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ARTICLE *in* PROTEIN SCIENCE · JANUARY 2008

Impact Factor: 2.85 · DOI: 10.1002/pro.5560060105 · Source: PubMed

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Protein Science (1997), 6:43–52. Cambridge University Press. Printed in the USA.

Probing the active site of α-class rat liver glutathione S-transferases using affinity labeling by monobromobimane

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(RECEIVED August 20, 1996; ACCEPTED October 9, 1996)

Abstract

Monobromobimane (mBBr) is a substrate of both μ- and α-class rat liver glutathione S-transferases, with K_m values of 0.63 μM and 4.9 μM for the μ-class isozymes 3-3 and 4-4, respectively, and 26 μM for the α-class isozymes 1-1 and 2-2. In the absence of substrate glutathione, mBBr acts as an affinity label of the 1-1 as well as μ-class isozymes, but not of the α-class 2-2 isozyme. Incubation of rat liver isozyme 1-1 with mBBr at pH 7.5 and 25 °C results in a time-dependent inactivation of the enzyme but at a slower (threefold) rate than for reactions with the μ-class isozyme 3-3 and 4-4. The rate of inactivation of 1-1 isozyme by mBBr is not decreased but, rather, is slightly enhanced by S-methyl glutathione. In contrast, 17β-estradiol-3,17-disulfate (500 μM) gives a 12.5-fold decrease in the observed rate constant of inactivation by 4 mM mBBr. When incubated for 60 min with 4 mM mBBr, the 1-1 isozyme loses 60% of its activity and incorporates 1.7 mol reagent/mol subunit. Peptide analysis after thermolysin digestion indicates that mBBr modification is equally distributed between two cysteine residues at positions 17 and 111. Modification at these two sites is reduced equally in the presence of the added protectant, 17β-estradiol-3,17-disulfate, suggesting that Cys 17 and Cys 111 reside within or near the enzyme's steroid binding sites. In contrast to the 1-1 isozyme, the other α-class isozyme (2-2) is not inactivated by mBBr at concentrations as high as 15 mM. The different reaction kinetics and modification sites by mBBr suggest that distinct binding site structures are responsible for the characteristic substrate specificities of glutathione S-transferase isozymes.

Keywords: affinity labeling; glutathione S-transferase; monobromobimane

Glutathione S-transferases (EC 2.5.1.18) catalyze the nucleophilic attack by the thiol of tripeptide glutathione on a variety of structurally diverse endogenous and xenobiotic substrates (Mannervik & Danielson, 1988; Pickett & Lu, 1989; Coles & Ketterer, 1990; Armstrong, 1991; Rushmore & Pickett, 1993; Wilce & Parker, 1994). This reaction serves as a detoxification pathway and is considered to be one of the biological defense mechanisms against toxic chemicals. In addition, glutathione S-transferases, especially the π class enzymes, are markers for malignant tumors and are also known to participate in mechanisms of their resistance to anticancer drugs (Lee et al., 1989; Sato, 1989; Nakagawa et al., 1990; Tsuchida & Sato, 1992). Therefore, specific glutathione S-transferase inhibitors might find applications in combating resistance of certain tumor cells to anticancer agents (Waxman, 1990; Rushmore & Pickett, 1993).

Glutathione S-transferases are classified into six classes, one microsomal and five cytosolic (α, μ, π, θ, and σ) (Mannervik & Danielson, 1988; Tsuchida & Sato, 1992; Rushmore & Pickett, 1993; Meyer & Thomas, 1995). Each class consists of two or more different subunits that combine to form homodimers or heterodimers. A glutathione binding site and a second xenobiotic binding site are present in every subunit of the dimeric enzyme, which tolerates structural variation in the xenobiotic electrophilic substrates (Mannervik & Danielson, 1988). Isozyme subunits within the same gene class share high sequence homology (≥75%), whereas between classes, the sequence homology is in the range of 25–45%.

In rat liver, there are four major cytosolic glutathione S-transferase subunits: 1, 2, 3, and 4, with 1 and 2 subunits belonging to the α-class, and 3 and 4 subunits to the μ-class. They combine to form four homodimeric isozymes—1-1, 2-2, 3-3, and 4-4—and can also form heterodimers within a given class (Mannervik & Danielson, 1988; Hayes & Pulford, 1995). Among the four rat liver isozymes,

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Abbreviations: mBBr, monobromobimane; mB-Cys, S-mB-cysteine; mB-SG, S-mB-glutathione; NEM-Cys, S-(N-ethylsuccinimido)cysteine; CDNB, 1-chloro-2,4-dinitrobenzene; PTH, phenylthiohydantoin; DPT, N,N′-diphenylthiourea; TFA, trifluoroacetic acid.

Table 1. *Comparison of amino acid sequences in selected regions of isozymes of glutathione S-transferase*

Subunit	Class	Source	Amino acid sequence
A1	α	Human	D^{100}-L-G-E-M-I-L-L-L-P-V^{110}-C^{111}-P-P-E-E-K-D-A-K^{119}
1	α	Rat	D^{100}-L-T-E-M-I-M-Q-L-V-I^{110}-C^{111}-P-P-D-Q-K-E-A-K^{119}
2	α	Rat	D^{100}-I-D-E-I-V-L-H-Y-P-Y^{110}-I^{111}-P-P-G-E-K-E-A-S^{119}
3	μ	Rat	D^{105}-N-R-M-Q-L-I-M-L-C-Y^{115}-N^{116}-P-D-F-E-K-Q-K-P^{124}
4	μ	Rat	D^{105}-T-R-L-Q-L-A-M-V-C-Y^{115}-S^{116}-P-D-F-E-R-K-K-P^{124}

only the crystal structure of the 3-3 isozyme is known (Ji et al., 1992, 1993, 1994); other isozymes are believed to assume similar three-dimensional structures to that of the 3-3 isozyme because X-ray crystallographic studies conducted on enzymes of various classes from different sources indicate that they all share similar topology in their tertiary and quaternary structures (Cowan et al., 1989; Reinemer et al., 1991, 1992; Ji et al., 1992, 1993, 1994; Wilce et al., 1995). However, each of them does have distinct substrate specificity (Mannervik & Danielson, 1988).

We reported recently that mBBr is a substrate of rat liver glutathione S-transferase 3-3 and also acts as an affinity label of this isozyme in the presence of a glutathione analogue, S-methylglutathione (Hu & Colman, 1995). Covalent modification of isozyme 3-3 with mBBr occurs initially at Tyr 115 and modification of this residue correlates with loss of enzymatic activity toward CDNB. The reaction is prevented by the presence of a xenobiotic substrate or analogue in the incubation mixture, suggesting that Tyr 115 is at or near the xenobiotic binding site of the 3-3 isozyme. It was also found that the activity toward mBBr as a substrate is retained by isozyme 3-3 after modification, indicating that rat liver isozyme 3-3 has two binding sites for mBBr: one site that is identical or overlaps with the CDNB site, and another site that is independent of the CDNB site and is catalytically active after modification of the first site. Even though the rat liver α- and μ-isozymes are believed to have similar tertiary and quaternary structures, they share only approximately 17% identity plus 13% similarity when their primary sequences are compared using the PC Gene CLUSTAL program (IntelliGenetics). As shown in Table 1, Tyr 115 is conserved in the glutathione S-transferase μ-class isozymes and in the 2 subunit, but not in the 1 subunit, of the α-isozymes. These differences suggest that the two classes of isozymes have distinct active site structures that are responsible for their characteristic substrate specificities.

