Resonance Energy Transfer between Sites in Rat Liver Glutathione *S*-Transferase, 1-1, Selectively Modified at Cysteine-17 and Cysteine-111†

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ABSTRACT: Monobromobimane (mBBr) can label both Cys^{111} and Cys^{17} of rat liver glutathione *S*-transferase, 1-1 (GST 1-1). However, selective modification of Cys^{111} was achieved by the maleimide-based sulfhydryl reagents *N*-ethylmaleimide (NEM) and fluorescein 5-maleimide (NFM). Incubation of GST 1-1 with 5 mM NEM for 30 min at pH 7.5 and 25 °C leads to the formation of modified enzyme with 92% residual activity toward 1-chloro-2,4-dinitrobenzene and completely blocks Cys^{111} from subsequent reaction with either NFM or mBBr. Reaction of GST 1-1 with 0.2 mM NFM under the same conditions affords a modified enzyme with only 14% residual activity even though NFM and NEM target the same Cys^{111}. The results indicate that when the bulky fluorescein is covalently bound to Cys^{111}, the ligand projects into both the xenobiotic binding site and the glutathione site. After NEM or NFM modification of GST 1-1, the enzyme was further modified by monobromobimane at Cys^{17} with loss of activity. Together with the only tryptophan (Trp^{20}), fluorescein linked to Cys^{111} and bimane to Cys^{17} provide three fluorescent probes to study the solution structure of GST 1-1. Fluorescence spectral analysis suggests that Trp^{20} and bimane linked to Cys^{17} are located in a relatively hydrophobic environment, while fluorescein linked to Cys^{111} is located in a charged environment. These fluorescent probes constitute three sets of donor−acceptor pairs for the measurement of fluorescence energy transfer, and distances calculated from such measurements are 20 Å between Trp^{20} and bimane at Cys^{17}, 19 Å between Trp^{20} and fluorescein at Cys^{111}, and <22 Å between bimane at Cys^{17} and fluorescein at Cys^{111}. Molecular modeling studies indicate that fluorescein lies between the two subunits, is surrounded by charged residues, and is extended into the xenobiotic binding site. They also suggest that mBBr must approach from the dimer interface in order to reach the reaction site at Cys^{17}.

Glutathione *S*-transferases (GSTs,[1] EC 2.5.1.18) are a family of dimeric isozymes involved in the detoxification of both xenobiotic and endogenous compounds. They catalyze the nucleophilic attack of the thiol of glutathione on a diverse range of substrates including aryl halides, alkyl halides, epoxides, and α,β-unsaturated ketones (Armstrong, 1991; Coles & Ketterer, 1990; Mannervik & Danielson, 1988; Rushmore & Pickett, 1993; Wilce & Parker, 1994). The glutathione adducts formed in this reaction are more water soluble and usually less toxic than the electrophilic substrates, thus facilitating their removal from the cell and providing a detoxification pathway for many toxic chemicals. Besides their role in detoxification, GSTs also bind a variety of other hydrophobic nonsubstrate ligands such as steroid hormones, metabolites, and drugs. Their importance as targets for drug design is implicated by their involvement in the mechanism of multiple drug resistance of tumor cells to anticancer drugs (Tsuchida & Sato, 1992).

GSTs exist in the form of homodimers or heterodimers. Cytosolic mammalian GSTs are generally grouped into five gene classes (α, μ, π, θ, and σ) on the basis of their primary sequence similarity, immunological reactivity, isoelectric point, inhibition properties, and substrate specificity (Mannervik & Danielson, 1988; Rushmore & Pickett, 1993; Hayes & Pulford, 1995). Rat liver GST, isozyme 1-1 (hereafter designated as GST 1-1), is a member of the α-class, which includes subunit types 1, 2, 8, and 10 (Armstrong, 1991; Mannervik & Danielson, 1988). GST 1-1 is closely related in its amino acid sequence (77% identity plus 9% similarity) to the human GST A1-1, which is also a member of the α-class. X-ray crystal structures have been reported of enzymes from various gene classes including the human GST A1-1 (Cowan et al., 1989; Ji et al., 1992; Reinemer et al., 1991; Sinning et al., 1993; Wilce et al., 1995). All crystallographic studies indicate that GSTs share similar topology in their tertiary and quaternary structures across gene classes (Sinning et al., 1993; Wilce et al., 1995), although each isozyme exhibits distinct substrate specificity (Mannervik & Danielson, 1988).

