiation effects

Cystolic GSTs§ (EC 2.5.1.18) are dimeric enzymes that conjugate a wide variety of endogenous electrophiles and xenobiotics with GSH for their elimination from cells, and, as such, the GSTs are important enzymes of detoxication [1–4]. The mammalian GSTs can be grouped into four classes, termed alpha, mu, pi, and theta [4, 5]. The GSTs exist as hetero- or homodimers with an active site present in each subunit [6–8]. Each active site contains a GSH binding site (G-site) and a hydrophobic binding site (H-site) for the electrophilic substrate [8].

The solutions of the crystalline structure of several GSTs have defined regions of the enzyme that are involved in the formation of the G- and H-sites. The G-site of human GST π is located in the N-terminal domain I (residues 1–76), whereas the H-site resides primarily in the C-terminal domain II [9]. The amino acids critical to the binding of GSH are well defined, whereas the particular amino acids that form the H-site are only partially known. Given the broad range of electrophilic substrates that bind to the H-site, it is not unexpected that the hydrophobic pocket would be extensive and that different hydrophobic electrophiles may interact with different amino acids. For example, the affinity labels AzGSH, S-(4-bromo-2,3-dioxobutyl)glutathione, and S-(2-nitro-4-azidophenacyl)glutathione label different areas within the H-site of rGSTA1-1, which probably reflects their different sizes and a different orientation of the labels within the hydrophobic pocket [10–12].

Although a pi class GST was the first isozyme for which a three-dimensional structure was determined, most of the studies to define the H-site of GSTs have used alpha and mu class isozymes [10–14]. The GST π enzyme is of particular interest because this form is the predominant isoenzyme expressed in some tissues and because it is expressed at abnormally high levels in transformed and malignant tissues [15]. To better define the H-site of human GST π, we obtained a partial GST π gene that had been cloned from human skin [16]. We completed the open reading frame

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§ *Abbreviations:* GST, glutathione S-transferase; GSH, glutathione; IPTG, isopropyl β-D-thiogalactopyranoside; AzGSH, S-(*p*-azidophenacyl)-glutathione; CDNB, 1-chloro-2,4,-dinitrobenzene; DCNB, 2,4-dichloronitrobenzene; and DTT, dithiothreitol.

Received 5 December 1995; accepted 2 February 1996.

and expressed the gene in bacteria. A photoaffinity label that we had used previously to identify portions of the H-site of two rat class alpha GSTs [10] was then used to label the H-site of human GST π. The importance of subunit interactions in the enzymatic function of the human GST π enzyme was also examined using radiation inactivation. The results of these studies are the subject of this report.

MATERIALS AND METHODS

Materials

Oligonucleotides based on the published sequence of human GST π [17] were synthesized at the University of California at San Francisco Protein Structure Laboratory or at the Emory University Microchemical Facility for Molecular Biology. Expression vector pPROK-1 was obtained from Clontech (Palo Alto, CA). IPTG was obtained from Fisher Scientific (Pittsburgh, PA). Rabbit anti-rat GST-Yp (MED 23 YP) polyclonal antiserum, which cross-reacts with human pi class enzymes but not with alpha or mu class enzymes, was obtained from Biotrin International (Dublin, Ireland). Human placental GST π, GSH, GSH-agarose, AzGSH, *p*-azidophenacyl bromide, CDNB, and activated charcoal were obtained from Sigma (St. Louis, MO). Dextran T70, PBE 94, and Polybuffer 74 were obtained from Pharmacia Biotech (Piscataway, NJ). Immobilon-P was obtained from Millipore (Bedford, MA). [*glycine*-2-^{3}H]-Glutathione (1 Ci/mmol) was purchased from Du Pont-NEN (Boston, MA). Other chemicals and reagents were of analytical grade or better. Dextran-coated charcoal was prepared as described previously [18].

Expression of Human GST π in Escherichia coli

We obtained a partial cDNA for GST π that had been cloned from human skin and that lacked 72 base pairs from the N-terminus of the open reading frame [16, 17]. Oligonucleotides containing the missing bases were synthesized and ligated to the partial GST π cDNA, and the complete gene was ligated into an expression plasmid containing the IPTG-inducible *tac* promoter (pPROK-1); the resulting plasmid was designated p2B2. The normal coding sequence of the gene was maintained, but silent substitutions were made with the synthetic oligonucleotides to avoid DNA secondary structure (a hairpin loop) that resulted in deletions of the 5′-end of the GST π gene by the bacteria. Cells of the *E. coli* strain JM 109 were then transformed with p2B2. Bacterial cultures were grown to $E_{1\ cm,\ 600} = 0.5$ and induced with IPTG (1 mM) for an additional 6–24 hr. GST enzymatic activity with the substrates CDNB and GSH and GST π immunoreactivity by western blotting were detectable in crude lysates from bacteria containing p2B2 induced by IPTG but not in lysates from similar bacteria not induced by IPTG or from lysates of bacteria transformed with pPROK alone.