The purpose of this study is to compare the α- and μ-class isozymes in their reactions with the affinity label mBBr. We report here that both μ-class isozymes, 4-4 and 3-3, react similarly with mBBr, whereas the α-class isozymes behave quite differently. The 2-2 isozyme is not inactivated by mBBr at concentrations as high as 15 mM, and the 1-1 isozyme is inactivated by mBBr, but at a slower rate and by reaction with different amino acids than the targets of mBBr reactions in the μ-class isozymes.

Results

mBBr as a substrate for rat liver glutathione S-transferases

The formation of the glutathione conjugate with mBBr is catalyzed by glutathione S-transferase 1-1, 2-2, 3-3, and 4-4. Table 2 lists the kinetic constants for mBBr as a substrate of each isozyme at pH 6.5 and 25 °C. mBBr is a substrate of all four rat liver isozymes, but the K_m and V_{max} values for the α-class isozymes are higher than those for the μ-isozymes. These results indicate that mBBr does bind and thus potentially can act as an affinity label for all four rat liver glutathione S-transferases. For comparison, the kinetic constants for CDNB as a substrate for rat liver glutathione S-transferase isozyme 1-1, 3-3, and 4-4 are also given in Table 2.

Inactivation of rat liver glutathione S-transferase 4-4 with mBBr

Incubation of rat liver glutathione S-transferase 4-4 with mBBr at pH 7.5 and 25 °C results in a time-dependent inactivation of the enzyme. Addition of 5 mM S-methyl glutathione increases the observed rate constant of inactivation approximately fivefold, and the combination of S-methyl glutathione and a xenobiotic substrate analogue such as bromosulfophthalein or 2,4-dinitrophenol protects the 4-4 isozyme against mBBr-inactivation approximately 20-fold (Table 3). These results are similar to those we reported for the 3-3 isozyme (Hu & Colman, 1995).

Table 2. *Apparent kinetic constants for monobromobimane as a substrate of rat liver glutathione S-transferases*[a]

Isozyme	K_{mapp} (μM)	V_{maxapp} (μmol/min/mg)	k_{catapp}[b] (s^{-1})	$(k_{cat}/K_m)_{app} \times 10^{-6}$ ($M^{-1}s^{-1}$)
mBBr				
1-1	26 ± 3	87 ± 4	36 ± 1.7	1.38
2-2	26 ± 3	27 ± 1	13 ± 0.5	0.50
3-3	0.63 ± 0.06	3.5 ± 0.1	1.5 ± 0.04	2.38
4-4	4.9 ± 0.5	9.5 ± 0.3	4.1 ± 0.13	0.86
CDNB[c]				
1-1	860	73	31	0.036
3-3	60	66	29	0.48
4-4	193	23	10	0.052

[a] Rat liver glutathione S-transferases (0.25–0.5 μg/mL) were incubated in the presence of 600 μM glutathione and various concentrations of mBBr at pH 6.5 and 25 °C and the product formation was monitored by fluorescence as described in Materials and methods. Data were fitted to the Michaelis–Menten equation to obtain the kinetic constants.

[b] $k_{cat\ app}$ is defined as moles of substrate converted by 1 mol of enzyme in 1 s.

[c] For comparison, the kinetic constants are given for CDNB as a substrate for isozyme 1-1 (Wang et al., 1996), for isozyme 3-3 (Habig et al., 1974), and for isozyme 4-4 (Barycki & Colman, 1993).

Table 3. *Effects of added ligands on the observed rate constant, k_{obs}, of inactivation of rat liver glutathione S-transferase 4-4 by 1 mM monobromobimane*[a]

Ligand added	k_{obs} (min^{-1})	k_{rel}[b]
1 mM mBBr	0.0277	0.18
1 mM mBBr + 5 mM S-methyl glutathione	0.150	0.049
1 mM mBBr + 5 mM S-methyl glutathione + 100 μM bromosulfophthalein	0.00735	0.049
1 mM mBBr + 5 mM S-methyl glutathione + 10 mM 2,4-dinitrophenol	0.00619	0.041

[a]Rat liver glutathione S-transferase 4-4 (0.3 mg/mL) was incubated with 1 mM mBBr in 100 mM potassium phosphate buffer containing 1 mM EDTA at pH 7.5 and 25 °C in the presence of the various ligands shown.
[b]The relative rate constant, k_{rel}, represents the observed pseudo first-order rate constants compared with that in the presence of 5 mM S-methyl glutathione, as described in Materials and methods.

Inactivation of rat liver glutathione S-transferase α-isozymes with mBBr

Isozyme 1-1 was inactivated by mBBr under identical conditions in a time-dependent manner (Fig. 1). Control enzyme, incubated under the same conditions but in the absence of the reagent, showed no change in activity during the same period. The observed rate constant, k_{obs}, for mBBr inactivation of the 1-1 isozyme is approximately threefold lower than that for the μ-class isozymes and exhibits a nonlinear dependence on the concentration of mBBr in the concentration range tested (Fig. 1, inset). Analysis of the concentration-dependence indicates a K_I value of approximately 5.5 mM and a k_{max} of 0.06 min^{-1}. This apparent dissociation constant (5.5 mM) is much greater than the 26 μM Michaelis constant (K_m), suggesting that the site of mBBr reaction in glutathione S-transferase 1-1 might not be the same site as the substrate site, which is reflected in the K_m. However, after isolation the mBBr-modified isozyme 1-1 was found to have the same residual activity toward both CDNB and mBBr as substrates (data not shown).