We recently reported that monobromobimane (mBBr) is a substrate for rat liver GSTs and acts as an affinity label of rat liver GST 1-1, 3-3, and 4-4 (Hu et al., 1996; Hu &

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[1] Abbreviations: GST, glutathione *S*-transferase; mBBr, monobromobimane; NFM, fluorescein 5-maleimide; NEM, *N*-ethylmaleimide; mB-Cys, *S*-mB-cysteine; mB-SG, *S*-mB-glutathione; NFM-Cys, *S*-[*N*-(5-fluoresceinyl)succinimido]cysteine; NEM-Cys, *S*-(*N*-ethylsuccinimido)cysteine; CDNB, 1-chloro-2,4-dinitrobenzene; DMF, dimethylformamide; PTH, phenylthiohydantoin; DPT, *N*,*N*′-diphenylthiourea; HPLC, high-performance liquid chromatography.

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Colman, 1995). In GST 1-1, mBBr reaction occurs at both cysteine residues at positions 17 and 111 with concomitant loss of enzyme activity toward 1-chloro-2,4-dinitrobenzene. Reactions at both cysteines are protected best by a steroid derivative, 17β-estradiol 3,17-disulfate, suggesting that Cys^{17} and Cys^{111} reside within or near a steroid binding site. No conditions were found which distinguished between the reactions of monobromobimane with these two cysteines of GST 1-1.

In this paper, we report that maleimide-containing sulfhydryl reagents, *N*-ethylmaleimide (NEM) and fluorescein 5-maleimide (NFM), react preferentially with Cys^{111} when GST 1-1 was incubated in the presence of these reagents at pH 7.5 and 25 °C. After modification of Cys^{111} with a maleimide reagent, the Cys^{17} residue can then be modified with mBBr. This procedure allows specific and selective introduction of fluorophores at each of the two cysteines. Together with the intrinsic fluorescent probe, the only tryptophan residue at position 20, they provide three different donor−acceptor pairs to study the solution structure properties of GST 1-1 using fluorescence resonance energy transfer. From the efficiency of such transfer between each donor−acceptor pair, distances between sites within the GST 1-1 enzyme were calculated. Results indicate that fluorescein linked to Cys^{111} is located in a charged environment between the two subunits and that mBBr is more likely to approach and react with Cys^{17} from the dimer interface.

EXPERIMENTAL PROCEDURES

Materials. Frozen Sprague-Dawley rat livers were purchased from Pel Freez Biologicals. Glutathione, *S*-hexylglutathione−Sepharose, Sephadex G-50, N−ethylmaleimide (NEM), and thermolysin were obtained from Sigma Chemical Co. 1-Chloro-2,4-dinitrobenzene (CDNB) was purchased from Aldrich Chemical Co., and guanidine hydrochloride and urea were from ICN Biochemicals, Inc. Monobromobimane (mBBr) and fluorescein 5-maleimide (NFM) were obtained from Molecular Probes, Inc. Polybuffer exchanger PBE 118 and Pharmalyte, pH 8−10.5, were purchased from Pharmacia Fine Chemicals. Bio-Rad Protein Assay dye reagent was supplied by Bio-Rad Laboratories.

Enzyme Preparation. Rat liver glutathione *S*-transferase, 1-1 (GST 1-1), was purified from Sprague-Dawley rat livers by a simplified procedure using only affinity column chromatography on *S*-hexylglutathione−Sepharose followed by chromatofocusing on PBE 118 resin in the pH range of 10.8−8 (Hu et al., 1996). The 1-1 isozyme was eluted first from the chromatofocusing column before other α- and μ-isozymes. In a typical experiment, about 20 mg of 1-1 isozyme was isolated from 160 g of rat liver. The protein concentration was measured using ϵ_{270nm} of 23 000 M^{-1} cm^{-1} (Katusz et al., 1992). A M_r of 25 400 per subunit was used in calculations (Mannervik, 1985). HPLC was used to assess the purity of the final preparation using a 30 min gradient of 30−48% acetonitrile containing 0.1% trifluoroacetic acid on a Vydac C_4 column. On the basis of the absorbance at 280 nm, the major protein peak constitutes more than 95% of each final preparation.