Purification of Expressed GST π

All protein extraction and purification steps were performed at 4° unless noted. After 6–24 hr of induction by IPTG, bacterial cells were separated from medium by centrifugation for 15 min at 4° and 6000 *g* (r_{av} 9.53 cm). Then a slurry of cells (2 g wet wt/10 mL) was sonicated (0.5 inch probe, power output 100–120 W) in PBS/EDTA/DTT buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4; 10 mM EDTA; 5 mM DTT; pH 7.00) for a total of 3–5 min in bursts of 30 sec with several minutes allowed between bursts for cooling. The lysate was centrifuged for 25 min at 4° and 15,000 *g* (r_{av} 6.88 cm), and the supernatant was recentrifuged for 60 min at 4° and 100,000 *g* (r_{av} 6.88 cm). The cleared lysate was filter-sterilized through a 0.22-μm nylon filter and applied to a GSH-agarose affinity column (2.2 × 3 cm) to purify GST π [19]. The column was washed with PBS/1 mM EDTA/1 mM DTT (pH 7.00) until the A_{280} of the eluant was unchanged, and then bound protein was eluted with 10 mM GSH in 50 mM Tris·HCl (pH 8.2); the eluant was adjusted immediately to pH 6.50 with HCl.

The affinity-purified GST π was purified further by column chromatofocusing. The GST π solution was concentrated in a stirred cell under N_2 pressure and diafiltered to remove GSH and to exchange the buffer with column chromatofocusing buffer (25 mM histidine·HCl, pH 6.20). The protein solution was applied to a PBE 94 column (1 × 17 cm) and eluted with Polybuffer 74 (diluted 1:8, pH 4.00). Fractions were monitored for A_{280}, pH, and GST enzymatic activity. Fractions with GST activity were associated with a peak of absorbance at about pH 4.80. The active fractions were pooled, affinity-purified on a GSH-agarose column (as above) to remove Polybuffer, made up to 30% glycerol, and stored at −75° until used. Protein concentrations were measured by the Bio-Rad Protein Assay with BSA as the standard.

GST Enzymatic Activity, SDS-PAGE, Western Blotting, Isoelectric Focusing, and Amino Acid Sequencing of GST π

GST enzymatic activity was routinely measured spectrophotometrically ($A_{1\ cm,\ 340}$) using 1 mM CDNB and 1 mM GSH as substrates at 25° [20]. Enzymatic activity of cloned GST π with DCNB (1 mM) and ethacrynic acid (0.2 mM) was also determined as described previously [21]. SDS–PAGE was performed using a 13.5% or 15% gel in a discontinuous buffer system [22], and proteins were detected with silver stain [23] or Coomassie blue. After destaining, the gels were scanned with a laser densitometer (Molecular Dynamics Computing Densitometer model 300A and ImageQuant V.3.15 software) for calculation of the amount of each GST subunit. Each gel was scanned twice, and the average volume of the protein band from each radiation dose was used to calculate the target size (see below). Western electrotransfers were made onto nitrocellulose or Im-

mobilon-P membranes at 12 V for 1–2 hr in 25 mM Tris base, 192 mM glycine, 15% methanol [24]. Proteins were immunodetected on membranes with either Bio-Rad colorimetric or Renaissance (Du Pont-NEN) chemiluminescence reagents used as recommended by the manufacturers. Isoelectric focusing of GSTs was performed on ultrathin polyacrylamide gels in a Bio-Rad 111 Mini IEF cell following the manufacturer's procedure, and proteins were detected with silver stain. For amino acid sequencing, bacterially expressed GST π was affinity purified and electrophoresed by SDS–PAGE and electrotransferred to Immobilon-P membrane [25]. GST π was localized on the membrane by staining with sulforhodamine-B [26], and a portion of the membrane containing GST π was excised and used for amino acid sequencing at the Emory Microchemical Facility for Molecular Biology.

Covalent Photoaffinity Labeling of GST π and Identification of the Labeled Region

Synthesis of the photoaffinity label [^{3}H]AzGSH and its characterization were done as described previously [10]. Covalent labeling of GST π, trypsin digestion of labeled GST π, and purification of labeled peptides were performed as described previously [10] with minor modifications. The molar ratio of [^{3}H]AzGSH to protein was 1:1 for each of three 10-min UV-irradiations. The dextran-coated charcoal used to absorb free label after each UV-irradiation was removed by centrifugation and filtration of the supernatant through 0.22-μm nylon membrane filters. Recovery of protein after each labeling reaction was 95–98%.

Radiation Target Size Analysis of GST π

Radiation target size analysis is a method to determine the smallest catalytically active unit of an oligomeric protein [6, 27]. To determine if the smallest catalytically active unit of human GST π is the monomer or the dimer, we performed radiation inactivation of the enzyme. Bacterially expressed, affinity purified, and chromatofocused GST π (110 μg/275 μL in 100 mM potassium phosphate buffer/20 mM DTT/2 mM EDTA, pH 6.50) was placed into glass ampoules and snap-frozen on dry ice. The ampoules were then flame-sealed and kept at −75° until irradiated. The ampoules of GST π were irradiated at −135° with high energy electrons (13 MeV) produced by a linear accelerator at the Armed Forces Radiobiology Research Institute (Bethesda, MD). Details of radiation exposure, dosimetry, and temperature control have been described previously [27]. For the assay of both surviving enzymatic activity and protein subunits, the ampoules were opened, purged with air, and thawed at 4°. Enzymatic activity was always measured on samples thawed for the first time after irradiation and was not done on refrozen samples. The amount of surviving enzymatic activity or protein subunits was fitted by least squares analysis to the relation

$$A/A_0 = e^{-kD}$$

where D is the dose in Mrad, A is the measured activity or protein, and A_0 is the unirradiated activity or protein. The molecular mass was calculated as described previously [6].