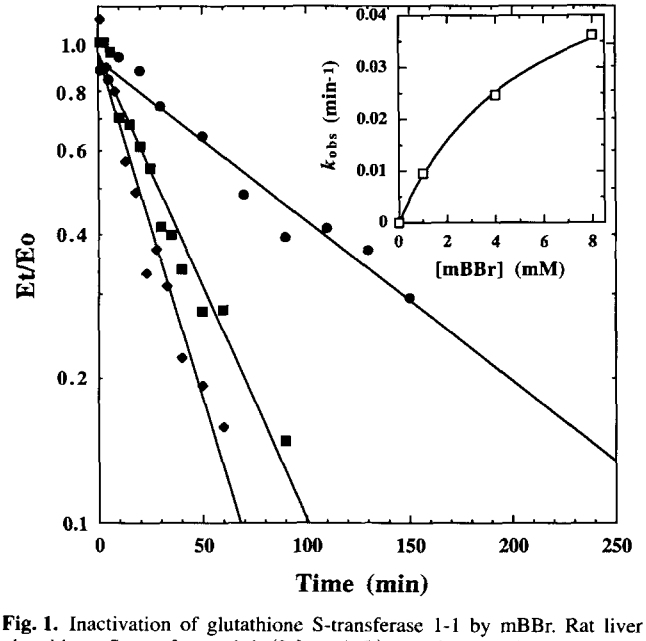

Fig. 1. Inactivation of glutathione S-transferase 1-1 by mBBr. Rat liver glutathione S-transferase 1-1 (0.3 mg/mL) was incubated with various concentrations of mBBr at pH 7.5 and 25 °C. Residual activity E_t/E_0 was measured as described in Materials and methods. The concentrations of mBBr shown are 1 mM (●), 4 mM (■), and 8 mM (◆). **Inset:** Plot of the observed rate constant of inactivation of glutathione S-transferase 1-1 by mBBr versus the concentration of mBBr.

Different from the 1-1 isozyme and the μ-class isozymes is the rat liver glutathione S-transferase 2-2 isozyme. When the 2-2 isozyme was incubated with mBBr at concentrations as high as 15 mM and for as long as 4 h at 25 °C, no loss of activity was observed. Inclusion in the incubation mixture of S-methyl glutathione, which increases the rate of inactivation of other isozymes by mBBr, did not have any effect (data not shown). These results indicate that mBBr is not an affinity label of the 2-2 isozyme, although mBBr does bind to and acts as a substrate of the 2-2 isozyme.

Effect of substrate analogues on the rate of inactivation of glutathione S-transferase 1-1 by mBBr

The results shown in Table 4A and B summarize the effects of substrate analogues on the reaction rate of rat liver glutathione S-transferase 1-1 with 4 mM mBBr. S-Methyl glutathione (5 mM) increases the rate of inactivation by approximately 38% (Table 4A, line 2), much less than the fivefold increase in rate constant of inactivation of rat liver glutathione S-transferase 4-4 (Table 3) and 3-3 isozymes (Hu & Colman, 1995). 17β-Estradiol-3,17-disulfate at 500 μM affords the best protection: a 12.5-fold decrease in the observed rate constant of inactivation by 4 mM mBBr (Table 4A, line 10), whereas other glutathione derivatives and xenobiotic substrates or analogues such as bromosulfophthalein, 2,4-dinitrophenol, Cibacron blue, or Δ^5-androstene-3,17-dione give only limited protection (Table 4A, lines 3–9). Furthermore, the presence of S-methyl glutathione does not enhance the ability of bromosulfophthalein, 2,4-dinitrophenol, or Cibacron blue to protect against inactivation by mBBr (Table 4B, lines 2–4). The fact that S-methyl glutathione does not protect the enzyme from inactivation by mBBr suggests that the reaction does not occur at the glutathione binding site. The best protection by 17β-estradiol-3,17-disulfate and limited protection by other xenobiotic substrates or analogues suggest that the reaction occurs at or near the steroid binding site and xenobiotic binding site, which may overlap with each other.

Incorporation of mBBr into glutathione S-transferase 1-1

The incorporation of bimane into glutathione S-transferase 1-1 was measured from the characteristic absorbance of bimane moiety at 390 nm as described in Materials and methods. Incubation of the enzyme with 4 mM mBBr for 60 min affords a modified enzyme that is 60% inactivated and contains 1.7 mol reagent/mol subunit. Incubation under the same conditions, but with the added protectant 17β-estradiol-3,17-disulfate, yields an enzyme that is only 17% inactivated and contains only 0.79 mol reagent/mol subunit. Incubation at a lower mBBr concentration (1 mM) for 30 min results in enzyme with approximately 0.39 mol reagent/mol subunit incorporation and 18% inactivation, whereas the addition of the protectant 17β-estradiol-3,17-disulfate under the same conditions leads to enzyme with no significant incorporation of mBBr and no loss of enzymatic activity. If the incorporation of mBBr into the enzyme is plotted against % inactivation, extrapolation to a

Table 4. *Effect of added ligands on the observed rate constant, k_{obs}, of inactivation of rat liver glutathione S-transferase 1-1 by 4 mM monobromobimane*[a]

	Ligand added	k_{obs} (min^{-1})	k_{+L}/k_{-L}[b]
A			
1	None	0.026	1.0
2	S-methyl glutathione (5 mM)	0.036	1.38
3	S-hexyl glutathione (5 mM)	0.010	0.37
4	S-(p-nitrobenzyl) glutathione (5 mM)	0.019	0.73
5	Bromosulfophthalein (400 μM)	0.0088	0.34
6	Cibacron blue F3GA (100 μM)	0.014	0.54
7	Cibacron blue F3GA (500 μM)	0.017	0.65
8	2,4-dinitrophenol (10 mM)	0.0072	0.28
9	Δ^5-androstene-3,17-dione (500 μM)	0.013	0.50
10	17β-estradiol-3,17-disulfate (500 μM)	0.002	0.08
B			
1	S-methyl glutathione (5 mM)	0.036	1.0
2	S-methyl glutathione (5 mM) + bromosulfophthalein (400 μM)	0.012	0.33
3	S-methyl glutathione (5 mM) + Cibacron blue F3GA (500 μM)	0.021	0.58
4	S-methyl glutathione (5 mM) + 2,4-dinitrophenol (10 mM)	0.012	0.33
5	S-methyl glutathione (5 mM) + 17β-estradiol-3,17-disulfate (500 μM)	0.002	0.06

[a]Rat liver glutathione S-transferase 1-1 (0.3 mg/mL) was incubated with 4 mM mBBr in 100 mM potassium phosphate buffer containing 1 mM EDTA at pH 7.5 and 25 °C in the presence of the various ligands shown.

[b]The observed pseudo first-order rate constants, k_{+L} and k_{-L}, represent, respectively, measurements in the presence and absence of the indicated ligand.

maximally inactivated enzyme yields an estimate of 2.6 mol reagent/mol subunit (Fig. 2). This plot suggests that inactivation may result from specific modification of two amino acid residues, but that a small amount of reaction occurs at other sites.

Isolation of thermolysin-digested peptides from modified glutathione S-transferase

Glutathione S-transferase (0.3–1.2 mg/mL) was inactivated for 60 min by 4 mM mBBr at pH 7.5 and 25 °C. The resulting modified enzyme, which is 60% inactivated, was isolated, incubated with N-ethylmaleimide, dialyzed, and then digested with thermolysin. The digest was subjected to HPLC separation using a C_{18} column and an acetonitrile gradient in 0.1% TFA, as illustrated in Figure 3A and B. Four peptide regions showing the characteristic bimane absorbance at 390 nm (Fig. 3B) are labeled Ia, IIa, Ib, and IIb.