Enzymatic Assays. Enzymatic activity was measured on a Gilford Model 240 spectrophotometer 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 containing 1 mM EDTA, 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 formation of the conjugate of glutathione and CDNB.

To determine the apparent K_m value of glutathione, a range of glutathione concentrations (40−2500 μM) were investigated at a constant CDNB concentration (3 mM). Similarly, the apparent K_m for CDNB was determined at a range of concentrations of CDNB (48−3000 μM) and a constant concentration of glutathione (2.5 mM). Data were analyzed using a nonlinear curve-fitting program by fitting directly to the Michaelis−Menten equation

$$V = \frac{V_{max}C}{K_m + C} \quad (1)$$

where v is the initial velocity, V_{max} is the maximum velocity, K_m is the Michaelis constant, and C is the substrate concentration.

Preparation of Bimane-GST 1-1. Rat liver GST 1-1 (0.3 mg/mL) was incubated for 60 min in 0.1 M potassium phosphate buffer containing 1 mM EDTA, pH 7.5, at 25 °C with 4 mM monobromobimane (mBBr) by the addition of a 40 mM stock solution of mBBr in dimethylformamide (DMF) as reported (Hu et al., 1996). Excess unreacted reagent was removed from the reaction mixture by the gel filtration procedure of Penefsky (1979). 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 purified GST 1-1 was used to establish the standard protein concentration curve for these determinations.

Preparation of N-Ethylmaleimide-Modified GST 1-1 (NEM-GST 1-1). GST 1-1 (1.5 mg/mL) was treated for 30 min with 5 mM NEM in 0.1 M potassium phosphate buffer containing 1 mM EDTA, pH 7.5, at 25 °C containing 10% DMF. NEM-modified enzyme was obtained by removing excess and decomposed reagent through two successive 5-mL columns of Sephadex G-50 equilibrated with 0.1 M potassium phosphate buffer containing 1 mM EDTA, pH 7.5. Where subsequent modification of NEM-GST 1-1 was needed, one Sephadex G-50 column was sufficient to remove excess reagents, and the isolated NEM-GST 1-1 was immediately subjected to a second modification.

Reaction of GST 1-1 with Fluorescein 5-Maleimide. GST 1-1 (0.3 mg/mL) was incubated in 0.1 M potassium phosphate buffer containing 1 mM EDTA, pH 7.5, at 25 °C with various concentrations of fluorescein 5-maleimide (NFM) by the addition of appropriate stock solutions of NFM 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 NFM. In control experiments, enzyme was incubated under the same conditions including 10% DMF, but without NFM. Aliquots of the reaction mixture were withdrawn at various times, diluted 25-fold with 0.1 M potassium phosphate buffer containing 1 mM EDTA, pH 6.5, at 0 °C, and assayed for residual activity toward CDNB. The rate constant of reaction of the enzyme with NFM was calculated by fitting data for $(E_t - E_\infty)/(E_0 - E_\infty)$ vs time to a pseudo-first-order kinetic equation

$$\frac{E_t - E_\infty}{E_0 - E_\infty} = e^{-k_{obs}t} \quad (2)$$

where E_0 is the activity of the enzyme at time zero, E_t represents the activity at a given time, t, E_∞ is the limiting residual activity at $t = \infty$, and k_{obs} is the observed pseudo-first-order rate constant.

In the preparation of fluorescein-GST, GST 1-1 (1.5 mg/mL) was treated for 30 min with 0.2 mM NFM in 0.1 M potassium phosphate buffer containing 1 mM EDTA and 10% DMF at pH 7.5 and 25 °C. The NFM-GST 1-1 was isolated using the same procedure used for NEM-GST 1-1.