RESULTS

Physical Properties of Bacterially Expressed Human GST π

The purification scheme for bacterially expressed GST π yielded up to 10 mg of pure enzyme/L of culture medium with a specific activity of up to 105 μmol/min/mg. The molecular mass of cloned GST π by SDS–PAGE was identical to that of placental GST π. Cloned GST π also cross-reacted with a polyclonal antiserum to human placental GST π on western blots. The amino acid sequence of the first 10 residues of the N-terminus of cloned GST π was identical to that of human placental enzyme except that some of the polypeptides had an N-terminal methionine residue. Removal of N-terminal methionine from proteins is normally a cotranslational event; however, incomplete removal frequently occurs in *E. coli* protein overexpression systems [28]. Cloned GST π behaved the same as placental GST π on isoelectric focusing gels or on a chromatofocusing column and had a pI = 4.7–4.9. The kinetic constants for the cloned and placental enzyme also were similar (Table 1). Activities with DCNB (placental 0.0036 vs cloned 0.0029 μmol/min/mg) and ethacrynic acid (placental 0.84 vs cloned 0.92 μmol/min/mg) also were similar.

Inhibition of GST π by AzGSH

To prove that AzGSH bound to the active site of GST π, inhibition kinetic studies were performed in the dark with CDNB and GSH as substrates and AzGSH as the inhibitor (Fig. 1). AzGSH behaved as a competitive inhibitor towards both GSH and CDNB.

Time Course of Inactivation of GST π

GST π was irradiated with UV light in the absence or presence of AzGSH for a total of 30 min (Fig. 2). In the absence of AzGSH, there was little or no decrease in enzymatic activity, indicating that the UV light did not denature the enzyme. In the presence of AzGSH, enzymatic

TABLE 1. Kinetic constants of placental and cloned human GST π

Enzyme	$K_{m(GSH)}$ (mM)	$K_{m(CDNB)}$ (mM)	V_{max} (μmol/min/mg)
Placental	0.22	2.57	846
Cloned	0.08	6.60	690

Each assay was performed in triplicate with various concentrations of CDNB (0.1 to 1.2 mM) and GSH (0.2 to 4 mM). K_m for GSH was determined at 1.2 mM CDNB, and K_m for CDNB was determined at 4 mM GSH. V_{max} was obtained at saturating concentrations of both substrates.