A substantially protected glutathione S-transferase 1-1 sample, which is 17% inactivated, was prepared by incubation of the rat liver enzyme with 4 mM mBBr in the presence of 500 μM 17β-estradiol-3,17-disulfate, treated, digested with thermolysin, and analyzed in the same way as above. The resulting HPLC pattern is shown in Figure 3C as monitored at 390 nm. The digest of this modified enzyme (Fig. 3C) contains the same four peptides, but in lower amounts, corresponding to peaks Ia, IIa, Ib, and IIb of the unprotected modified enzyme digest (Fig. 3B). These results indicate that peaks Ia, IIa, Ib, and IIb contain modified peptides, whose appearance correlates with inactivation.

The peptides were also analyzed as a function of time of reaction and corresponding degree of inactivation, and in the presence of different protectants. When the reaction time increases, there is an increased degree of inactivation and corresponding increase in amount of modified peptides in peaks Ia, IIa, Ib, and IIb. Various ligands added to the reaction mixture lead to a decreased degree of inactivation and a concomitant decrease in modified peptides (data not shown). In all these cases, the magnitude of the decrease in modified peptide peaks seems to be the same for all four of them.

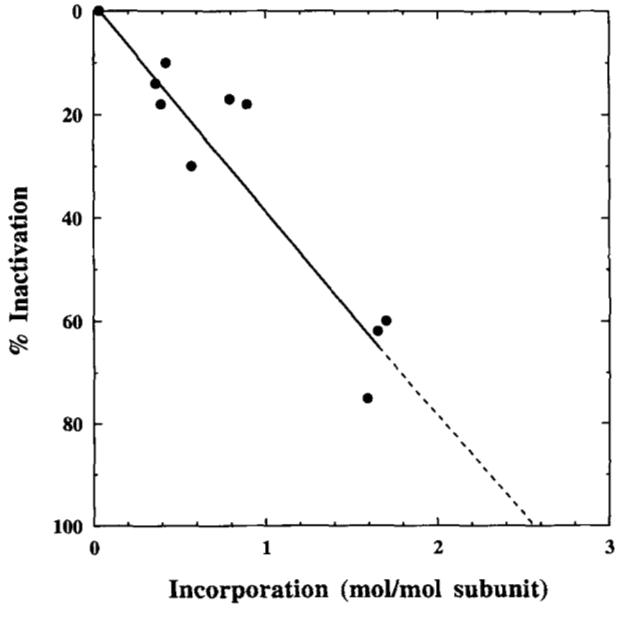

Fig. 2. Inactivation (%) as a function of incorporation for the modification of glutathione S-transferase 1-1 by mBBr. Extrapolation to complete inactivation gives a maximum incorporation of 2.6 mol of reagent/mol of enzyme subunit.

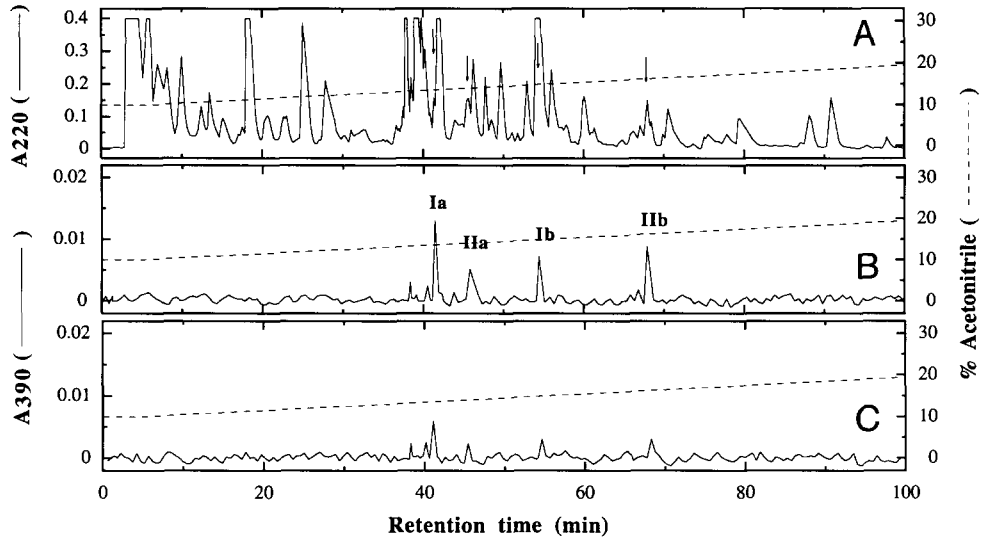

Fig. 3. HPLC separation of proteolytic digests of proteins resulting from the 60-min modification of glutathione S-transferase 1-1 by 4 mM mBBr. The enzyme was modified by 4 mM mBBr at pH 7.5 and 25 °C for 60 min, digested, and separated as described in Materials and methods. **A:** A_{220nm} and **B:** A_{390nm} profiles of digest of the modified enzyme, which is 60% inactivated by mBBr, prepared in the absence of protectant β-estradiol-3,17-disulfate. **C:** A_{390nm} profile of digest of the protected enzyme, which is 17% inactivated by mBBr, prepared in the presence of protectant β-estradiol-3,17-disulfate.

Characterization of modified peptides

As shown in Table 5, peptides Ia and Ib represent different proteolytic products of the same peptide region, in which Cys 17 is modified by mBBr. Peptides IIa and IIb are different proteolytic products, derived from a different region in the sequence, in which Cys 111 is modified by mBBr. To confirm our assignment of modified residues, we synthesized mB-modified cysteine. The PTH derivative of the synthetic mB-Cys yields a characteristic peak appearing between PTH-Tyr and PTH-Pro on a gas-phase sequencer. In addition, there is fluorescence characteristic of a bimane derivative (excitation at 395 nm and emission at 480 nm) in the effluent from the sequencer with the PTH derivative of mB-Cys, but not with other PTH amino acids. Cysteine modification

Table 5. *Representative amino acid sequences of modified peptides present for the inactivated enzyme*[a]

	Amino acid (pmol)			
Cycle #	Peptide Ia	Peptide IIa	Peptide Ib	Peptide IIb
1	Ala 11 (434)	Val 109 (465)	Phe 9 (322)	Leu 108 (336)
2	Arg (60)	Ile (464)	Asn (231)	Val (178)
3	Gly (245)	**mB-Cys 111 (217)**	Ala (218)	Ile (157)
4	Arg (79)	Pro (336)	Arg (57)	**mB-Cys 111 (158)**
5	Met (444)	Pro (302)	Gly (96)	Pro(151)
6	Glu (221)	Asp (254)	Arg (82)	Pro (88)
7	**mB-Cys 17 (82)**[b]	Gln (326)	Met (176)	Asp (117)
8		Arg(110)Lys(18)[c]	Glu (77)	Gln (68)
9		Glu (226)	**mB-Cys 17 (47)**[b]	Lys (70)
10		Ala (274)		Glu 117 (30)[b]
11		Lys (238)		
12		Thr (88)		
13		Ala 121 (37)[b]		
14				

[a]Rat liver glutathione S-transferase 1-1 (0.3 mg/mL) was incubated for 60 min with 4 mM mBBr at pH 7.5 and 25 °C. The modified enzyme was isolated, treated with *N*-ethylmaleimide and digested with thermolysin. Peptides were separated by HPLC on a C_{18} column with 0.1% TFA/acetonitrile/H_2O system (Fig. 3A,B). These sequences are representative and were not all derived from the same thermolysin digest. Thus, the amounts of peptides do not represent the relative magnitude of the peaks shown in Figure 3B.