Reaction of NEM-Modified or Fluorescein-GST 1-1 with Monobromobimane. NEM- or fluorescein-modified GST 1-1 (0.3 mg/mL) was incubated in 0.1 M potassium phosphate buffer containing 1 mM EDTA, pH 7.5, at 25 °C with 4 mM mBBr. 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. Aliquots of the reaction mixture were withdrawn at various times, diluted 25-fold with 0.1 M potassium phosphate buffer containing 1 mM EDTA, pH 6.5, at 0 °C, and assayed for residual activity toward CDNB. In the preparation of mBBr-modified NEM- or fluorescein-GST 1-1, the same procedure was used as for the preparation of bimane-GST 1-1.

Measurement of Incorporation of mBBr and NFM into GST 1-1. The amount of mBBr and NFM incorporated was determined from the absorbance at 390 and 490 nm, respectively, using $\epsilon_{390nm} = 5360\ M^{-1}\ cm^{-1}$ for mBBr derivatives (Kosower & Kosower, 1987) and $\epsilon_{490nm} = 70\,800\ M^{-1}\ cm^{-1}$ for NFM derivatives (Richter et al., 1985). Similar results were obtained when measurements were performed under nondenaturing and denaturing conditions, except for mB-NFM-GST 1-1, which was done under denaturing conditions. The incorporation of reagents into the enzyme was calculated as the number of moles of reagent per mole enzyme subunit.

Proteolytic Digestion of Modified Glutathione S-Transferase. Modified glutathione *S*-transferase was 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 urea and then digested with thermolysin at 37 °C as described previously (Hu et al., 1996).

Separation of Modified Peptides by HPLC. The peptides after thermolysin digestion were 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% trifluoroacetic acid in water (solvent A) and acetonitrile containing 0.07% trifluoroacetic acid (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 for mBBr-modified peptides; excitation at 440 nm and emission at 530 nm for fluorescein-modified peptides). The excitation maximum of NFM- and NFM-modified compounds is 440 nm at pH 2, which is blue shifted 55−60 nm from that at pH 7.5.

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 (NEM-Cys) was identified by the doublet migrating on the HPLC column of the sequencer between the PTH derivatives of Pro and Met (Smyth & Colman, 1991) and mB-Cys by a distinct peak appearing between PTH derivatives of Tyr and Pro (Hu & Colman, 1995). 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. NFM-Cys is indicated by the absence of amino acid peaks since it remains on the filter and is not detected in the HPLC of the sequencer.

Fluorescence Measurements and Distance Calculations. Fluorescence measurements were made with a Perkin-Elmer MPF-44B fluorescence spectrophotometer thermostated at 25 °C and equipped with a DCSU-2 differential corrected spectra unit and a Hitachi 057 X-Y recorder. Fluorescence spectra were corrected for instrumental errors and buffer background. The quantum yields (ϕ) were determined by the ratio procedure of Chen (1965) using quinine sulfate in 0.1 N H_2SO_4 ($\phi = 0.70$) (Scott et al., 1970) as the fluorescence standard. Another value of 0.55 for the quantum yield of quinine sulfate exists in the literature (Demas, 1982). However, the most commonly adopted value for the quantum yield of quinine sulfate is 0.70 (First & Johnson, 1989; Kung et al., 1994; Bujalowski & Klonowska, 1994).

The steady-state fluorescence polarization of modified enzyme and model compounds was measured at 25 °C with an SLM 4800 spectrophotometer (SLM Instruments, Urbana, IL). The fluorescence polarization, P, is defined from the equation (Chen & Bowman, 1965)

$$P = \frac{I_{vv} - G(I_{vh})}{I_{vv} + G(I_{vh})} \quad (3)$$

where I_{vv} is the fluorescence intensity when both the excitation and emission polarization filters are in the vertical positions, I_{vh} is the intensity when the excitation polarization filter is in the vertical position and the emission polarization filter is in the horizontal position, and $G = I_{hv}/I_{hh}$. I_{hv} is the fluorescence intensity when the excitation polarization filter is in horizontal position and the emission polarization filter is in the vertical position, and I_{hh} is the intensity when both the excitation and emission polarization filters are in the horizontal positions.