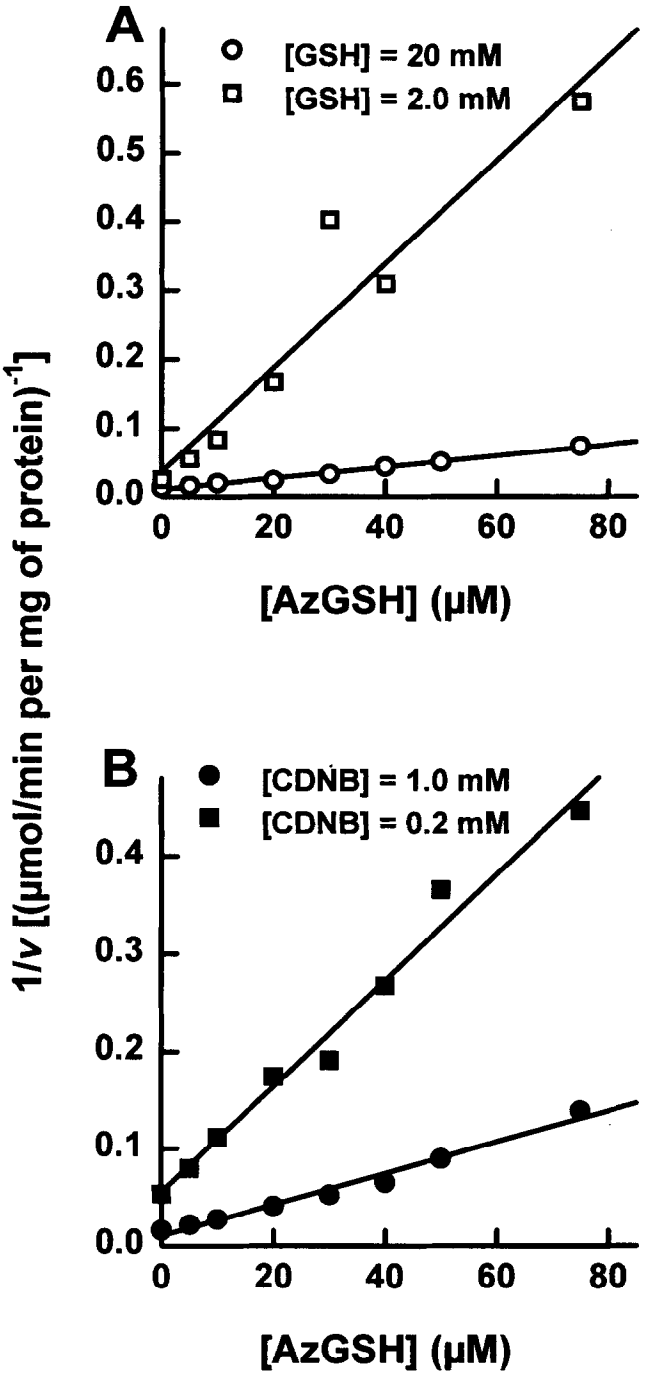

FIG. 1. Inhibition of human GST π enzymatic activity by AzGSH. (A) Inhibition of GST π activity by increasing [AzGSH] with constant [GSH] at 2 or 20 mM with [CDNB] at 1 mM. (B) Inhibition of GST π activity by increasing [AzGSH] with constant [CDNB] at 1 or 0.2 mM with [GSH] at 20 mM. Each assay was performed in duplicate and contained 0.42 μg of GST π in 100 mM phosphate buffer (pH 6.50, 25°) and was performed in the dark; the data were fitted by least squares regression analysis.

activity decreased to 73% of initial activity by 5 min of UV-irradiation with no further loss of activity over 25 min of additional UV-irradiation. Addition of more AzGSH led

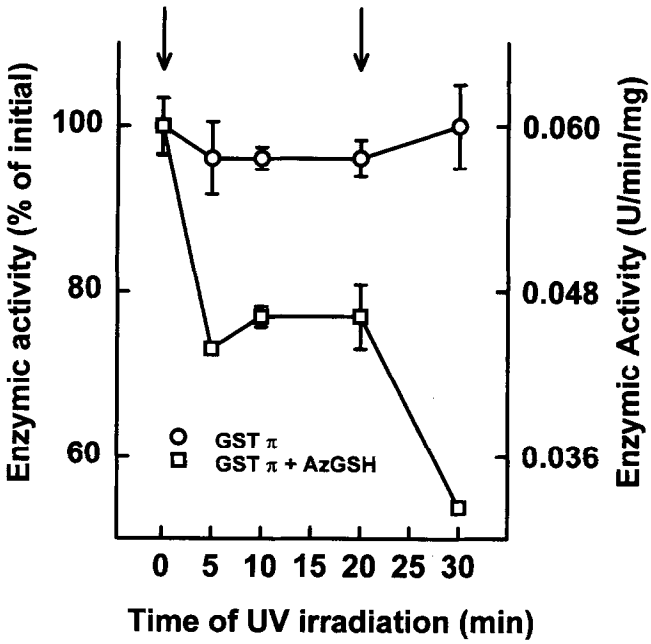

FIG. 2. Time course of inactivation of human GST π by AzGSH. GST π (10 μM) was UV-irradiated alone or in the presence of 10 μM AzGSH. Enzyme activities were measured in triplicate and are shown as means ± SEM. Arrows indicate additions of AzGSH.

to a further decrease in activity upon additional UV-irradiation (Fig. 2).

Covalent Labeling of Human GST π with AzGSH

To minimize non-specific labeling, human GST π was radiolabeled three times for 10 min each in the presence of a low (equimolar) concentration of [^{3}H]AzGSH. The labeled enzyme was then applied to a GSH-agarose affinity column, and two peaks of radioactive protein were eluted. The first peak of radioactive protein did not bind to the affinity column and eluted in the flow-through fraction. This peak represents enzyme with active sites blocked by covalently and noncovalently bound label. The second peak of radioactive protein bound to the affinity column. This peak represents non-specifically labeled and unlabeled enzyme, since the active sites were not blocked by the label and the enzyme eluted only after the addition of GSH (Fig. 3). Enzymatic activity was associated only with the second peak of radioactive protein. Protein in the first peak was dialyzed against 8 M urea for 24 hr to denature the enzyme and to permit removal of any label that was not attached covalently. Approximately 45% of the radioactivity present before dialysis remained with the protein after denaturation and dialysis. After dialysis, the molar ratio of radiolabel to enzyme dimer was 1:1, and therefore the ratio of label to active sites was 0.5:1. In a separate experiment S-hexylglutathione was added to the reaction mixture before irradiation in an amount equimolar to AzGSH, and the enzyme