[b]Peptide ends.

[c]Heterogeneity at position 116 is one of only nine variations found between two clones of rat liver isozyme subunit 1, which are present in the preparations.

by N-ethylmaleimide is demonstrated as a distinct doublet appearing between PTH-Pro and PTH-Met, as reported previously by Smyth and Colman (1991).

Discussion

mBBr acts as a substrate for all four rat liver glutathione S-transferases tested. Overall, mBBr binds better to the enzymes as reflected by their apparent K_ms (Table 2) than the often used xenobiotic substrate CDNB. Like CDNB, mBBr has a higher affinity for the μ-class rat liver isozymes 3-3 and 4-4 than for the α-class isozymes 1-1 and 2-2. These differences in substrate specificity suggest that isozymes of different gene classes have different active site structures. Affinity labeling provides a way of looking at the active site structures chemically (Colman, 1990, 1995).

We reported previously that mBBr acts as an affinity label of a μ-class rat liver glutathione S-transferase 3-3 and that Tyr 115 is the target residue whose modification correlates with loss of enzyme activity (Hu & Colman, 1995).[2] In this paper, we present evidence that mBBr behaves also as an affinity label of both rat liver 4-4 and 1-1 isozymes, but not of the 2-2 isozyme. Because sequence homology between the two μ-class isozymes is very high (78% identity + 9% similarity), and because the active site residue Tyr 115, the target of mBBr modification in isozyme 3-3, is also conserved in the 4-4 isozyme (Table 1), it is not surprising that the kinetics of mBBr reaction with the 4-4 isozyme is similar to that with the 3-3 isozyme. The two tyrosine residues (Tyr 115 in 3-3 and 4-4 isozymes) are, therefore, functionally and structurally equivalent. This result is consistent with other affinity labeling and mutational studies. Affinity labeling studies using S-(4-bromo-2,3-dioxobutyl)glutathione (Katusz & Colman, 1991; Katusz et al., 1992b), 4-(fluorosulfonyl)benzoic acid (Barycki & Colman, 1993), and 2-(S-glutathionyl)-3,5,6-trichloro-1,4-benzoquinone (Ploemen et al., 1994) all implicated the importance of Tyr 115 in the function of rat liver glutathione S-transferases 3-3 and 4-4. Substitution in rat liver 3-3 isozyme of phenylalanine for Tyr 115 by site-directed mutagenesis also suggests that Tyr 115 is a key residue in the enzyme active site, especially during epoxide ring opening and Michael addition reactions (Johnson et al., 1993). For aromatic substitution reactions, Tyr 115 exerts its effect on the rate of product release, which is the rate-limiting step for the reaction of CDNB with glutathione. Johnson et al. (1993) observed in their mutational analysis that replacement of Tyr 115 by phenylalanine leads to a slight increase in enzymatic activity toward CDNB. They postulated that Tyr 115 forms a hydrogen bond to Ser 209, thus blocking product release. In our affinity labeling experiments, modification of this residue in both μ-class isozymes leads to loss of enzymatic activity toward CDNB, suggesting that, due to the size of bimane moiety, the modification blocks CDNB binding to the active site thus preventing the catalytic reaction.

Sequence alignment of rat liver isozymes indicates that the Tyr 115 is also conserved in the 2-2 isozyme and the equivalent residue is Tyr 110 (Table 1). However, the 2-2 isozyme is not inactivated by mBBr at concentrations as high as 15 mM, even though mBBr is a substrate of the enzyme. Therefore, binding to an enzyme is necessary, but not sufficient for the label to act as an affinity label. Our molecular modeling analysis indicates that, although Tyr 110 in the 2-2 isozyme is in approximately the same position at the C-terminal end of helix 4, the side-chain phenolic hydroxyl group of Tyr 110 in the 2-2 isozyme is oriented away from the bromine-bearing carbon center of mBBr, as shown in an overlay of the 2-2 model on the mBBr-docked 3-3 structure (Fig. 4). The distance between the -CH_2Br of mBBr and the -OH of Tyr 115 in the 3-3 isozyme is approximately 3 Å, whereas it is about 5 Å to the -OH of Tyr 110 (2-2 isozyme), which is pointed in the wrong direction for reaction (Fig. 4). The longer distance and unfavorable orientation of Tyr 110 explains why the 2-2 isozyme is not inactivated by mBBr in spite of the fact that mBBr does bind and act as a substrate for this isozyme.

Tyr 115 is not conserved in rat liver 1-1 isozyme and the equivalent residue in this isozyme is Ile 110 (Table 1). Although Ile 110 is not expected to react with mBBr, the neighboring Cys 111 is likely to be modified by mBBr because of the high nucleophilicity of the sulfhydryl group and its exposed orientation, as shown in its three-dimensional model (Fig. 5). Indeed, modification of the 1-1 isozyme by mBBr occurs at this Cys residue (Cys 111) and, surprisingly, also at another cysteine residue at position 17. Cys 17 and Cys 111 are about equally modified by mBBr and there were no other predominant modified residues.

Attempts to react mBBr with isozyme 1-1 under various conditions and in the presence of various protecting ligands failed to give selective modification of one of the two cysteine residues. However, selective modification of Cys 111 can be achieved by maleimide-based reagents such as N-ethylmaleimide and fluorescein-5-maleimide (L. Hu & R.F. Colman, in prep.). Results indicate that the degree of inactivation when modified depends on the size of the modifying reagent. The enzyme, modified by the small reagent, NEM, at Cys 111, only loses approximately 10% of its enzymatic activity, whereas that modified on the same residue by a much bulkier reagent, fluorescein-5-maleimide, loses approximately 85% activity. In a site-directed mutagenesis study (Wang et al., 1992), C17A mutation decreases the catalytic efficiency by lowering the k_{cat}, whereas the C111A mutant has a slightly better catalytic efficiency compared with the wild-type enzyme, indicating that the cysteines are not essential for catalysis. This is consistent with our result that the effects of modification of Cys 17 and Cys 111 on the catalytic activity depend on the size of chemical reagent used. Perhaps large substituents on Cys 17 and Cys 111 protrude into the xenobiotic substrate site or block access to that site.