The distances between different fluorophores in modified glutathione *S*-transferases were measured in accordance with

the Förster theory of energy transfer. The distance R is related to the efficiency of energy transfer E according to

$$R = R_0\left(\frac{1}{E} - 1\right)^{1/6} \quad (4)$$

where R_0 is the "Förster critical transfer distance" at which E is 50%. The value of R_0 is calculated from specific properties of the energy donor and acceptor by

$$R_0 = (9.79 \times 10^3)(J\kappa^2\phi_D n^{-4})^{1/6} \quad (5)$$

where n is the refractive index of the medium between the donor and acceptor, ϕ_D is the quantum yield of the donor in the absence of acceptor, κ^2 is the orientation factor and accounts for the relative orientation of the donor emission and acceptor absorption transition dipoles, and J is the overlap integral of donor emission and acceptor absorption. The spectral integral J is calculated by using the equation

$$J = \frac{\sum F_D(\lambda)\epsilon_A(\lambda)\lambda^4\Delta\lambda}{\sum F_D(\lambda)\Delta\lambda} \quad (6)$$

and integrating at 2-nm intervals. In the calculation of R_0, the refractive index n has been taken to be 1.4 for the protein solution. The orientation factor κ^2 was assumed to be $^2/_3$, which is a calculated value for donor and acceptor dipoles rotating rapidly compared to the fluorescence lifetime of the donor (Förster, 1959). The efficiency of energy transfer was measured from the quenching of the donor fluorescence in the presence of the acceptor by

$$E = 1 - \frac{\phi_{DA}}{\phi_D} \quad (7)$$

where ϕ_{DA} and ϕ_D are the quantum yields of donor fluorescence in the modified enzyme in the presence and absence of an acceptor, respectively (Fairclough & Cantor, 1978). Corrections were made for less than 1 mol of incorporated acceptor fluorophore per mole of subunit.

Molecular Modeling. Modeling was conducted using the program Insight II from Biosym Technologies on a Silicon Graphics workstation. The molecular models of monobromobimane and NFM were built and energy minimized using the Builder module of the Insight II program. The atomic coordinates of the human GST A1-1 (1GUH) were obtained from the Brookhaven Protein Data bank (Sinning et al., 1993). The three-dimensional structure of rat liver GST 1-1 was constructed using the Homology module of the Insight II program, as described previously (Hu et al., 1996).

Once the GST 1-1 model was obtained, the substrate analogue *S*-benzylglutathione was removed from the model and one fluorescein 5-maleimide was docked into the pocket next to Cys^{111} between the two subunits, while one monobromobimane was docked into the cleft next to Cys^{17} approaching from the dimer interface. During the docking process, intermolecular energy in terms of both van der Waals' and electrostatic interactions and the interatomic distance between respective target sites and reactive centers were continuously monitored for conformations with reasonable distances and potential energies constituting possible productive interactions for chemical modification of respective cysteine sulfhydryl groups. The manually docked complex was merged and bonds formed between respective sites. It was then subjected to the Discover module for extensive energy minimization using the steepest descent and conjugate gradient methods. From inspection of the model, it was clear that a second molecule of NFM and mBBr could be docked into the other subunit.

Table 1: Effect of Substrate Analogues on the Rate Constant for Inactivation of NEM-GST 1-1 by 4 mM Monobromobimane[a]

ligands added to reaction mixture	k_{obs} (min^{-1})	k_{+L}/k_{-L}[b]
1. none	0.032	1.0
2. *S*-methylglutathione (5 mM)	0.041	1.28
3. *S*-hexylglutathione (5 mM)	0.0073	0.23
4. 2,4-dinitrophenol (10 mM)	0.0090	0.28
5. Δ^5-androstene-3,17-dione (500 μM)	0.031	0.97
6. 17β-estradiol 3,17-disulfate (500 μM)	0.0027	0.08

[a] NEM-GST 1-1 (0.3 mg/mL) was incubated with 4 mM mBBr in 0.1 M potassium phosphate buffer containing 1 mM EDTA at pH 7.5 and 25 °C in the presence of the various ligands shown. k_{obs} represents the observed rate constant of inactivation of NEM-GST 1-1 by mBBr assuming a limiting residual activity of 30% as described under Experimental Procedures. [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 as described in Experimental Procedures.