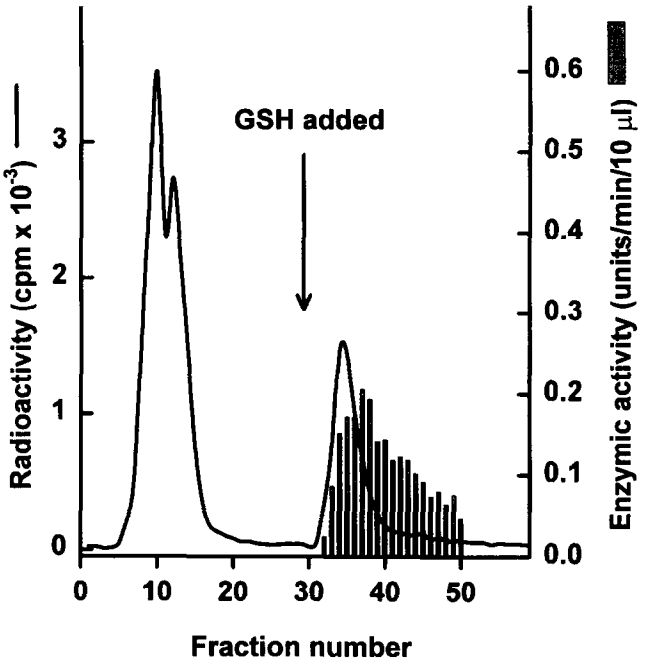

FIG. 3. Elution profile of human GST π labeled with [^{3}H]AzGSH from a GSH-agarose affinity column. After labeling with [^{3}H]AzGSH, GST π was applied to a GSH-agarose affinity column. The protein was eluted with buffer alone up to fraction 30 and then with buffer plus 25 mM GSH (arrow). Fractions were assayed for both radioactivity and enzymatic activity.

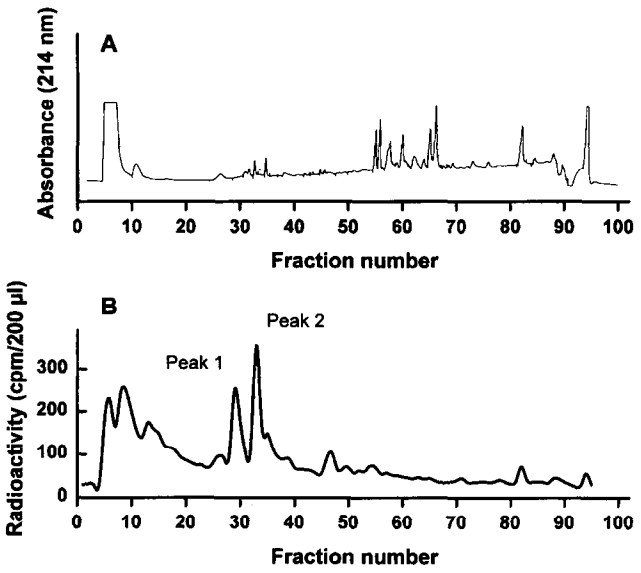

FIG. 4. HPLC profile of a tryptic digest of [^{3}H]AzGSH-labeled human GST π. (A) Absorbance profile of peptides separated on a C_{18} reversed-phase column. Lyophilized protein digest was made up with 0.1% triflouroacetic acid (TFA). After running for 10 min with 0.1% TFA, peptides were eluted with a gradient from 0 to 100% of 0.1% TFA in acetonitrile over 70 min at a flow rate of 0.9 mL/min at room temperature; the detector range was 0.5 absorbance units full scale. (B) Radioactive profile of 1-min (0.9 mL) fractions. Fractions from Peak 1 and Peak 2 were lyophilized and further purified on a C_8 column.

was UV-irradiated three times with AzGSH as described above. The labeled protein was purified and dialyzed as above and the molar ratio of radiolabel to enzyme dimer was 0.07:1, indicating that the enzyme is not labeled when the active sites are occupied by a competitive inhibitor.

Purification and Sequencing of Radiolabeled Peptides from Human GST π

Labeled GST π was hydrolyzed by trypsin, and the digest was chromatographed by reversed-phase-HPLC. Two different labeled peptides were purified from the tryptic digest on a C_{18} reversed-phase column (Fig. 4). Fractions containing the labeled peptides (named Peak 1 and Peak 2 in order of their elution) were pooled separately, and each pool was lyophilized and applied to a C_8 reversed-phase column for further purification. Single peaks of absorbance and radioactivity were obtained for each pool from the C_8 column. The purified peptides were sequenced at the Emory University Microchemical Facility for Molecular Biology. The amino acid sequence of Peak 1 was Leu-Ser-Ala-Arg which corresponds to residues 183–186 of human GST π, and the amino acid sequence of Peak 2 was Leu-Ser-Ala-Arg-Pro-Lys which corresponds to residues 183–188 of GST π (Table 2). We were unable to determine which amino acid(s) had been labeled because the label is lost during sequencing and does not elute in association with any particular amino acid.

Target Size Analysis of Human GST π

The calculated target size for GST π by measurement of loss of enzymatic activity was 22.9 ± 1.3 kDa and by loss of protein was 24.1 ± 6.0 kDa. These two values did not differ significantly (Fig. 5).

DISCUSSION

The physical and kinetic data presented above demonstrated that cloned GST π expressed by and purified from *E. coli* bacteria is identical to GST π isolated from human placentas except that the N-terminal methionine is incom-

TABLE 2. Amino acid sequences of labeled peptides

	Identity (pmol)	
Edman cycle	Peptide Peak 1	Peptide Peak 2
1	Leu (38)	Leu (629, 814)
2	Ser (5.7)	Ser (236, 213)
3	Ala (14)	Ala (373, 676)
4	Arg (2.1)	Arg (184, 307)
5		Pro (412, 308)
6		Lys (243, 114)

Purified radiolabeled peptides from photoaffinity-labeled and trypsin-digested human GST π were identified. Peptide from Peak 2 was sequenced twice, but there was only enough material to sequence peptide from Peak 1 once.