Because reactions of mBBr at both Cys 111 and Cys 17 are significantly prevented in the presence of 17β-estradiol-3,17-disulfate, these residues are postulated to be near or at the steroid binding site(s). Furthermore, Cys 111 and Cys 17 are also labeled specifically by 3β-(iodoacetoxy)-dehydroisoandrosterone, which acts as a steroid affinity label (Barycki & Colman, 1996). The proposed steroid sites may be close to or overlap the classically defined xenobiotic binding site(s). Results from this laboratory and others support the existence of more than one xenobiotic binding site for glutathione S-transferases (Bhargava et al., 1978; Vander Jagt et al., 1985; Barycki & Colman, 1993; Hu & Colman, 1995).

The recently solved crystal structure of a glutathione S-transferase from parasitic worm *Schistosoma japonica* with an anti-schistosomal drug praziquantel bound establishes that inhibition by the drug praziquantel is not due to binding at the classic substrate binding site, but rather to binding in the dimer interface groove adjoining

[2] In the 3-3 isozyme, we showed previously that mBBr also modifies Cys 114, but this reaction occurs more slowly than the inactivation. The distance between the -CH_2Br of mBBr and the -SH of Cys 114 is 11.2 Å (Fig. 4), and the -SH points away from the active site cavity, making it inaccessible to the active site-bound reagent. We have proposed that Cys 114 may react with another mBBr coming from the outside of the protein molecule (Hu & Colman, 1995).

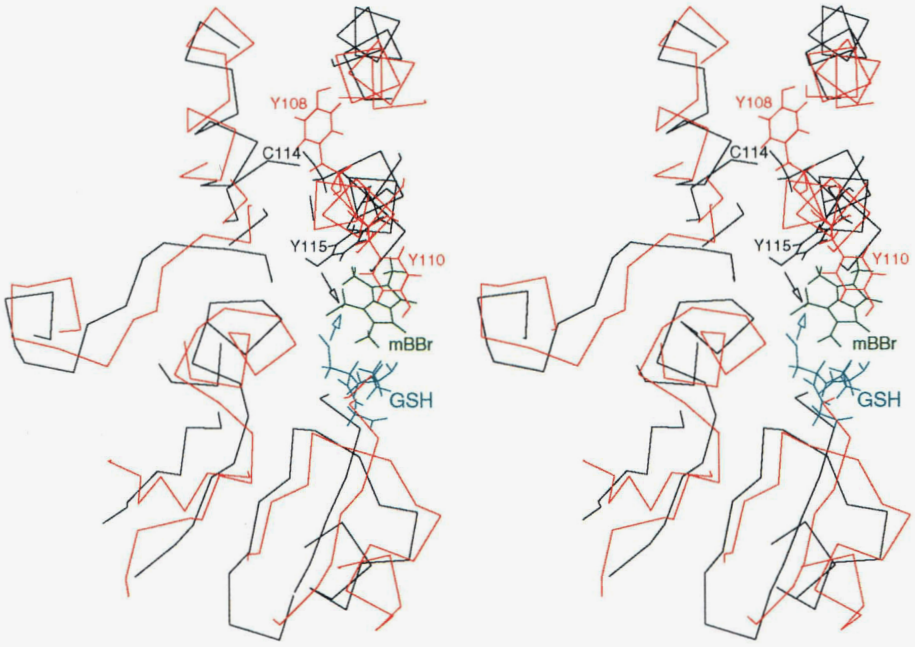

Fig. 4. Stereo view showing the superposition of the structural model of rat liver glutathione S-transferase 2-2 model and the mBBr-docked rat liver 3-3 isozyme structure. The C_α-trace of isozyme subunit 2 (red lines) is superimposed over that of isozyme subunit 3 (black lines) by performing a structure alignment between the C_α-atoms (100 pairs) of residues in the α-helices (RMSD = 2.18 Å for the 100 pairs). Glutathione (GSH) is shown in blue and mBBr in green. Arrows indicate the attack of glutathione on the bromine-bearing carbon of mBBr during catalysis (blue arrow) and of the phenolic hydroxyl of Tyr 115 in isozyme 3-3 during chemical modification (black arrow).

the two catalytic sites (McTigue et al., 1995). This study locates an additional binding site in the glutathione S-transferases. Molecular modeling studies performed in this laboratory indicate that binding to the interface between the two subunits may lead to reactions at the cysteine residues at positions 17 and 111.

mBBr modification of the 3-3 isozyme yields an enzyme that loses its ability to catalyze the glutathione-CDNB reaction, but retains its activity in catalyzing the conjugation between glutathione and mBBr; these results suggest that the 3-3 isozyme has two binding sites for mBBr (Hu & Colman, 1995). In the case of isozyme 1-1, modification by mBBr results in the same degree of inactivation toward both CDNB and mBBr, indicating that CDNB and mBBr share the same substrate binding site in this isozyme. The large difference between the apparent K_m for mBBr as a substrate and the K_I obtained from the inactivation data suggests that the site of inactivation is different from the site for substrate binding, a result consistent with our protection studies discussed above. In Figure 5, the distances between the -CH_2Br of mBBr bound in the active site and the -SH of Cys 111 and Cys 17 (1-1 isozyme) are approximately 10.1 Å and 13.9 Å, respectively. These long distances also indicate that reaction of mBBr with Cys 111 and Cys 17 of the 1-1 isozyme does not occur at the substrate binding site; rather, the reaction targets are more consistent with a location of mBBr at the steroid binding sites of the 1-1 enzyme.

In summary, mBBr, in addition to serving as a substrate for rat liver glutathione S-transferases, also acts as an affinity label for the α-class 1-1 isozyme and the μ-class isozymes 3-3 and 4-4, but not for the α-class 2-2 isozyme. Modification of the 1-1 isozyme occurs at Cys 17 and Cys 111, with concomitant loss of enzymatic activity toward both CDNB and mBBr. Protection against mBBr inactivation of the 1-1 isozyme is best provided by 17β-estradiol-3,17-disulfate, suggesting that Cys 17 and Cys 111 may be within or near the steroid binding sites of glutathione S-transferase isozyme 1-1.

Materials and methods

Materials

Frozen Sprague–Dawley rat livers were purchased from Pel Freez Biologicals, Rogers, Arkansas. Cysteine hydrochloride, glutathione, S-hexyl glutathione, S-methyl glutathione, S-(nitrobenzyl)glutathione, S-hexyl glutathione-Sepharose, bromosulfophthalein, 2,4-dinitrophenol, Sephadex G-50, N-ethylmaleimide, thermolysin, Δ^5-androstene-3,17-dione, 17β-estradiol-3,17-disulfate, and Cibacron blue F3GA were obtained from Sigma Chemical Co., St. Louis, Missouri. CDNB was purchased from Aldrich Chemical Co., Milwaukee, Wisconsin and guanidine·HCl and urea were from ICN Biochemicals, Inc., Irvine, California. mBBr was obtained from Molecular Probes, Inc. Polybuffer exchanger PBE 118 and Pharmalyte® pH 8–10.5 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Hydroxylapatite (Bio-Gel HT) and Bio-Rad Protein Assay dye reagent were supplied by Bio-Rad Laboratories, Hercules, California.