RESULTS

N-Ethylmaleimide (NEM) Inactivation of Glutathione S-Transferase, 1-1 (GST 1-1), and Preparation of NEM-Modified GST 1-1 (NEM-GST 1-1). Rat liver GST 1-1, incubated at pH 7.5 with 5 mM sulfhydryl-selective reagent NEM (Lundblad & Noyes, 1984), undergoes slight inactivation of the enzyme with maximum inactivation reached within 30 min of incubation. This condition was used to prepare NEM-GST 1-1 which was found, after isolation, to have a residual activity of 92% toward CDNB.

Monobromobimane Reaction with NEM-GST 1-1 and Effect of Added Protecting Ligands. The NEM-GST 1-1 was further treated with 4 mM mBBr under conditions in which both Cys^{17} and Cys^{111} in the native GST 1-1 are modified by mBBr (Hu et al., 1996). This treatment of NEM-GST 1-1 leads to further inactivation of the enzyme, yielding enzyme with 30% residual activity. The rate constant of inactivation of 0.032 min^{-1} could be calculated assuming a limiting residual activity of 30%. [The initial rate constant of inactivation, assuming an end point of 0, is 0.022 min^{-1}, about the same as the rate constant of inactivation of native GST 1-1 by 4 mM mBBr under identical conditions (Hu et al., 1996)]. The effect of added protecting ligands into the reaction mixture of mBBr and NEM-GST 1-1 was also investigated. As shown in Table 1, addition of *S*-methylglutathione slightly enhances the rate of inactivation; *S*-hexylglutathione and the xenobiotic substrate analogue 2,4-dinitrophenol afford only limited protection; Δ^5-androstene-3,7-dione has no effect, while 17β-estradiol 3,17-disulfate produces a 12.5-fold decrease in the rate constant of inactivation. This protection pattern is similar to that we observed for the inactivation of native GST 1-1 by mBBr (Hu et al., 1996), indicating that the inactivation is due to reaction at the same site(s) in GST 1-1. Figure 1A (solid line) shows the UV/vis absorption spectrum of mBBr-modified NEM-GST 1-1 (mB-NEM-GST 1-1), which has an absorption maximum around 390 nm, characteristic of the bimane chromophore.

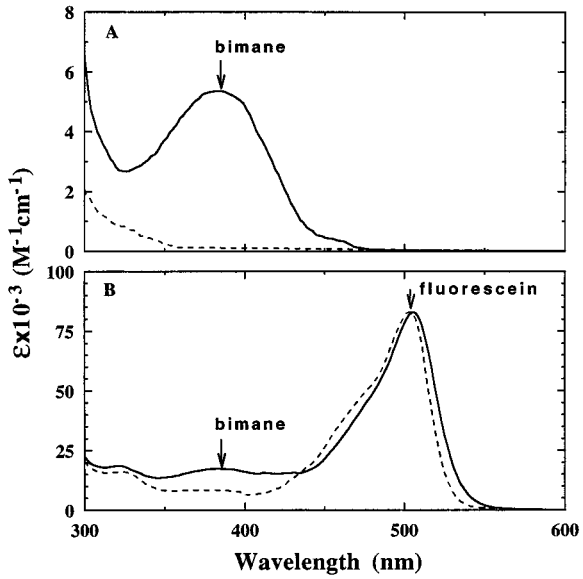

FIGURE 1: UV/vis absorption spectra of modified GST 1−1 in 0.1 M potassium phosphate buffer, pH 7.5 at 25 °C. Panel A: Absorption spectra of mB-NEM-GST 1-1 (—) and NEM-GST 1-1 (- - -) at a concentration of 60 μM. Panel B: Absorption spectra of mB-NFM-GST 1-1 (—) and NFM-GST 1-1 (- - -) at a concentration of 20 μM.