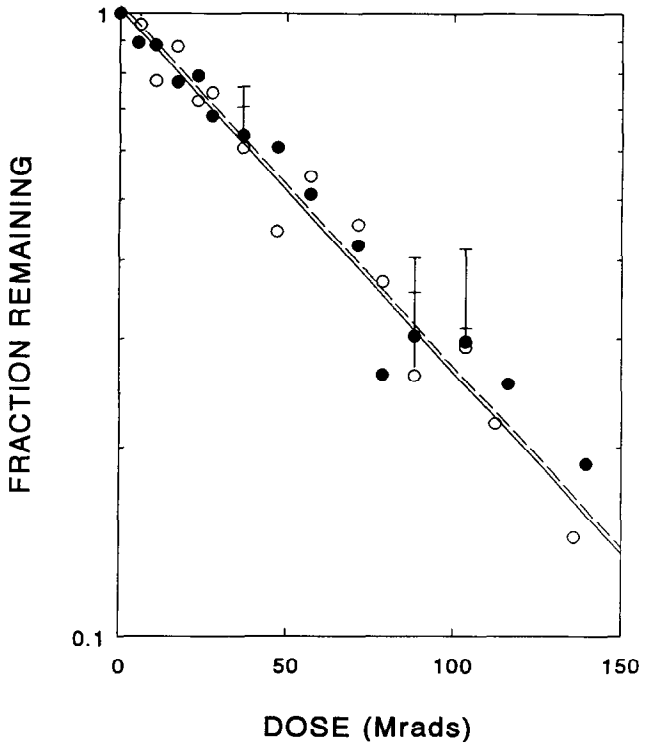

FIG. 5. Radiation target size analysis of human GST π. Purified human GST π was exposed at −135° to various doses of radiation. Enzymatic activity (○) was measured as described in Materials and Methods using 1 mM CDNB and 1 mM GSH as substrates, and protein subunit (●) was measured by SDS–PAGE as described in Materials and Methods. Each value is the mean + SD for three separate experiments. The fractional remaining enzymatic activity or protein monomer has been plotted versus dose of radiation. The data-fitted lines were derived from a least squares analysis as described in Materials and Methods.

pletely removed. We base this conclusion on the similarity of the molecular mass, pI, substrate specificities, and kinetic constants. Expression of other forms of GST in bacteria have also yielded enzymes that are similar to the isozymes extracted from tissues [29]. The affinity-labeled peptide sequence (residues 183–186) purified from human GST π appears to be part of the H-site or hydrophobic substrate binding site of the enzyme. This conclusion is based on the following evidence: (1) AzGSH was a competitive inhibitor of GST π towards both GSH and CDNB, which suggests that AzGSH is binding at the active site, (2) the covalent photoaffinity labeling of GST π by AzGSH was blocked by S-hexyglutathione, which is known to bind to the active site of GST π, (3) the photoaffinity-labeled GST π did not bind to a GSH-agarose affinity column, which suggests that access to the active site is blocked by the affinity label, and (4) the peptides of rat alpha class GSTs labeled previously with AzGSH have been shown subsequently to form part of the H-site of these forms of GST [8, 10, 30–34].

Until recently, the H-sites of the GSTs were not well characterized [8]. The photo-reactive end of AzGSH is attached to a large and very hydrophobic (phenyl) structure that reacts with residues within the H-site of the GSTs. Previously, we used AzGSH to label the active sites of two rat alpha class GSTs, YaYa and YcYc, and found that a portion of the C-terminus of both enzymes (residues 212–218 in YaYa and residues 206–218 in YcYc) was labeled by AzGSH [10]. Confirmation that this region was part of the H-site of alpha class enzymes as well as mu class enzymes was provided by solution of the three-dimensional structures of alpha and mu class proteins [30–34].

In human GST π, the H-site, as determined by crystallography with S-hexylglutathione, is a relatively hydrophobic pocket bounded by residues 8–14 which connect strand β1 to helix αA, the C terminus of the polypeptide, and by residues 33–34 which connect strand β2 to helix αB [9]. The region of GST π labeled in the current study is at the base of the C-terminus region and links the αG and αH helices. In fact, this segment is partially responsible for creating a separation of the αH helix from the other helices in domain II, forming a cavity in domain II. The cavity in domain II was one of three possible H-sites proposed by Reinemer *et al.* [35] based on the three-dimensional structure of porcine lung GST π. The labeling of only this region by AzGSH in the current study establishes with certainty that this region is indeed a portion of the H-site of human GST π. It is interesting that there is an arginine residue (Arg-186) in the peptide purified from photoaffinity-labeled human GST π that was only partially hydrolyzed by trypsin, suggesting that it may have been modified by the affinity label. Also, mutational substitution of Arg-183 of human GST π reduces the enzymatic activity of the mutant to 11% of the wild type, providing further support to the suggestion that this region is involved in the formation of the active site of human GST π [36].