Preparation of S-mB-Cys

mB-Cys was prepared by reacting mBBr with 20-fold excess cysteine at pH 8 as reported previously (Hu & Colman, 1995). The PTH derivative of mB-Cys is a distinct peak appearing between PTH-Tyr and PTH-Pro on an Applied Biosystems gas-phase protein (peptide) sequencer; the amount of mB-Cys was estimated using Met as a standard.

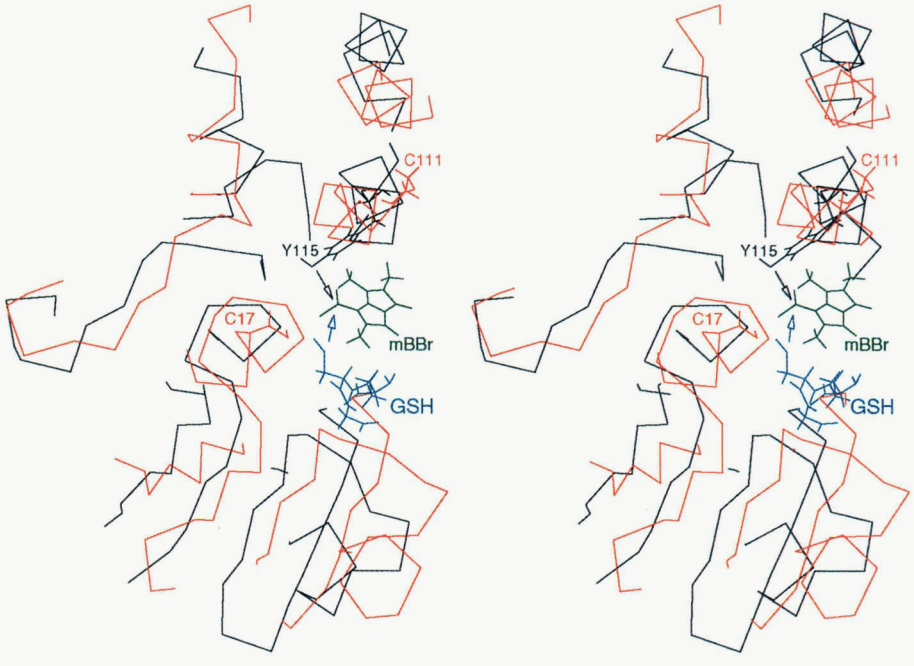

Fig. 5. Stereo view showing the superposition of the rat liver glutathione S-transferase 1-1 model and the mBBr-docked rat liver 3-3 isozyme structure. The C_α-trace of isozyme subunit 1 structure (red lines) is superimposed over that of isozyme subunit 3 (black lines) by performing a structure alignment between the C_α-atoms (100 pairs) of residues in the α-helices (RMSD = 2.67 Å for the 100 pairs). Glutathione (GSH) is shown in blue and mBBr in green.

Enzyme preparation

Rat liver glutathione S-transferases were purified from Sprague–Dawley rat livers by a modified procedure, reported previously (Hu & Colman, 1995), using only affinity column chromatography on S-hexyl glutathione-Sepharose followed by chromatofocusing on PBE 118 resin in the pH range of 10.8–8. The pool of glutathione S-transferases, eluted by S-hexyl glutathione from the affinity column, was loaded, after dialysis against 10 mM Tris·HCl, pH 8.0, onto a chromato-focusing column of PBE 118 equilibrated at pH 10.8. The column was then washed with a Pharmalyte® pH 8–10.5 solution adjusted to pH 8.0 and the isozymes were eluted from the column in the following sequence: 1-1, 1-2, 2-2, 3-3, 3-4, and 4-4. For the 1-1, 3-3, and 4-4 isozymes, protein concentration was measured using ϵ_{270} nm values of 23,000 (Katusz et al., 1992a), 37,700, and 36,700 M^{-1} cm^{-1}, respectively (Graminski et al., 1989). M_r of 25,400, 25,800, and 25,600 per subunit were used in calculations for the 1-1, 3-3, and 4-4 isozymes, respectively (Mannervik, 1985). For the 2-2 isozyme, the protein concentration was determined by the Bio-Rad method using the 1-1 isozyme as the standard. HPLC was used to assess the purity of the final preparation using a 30 min gradient of 30–48% acetonitrile containing 0.1% TFA on a Vydac C_4 column. Based on the absorbance at 280 nm, the major protein peak constitutes more than 95% of each final preparation. The amino-terminal sequences were determined on a gas-phase protein (peptide) sequence analyzer for at least 17 cycles to confirm the identity of each isozyme (Ding et al., 1985; Mannervik, 1985).

Enzymatic assays

Unless otherwise indicated, enzymatic activity was measured using a Gilford model 240 spectro-photometer by monitoring the formation of the conjugate of CDNB (1 mM) and glutathione (2.5 mM) at 340 nm ($\Delta\epsilon$ = 9.6 mM^{-1} cm^{-1}) in 0.1 M potassium phosphate buffer, pH 6.5, at 25 °C according to the method of Habig et al. (1974). All measurements were corrected for the spontaneous nonenzymatic rate of the formation of the conjugate of glutathione and CDNB.

When mBBr was used as a substrate, the enzymatic activity was measured using a Perkin-Elmer MPF-3 fluorescence spectrophotometer (excitation at 395 nm and emission at 480 nm) by monitoring formation of the conjugate of mBBr (30 μM) and glutathione (600 μM) in 0.1 M potassium phosphate buffer, pH 6.5, at 25 °C according to the method of Hulbert and Yakubu (1983) (Hu & Colman, 1995). A lower glutathione concentration was chosen because of the relatively large spontaneous nonenzymatic rate of reaction between glutathione and mBBr. For each experiment, a known amount of mB-SG was prepared in the presence of glutathione S-transferase (0.5 μg/mL) from mBBr (5 μM) and glutathione (600 μM), and used as a fluorescence standard to calibrate and calculate the initial rate of product formation.

The apparent K_m for mBBr was determined at a range of concentrations of mBBr (0.4–100 μM) and a constant concentration of glutathione (600 μM). Data were analyzed using a nonlinear curve-fitting program by fitting directly to the Michaelis–Menten equation.