Table 2: Effect of Substrate Analogues on the Rate Constant for Inactivation of NFM-GST 1-1 by 4 mM Monobromobimane[a]

ligands added to reaction mixture	k_{obs} (min^{-1})	k_{+L}/k_{-L}[b]
1. none	0.33	1.0
2. preincubation of NFM at pH 7.5, 25 °C, for 20 min	0.27	0.82
2. *S*-methylglutathione (5 mM)	0.058	0.18
3. *S*-hexylglutathione (5 mM)	0.063	0.19
4. 2,4-dinitrophenol (10 mM)	0.057	0.17
5. Δ^5-androstene-3,17-dione (500 μM)	0.26	0.79
6. 17β-estradiol 3,17-disulfate (500 μM)	0.015	0.045

[a] Rat liver GST 1-1 (0.3 mg/mL) was incubated with 0.2 mM NFM in 0.1 M potassium phosphate buffer containing 1 mM EDTA at pH 7.5 and 25 °C in the presence of the various ligands shown. k_{obs} represents the observed rate constant of inactivation of GST 1-1 by NFM assuming a limiting residual activity of 14% as described under Experimental Procedures. [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 as described in Experimental Procedures.

Fluorescein 5-Maleimide (NFM) Modification of GST 1-1 and Effect of Added Protectant. NFM, an analogue of NEM bearing a bulky fluorescent label, fluorescein, instead of the ethyl group in NEM, inactivates rat liver GST 1-1 to a much greater extent than NEM does. At concentrations as low as 0.2 mM, NFM inactivates GST 1-1 at pH 7.5 and 25 °C with an initial rate constant of 0.33 min^{-1}. The NFM inactivation reaches a limiting residual activity of about 14%; incubation of GST 1-1 in the presence of 0.2 mM NFM for 20 min reaches this limiting residue activity. To ascertain that this limiting residual activity is not due to decomposition of the reagent, we preincubated NFM under the same reaction conditions for 20 min prior to the addition of enzyme and found that this preincubation did not significantly affect the rate of inactivation and the limiting residual activity of the modified enzyme (Table 2, entry 2). As shown in Table 2, *S*-methylglutathione as well as *S*-hexylglutathione and 2,4-dinitrophenol produce a 5-fold decrease in the rate of inactivation by NFM. Δ^5-Androstene-3,17-dione does not significantly affect the inactivation, while 17β-estradiol 3,-17-disulfate causes a 22-fold decrease in the rate constant of inactivation.

Table 3: Residual Activity of Modified GST 1-1 and Incorporation of mBBr and NFM[a]

modified enzyme	residual activity (%)	incorporation (mol/subunit)	
		mBBr	NFM
1. NEM-GST 1-1	92	0	0
2. mB-NEM-GST 1-1	45	0.72	0
3. NFM-GST 1-1	15	0	0.94
4. mB-NFM-GST 1-1	8.7	1.1	0.94

[a] Modified GST 1-1's were prepared by reacting rat liver GST 1-1 with various reagents in 0.1 M potassium phosphate buffer containing 1 mM EDTA at pH 7.5 and 25 °C followed by removal of excess reagent as described in Experimental Procedures.

Since NEM and NFM are both substituted maleimides, we postulated that they would target the same site in GST 1-1. To evaluate this point, we incubated NEM-GST 1-1 (92% active) with 0.2 mM NFM under the same conditions used for the NFM inactivation of native GST 1-1. No further inactivation of the NEM-GST 1-1 occurred, indicating that the target site of NFM in GST 1-1 was previously blocked by NEM.

To prepare fluorescein-GST 1-1 (NFM-GST 1-1), enzyme was treated with 0.2 mM NFM for 30 min and the excess reagent was removed; the isolated fluorescein-GST 1-1 has a residual activity of 15%. Its UV/vis absorption spectrum is shown in Figure 1B (broken line) with an absorption maximum around 505 nm, which is red shifted about 10 nm as compared with that of the mercaptoethanol adduct of NFM at pH 7.5. The unusual absorption spectrum of fluorescein-GST 1-1 must reflect the environment of the chromophore in the native structure of glutathione *S*-transferase, since λ_{max} shifts to about 494 nm upon denaturation of the modified enzyme in 4 M guanidine hydrochloride.