We did not attempt to quantitate rigorously the effect of label incorporation on enzyme inactivation in the present study. Rather, the labeling and purification schemes were designed to ensure specificity of the labeling while reducing nonspecific labeling. Significant label bound noncovalently to the enzyme and required dialysis under denaturing conditions to remove it. Unfortunately, the enzyme failed to renature in an active form so that the relationship between the number of active sites labeled covalently and activity could not be determined. Therefore, to answer the question of whether one active site of GST π can retain catalytic activity if the other site is inactivated, we employed a totally different method, target size analysis by radiation inactivation.

The target size analysis of human GST π demonstrated that the loss of enzymatic activity parallels the loss of individual protein subunits. This finding indicates that individual subunits retain catalytic activity even when the activity of the other subunit has been destroyed by radiation. These results suggest that the complete active site is present in individual subunits, which is consistent with published

structural data [35]. The target size results obtained with human GST π resembled those found with the rat mu class and differed from those found with the rat alpha class, i.e. irradiation of the alpha class enzymes yielded an activity target size of the dimer. Thus, for maintenance of enzymatic activity, the rat alpha class GSTs require two intact subunits [6]. It is important to note that damage to one subunit by radiation does not mean that the two subunits dissociate. In fact, under nondenaturing conditions, the subunits appear to remain associated in a conformation that is close to that of the native enzyme [37]. Thus, the subunit interactions that hold the damaged and undamaged subunits together may be critical to the maintenance of catalytic activity in the undamaged subunit. The difference between the rat alpha and the rat mu and human pi classes of GST does not reflect a simple difference in the extent of the subunit interactions. In the alpha and pi forms of GST, <10 amino acids form salt bridges or hydrogen bonds with the opposite subunit, whereas in a GST mu isozyme 14 amino acids from subunit A interact with 19 amino acids from subunit B [9, 30, 31]. Alternatively, there may be critical amino acids, that are lacking in the alpha class enzymes, which are at the subunit interface of mu and pi class GSTs and which help maintain the undamaged subunit in an active conformation.

Now that the amino acid contacts between subunits of several GSTs have been determined by crystallography, the effects of substitutions of these amino acids on target size, and therefore their importance in maintaining catalytic activity of the subunit, can be determined.

We appreciate the skilled secretarial assistance of L. T. Tucker and the skilled technical assistance of C-O. Evans. Work was supported, in part, by a grant from the NIH (GM-31555) To T. D. Boyer.

References

1. Boyer TD, The glutathione S-transferases: An update. *Hepatology* **9:** 486–496, 1989.
2. Pickett CB and Lu AYH, Glutathione S-transferases: Gene structure, regulation, and biological function. *Annu Rev Biochem* **58:** 734–764, 1989.
3. Coles B and Ketterer B, The role of glutathione and glutathione transferases in chemical carcinogenesis. *CRC Crit Rev Biochem* **25:** 47–70, 1990.
4. Mannervik B and Danielson UH, Glutathione transferases: Structure and catalytic activity. *CRC Crit Rev Biochem* **23:** 283–337, 1988.
5. Mannervik B, Awasthi YC, Board PG, Hayes JD, Di Ilio C, Ketterer B, Listowsky I, Morgenstern R, Muramatsu M, Pearson WR, Pickett CB, Sato K, Wildersten M and Wolf CR, Nomenclature for human glutathione transferases. *Biochem J* **282:** 305–308, 1992.
6. Boyer TD and Kempner ES, Effect of subunit interactions on enzymatic activity of glutathione S-transferases: A radiation inactivation study. *Anal Biochem* **207:** 51–57, 1992.
7. Jakobson I, Warholm M and Mannervik B, The binding of substrates and a product of the enzymatic reaction to glutathione S-transferase A. *J Biol Chem* **254:** 7085–7089, 1979.
8. Dirr H, Reinemer P and Huber R, X-ray crystal structures of cytosolic glutathione S-transferases. Implications or protein architecture, substrate recognition and catalytic function. *Eur J Biochem* **220:** 645–661, 1994.
9. Reinemer P, Dirr HW, Ladenstein R, Huber R, Lo Bello M, Federici G and Parker MW, Three-dimensional structure of class π glutathione S-transferase from human placenta in complex with S-hexlglutathione at 2.8 Å resolution. *J Mol Biol* **227:** 214–226, 1992.
10. Hoesch RM and Boyer TD, Localization of a portion of the active site of two rat liver glutathione S-transferases using a photoaffinity label. *J Biol Chem* **264:** 17712–17717, 1989.
11. Katusz RM, Bono B and Colman RF, Affinity labeling of Cys^{111} of glutathione S-transferase, isoenzyme 1-1, by S-(4-bromo-2,3-dioxobutyl)glutathione. *Biochemistry* **31:** 8984–8990, 1992.
12. Cooke RJ, Bjornestedt R, Douglas KT, McKie JH, King MD, Coles B, Ketterer B and Mannervik B, Photoaffinity labelling of the active site of the rat glutathione transferases 3-3 and 1-1 and human glutathione transferase A1-1. *Biochem J* **302:** 383–390, 1994.
13. Katusz RM and Colman RF, S-(4-Bromo-2,3,-dioxobutyl)-glutathione: A new affinity label for the 4-4 isozyme of the rat liver glutathione S-transferase. *Biochemistry* **30:** 11230–11238, 1991.
14. Katusz RM, Bono B and Colman RF, Identification of Tyr^{115} labeled by S-(4-bromo-2,3-dioxobutyl)glutathione in the hydrophobic substrate binding site of glutathione S-transferase, isoenzyme 3-3. *Arch Biochem Biophys* **298:** 667–677, 1992.
15. Sato K, Satoh H, Tsuchida S, Hatayama I, Shen H, Yokoyama Y, Yamada Y and Tamai K, Specific expression of glutathione S-transferase pi forms in (pre)neoplastic tissues: Their properties and functions. *Tohoku J Exp Med* **168:** 97–103, 1992.
16. Konohana A, Konohana I, Schroeder WT, O'Brien WR, Amagai M, Greer J, Shimizu N, Gammon WR, Siciliano MJ and Duvic M, Placental glutathione-S-transferase-pi mRNA is abundantly expressed in human skin. *J Invest Dermatol* **95:** 119–126, 1990.
17. Kano T, Sakai M and Muramatsu M, Structure and expression of a human class pi glutathione S-transferase messenger RNA. *Cancer Res* **47:** 5626–5630, 1987.
18. Armaud CD, Tsao HS, and Littledike T, Radioimmunoassay of human parathyroid hormone in serum. *J Clin Invest* **50:** 21–34, 1971.
19. Simons PC and Vander Jagt DL, Purification of glutathione S-transferases from human liver by glutathione-affinity chromatography. *Anal Biochem* **82:** 334–341, 1977.
20. Habig WH, Pabst MJ and Jakoby WB, Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* **249:** 7130–7139, 1974.
21. Hoesch RM and Boyer TD, Purification and characterization of hepatic glutathione S-transferases of rhesus monkeys. *Biochem J* **251:** 81–88, 1988.
22. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685, 1970.
23. Morrissey JH, Silver stain for proteins in polyacrylamide gels: A modified procedure with enhanced sensitivity. *Anal Biochem* **117:** 307–310, 1981.
24. Towbin H, Staehelin T and Gordon J, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci USA* **76:** 4350–4354, 1979.
25. Matsudaira P, Sequence from picomole quantities of proteins electroblotted onto polyvinylidene diflouride membranes. *J Biol Chem* **262:** 10035–10038, 1987.