Reaction of mBBr with glutathione S-transferases

Glutathione S-transferase (0.3 mg/mL) was incubated in 0.1 M potassium phosphate buffer, pH 7.5, at 25 °C with various concentrations of mBBr by the addition of appropriate stock solutions of mBBr in DMF. The volume of DMF was always 10% of the total volume of the reaction mixture. When the effect of ligands on the rate of inactivation was studied, the enzyme was preincubated with the ligands for 10 min prior to the addition of mBBr. In control

experiments, enzyme was incubated under the same conditions, including 10% DMF, but without mBBr. Aliquots of the reaction mixture were withdrawn at various times, diluted 25-fold with 0.1 M potassium phosphate buffer, pH 6.5, at 0 °C, and assayed for residual activity toward CDNB. The rate constant of reaction of the enzyme with mBBr was calculated by fitting data for E_t/E_0 versus time to the pseudo first-order kinetic equation:

$$\frac{E_t}{E_0} = e^{-k_{obs} \cdot t},$$

where E_0 is the activity of the enzyme at time zero, E_t represents the activity at a given time, t and k_{obs} is the observed pseudo first-order rate constant.

In the preparation of modified and control enzyme, excess unreacted reagent was removed from the reaction mixture by the gel filtration procedure of Penefsky (1979). Aliquots (0.5 mL) of the reaction mixture at the end of reaction were applied to two successive 5-mL columns of Sephadex G-50 equilibrated with 0.1 M potassium phosphate buffer, pH 7.5. The protein concentration in the filtrate was determined by the Bio-Rad protein assay, which is based on the dye-binding method of Bradford (1976), using a Bio-Rad 2550 RIA reader (600-nm filter). The corresponding purified glutathione S-transferase isozyme was used to establish the standard protein concentration curve for these determinations.

Measurement of incorporation of mBBr into glutathione S-transferase 1-1

Glutathione S-transferase 1-1 (0.3 mg/mL) was incubated for the indicated time with 1 or 4 mM mBBr in the presence or absence of 500 μM 17β-estradiol-3,17-disulfate or other protectants under standard reaction conditions. Excess reagents were removed by gel filtration 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 390 nm using ϵ_{390} nm = 5,360 M^{-1} cm^{-1}, which is the characteristic absorptivity for the bimane moiety in model compounds such as mB-SG (Kosower & Kosower, 1987). Similar results were obtained when measurements were performed under nondenaturing and denaturing conditions. Incorporation of mBBr into the enzyme was calculated as the number of moles of bimane per mole of enzyme subunit.

Preparation of proteolytic digest of modified glutathione S-transferase

Glutathione S-transferase (0.3–1.2 mg/mL) was incubated for the indicated time with 4 mM mBBr in the presence or absence of 500 μM 17β-estradiol-3,17-disulfate or other protectants under standard reaction conditions. The reaction mixture was then divided into 0.5-mL aliquots and excess reagent was removed by gel filtration as described above. Thiol groups of free cysteine residues in the enzyme were blocked by reaction with N-ethylmaleimide (10 mM) for 5 min under nondenaturing conditions, pH 7.5, at 25 °C, and for an additional 30 min under denaturing conditions in 9 M urea, pH 7.5, at 25 °C. The solution was then dialyzed against 6 L of 50 mM ammonium bicarbonate, pH 8.0, at 4 °C with one change for a total of 20 h.

After dialysis, the solution of modified enzyme was lyophilized. The lyophilized enzyme was solubilized in 8 M urea in 50 mM ammonium bicarbonate (250 μL) by incubation at 37 °C for 2 h, after which 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 thermolysin (5% w/w) for 2 h, followed by an additional 3 h incubation after a second addition of thermolysin (5% w/w). After thermolysin digestion, the digest was lyophilized and stored at −20 °C if not used immediately for HPLC separation of peptides.

Separation of modified peptides by HPLC

The thermolysin digest was separated by HPLC on a Varian 5000 LC equipped with a Vydac C_{18} column (0.46 × 25 cm) and two consecutive UV detectors, one UV-100 detector set at 390 nm and one Vari-Chrom UV detector set at 220 nm. The solvent system used was 0.1% TFA in water (solvent A) and acetonitrile containing 0.07% TFA (solvent B). After elution with 10% solvent B for 5 min, a linear gradient was run to 20% solvent B at 105 min followed by successive linear gradients to 40% solvent B at 135 min, 95% solvent B at 165 min (chromatography system 1). The flow rate was 1 mL/min. The effluent was monitored continuously at both 220 and 390 nm; 1-mL fractions were collected and checked for fluorescence (excitation at 395 nm and emission at 480 nm).

When further purification of peptides was needed, samples were separated using a second solvent system with 20 mM ammonium acetate in water, pH 6.0, as solvent A, and 20 mM ammonium acetate in 90% acetonitrile, pH 6.0, as solvent B. Elution was started with isocratic 10% solvent B for 5 min followed by a linear gradient to 40% solvent B for a total of 125 min at a flow rate of 1 mL/min (chromatography system 2).

Analysis of isolated peptides

An Applied Biosystems gas-phase protein (peptide) sequencer, model 470, equipped with a model 120 phenylthiohydantoin analyzer and a model 900A computer, was used to determine the amino acid sequence of peptides. Cysteine modified by N-ethylmaleimide was identified by a doublet migrating on the HPLC column of the sequencer between the PTH derivatives of Pro and Met (Smyth & Colman, 1991), mB-Cys by a distinct peak appearing between PTH derivatives of Tyr and Pro. In addition, there is measurable fluorescence associated with the PTH derivatives of mB-Cys. The amount of mB-Cys in picomoles was estimated using PTH derivatives of Met as standards.

Molecular modeling

Modeling was conducted using the program Insight II from Biosym Technologies on a Silicon Graphics work station. The molecular model of mBBr was built and energy minimized using the Builder module of the Insight II program. The atomic coordinates of the human glutathione S-transferase A1-1 (1GUH) and rat glutathione S-transferase 3-3 (1GST) were obtained from the Brookhaven Protein Data Bank (Ji et al., 1992; Sinning et al., 1993). The three-dimensional structures of rat liver glutathione S-transferase 1-1 and 2-2 were constructed using the Homology module of the Insight II program to replace residues in the human A1-1 sequence with those of the rat sequence. Various side chains were replaced and positioned in a local energy minimum without disrupting the peptide backbone of the human structure. Once all substitutions were complete, the structures were submitted to the Discover® module for extensive energy minimization using the steepest de-

scent and conjugate gradient methods to relieve residual van der Waals overlaps and to optimize the structures. The docking of mBBr into the rat liver glutathione S-transferase 3-3 isozyme was performed as reported previously (Hu & Colman, 1995).

Acknowledgments

We thank Dr. Yu-Chu Huang for obtaining peptide sequences. This work was supported by United States Public Health Service grant CA 66561. L.H. is the recipient of individual National Research Service Award postdoctoral fellowship F32 CA 66276 from United States Public Health Service.

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