26. Coull JM and Pappin DJC, A rapid flourescent staining procedure for proteins electroblotted onto PVDF membranes. *J Protein Chem* **9:** 259–260, 1990.
27. Harmon JT, Nielsen TB and Kempner ES, Molecular weight determinations from radiation inactivation. *Methods Enzymol* **117:** 65–94, 1985.
28. Kendall RL, Yamada R and Bradshaw RA, Cotranslational amino-terminal processing. *Methods Enzymol* **185:** 398–407, 1990.
29. Wang RW, Pickett CB and Lu AYH, Expression of a cDNA encoding a rat liver glutathione S-transferase Ya subunit in *Escherichia coli*. *Arch Biochem Biophys* **269:** 536–543, 1989.
30. Sinning I, Kleywegt GJ, Cowan SW, Reinemer P, Dirr HW, Huber R, Gilliland GL, Armstrong RN, Ji X, Board PG, Olin B, Mannervik B and Jones TA, Structure determination and refinement of human alpha class glutathione transferase. A1-1, and a comparison with the mu and pi class enzymes. *J Mol Biol* **232:** 192–212, 1993.
31. Ji X, Zhang P, Armstrong RN and Gilliland GL, The three-dimensional structure of a glutathione *S*-transferase from the mu gene class. Structural analysis of the binary complex of isoenzyme 3-3 and glutathione at 2.2-Å resolution. *Biochemistry* **31:** 10169–10184, 1992.
32. Raghunathan S, Chandross RJ, Kretsinger RH, Allison TJ, Penington CJ and Rule GS, Crystal structure of human class mu glutathione transferase GSTM2-2. Effects of lattice packing on conformational heterogeneity. *J Mol Biol* **238:** 815–832, 1994.
33. Penington CJ and Rule GS, Mapping the substrate-binding site of a human class mu glutathione transferase using nuclear magnetic resonance spectroscopy. *Biochemistry* **31:** 2912–2920, 1992.
34. Board PG and Mannervik B, The contribution of the C-terminal sequence to the catalytic activity of GST2, a human alpha-class glutathione transferase. *Biochem J* **275:** 171–174, 1991.
35. Reinemer P, Dirr HW, Ladenstein R, Schäffer J, Gallay O and Huber R, The three-dimensional structure of class π glutathione *S*-transferase in complex with glutathione sulfonate at 2.3 Å resolution. *EMBO J* **10:** 1997–2005, 1991.
36. Manoharan TH, Gulick AM, Puchalski RB, Servais AL and Fahl WE, Structural studies on human glutathione *S*-transferase π. Substitution mutations to determine amino acids necessary for binding glutathione. *J Biol Chem* **267:** 18940–18945, 1992.
37. Potier M, Thauvette L, Michaud L, Giroux S and Beauregard G, Inactivation mechanism of tetrameric β-galactosidase by γ-rays involves both fragmentation and temperature-dependent denaturation of protomers. *Biochemistry* **30:** 8151–8157, 1991.