Monobromobimane Reaction with Fluorescein-GST 1-1. Like NEM-GST 1-1, the fluorescein-GST 1-1 (15% active), isolated after incubation of GST 1-1 with 0.2 mM NFM for 30 min, is further inactivated by 4 mM mBBr. Treatment of NFM-GST 1-1 by 4 mM mBBr for 60 min leads to a doubly modified enzyme (mB-NFM-GST 1-1) with 8.7% residual activity. mB-NFM-GST 1-1 is shown to have the absorption characteristics of both bimane and fluorescein (Figure 1B, solid line).

Incorporation of mBBr and NFM. Using the characteristic absorption spectra of bimane and fluorescein, each modified enzyme was measured for the incorporation of reagents under native and/or denaturing conditions. As shown in Table 3, mBBr reaction with NEM-pretreated GST 1-1 leads to a 45% active enzyme (mB-NEM-GST 1-1) with 0.72 mol of bimane incorporated/mol of subunit. NFM modification of GST 1-1 affords a fluorescein-GST 1-1 with 15% residual activity and 0.94 mol of fluorescein incorporated/mol of subunit. Further treatment of the NFM-GST 1-1 with mBBr leads to an additional mole of bimane incorporated per mole of subunit to give the 8.7% active mB-NFM-GST 1-1. These results are consistent with the label being predominantly localized in one single residue in every modified enzyme.

Isolation of Thermolysin-Digested Peptides from Modified GST 1-1 and Identification of Modified Peptides. The thermolysin digests were subjected to HPLC separation using a C_{18} column and an acetonitrile gradient in 0.1% trifluo-

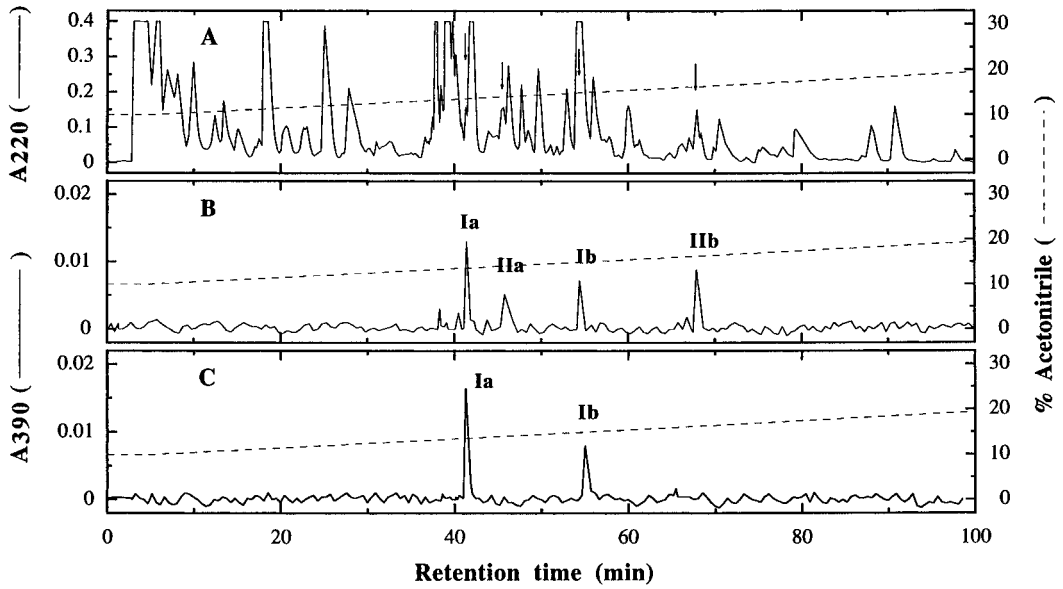

FIGURE 2: HPLC separation of proteolytic digests of mB-GST 1-1 and mB-NEM-GST 1-1. The enzyme without or with NEM treatment prior to modification by 4 mM mBBr at pH 7.5 and 25 °C for 60 min was digested and separated using a C_{18} column and an acetonitrile gradient containing 0.1% trifluoroacetic acid as described under Experimental Procedures. (A) A_{220nm} and (B) A_{390nm} profiles of a digest of mB-GST 1-1, which was prepared without NEM treatment. (C) A_{390nm} profile of a digest of mB-NEM-GST 1-1, which was prepared with NEM treatment prior to mBBr modification.

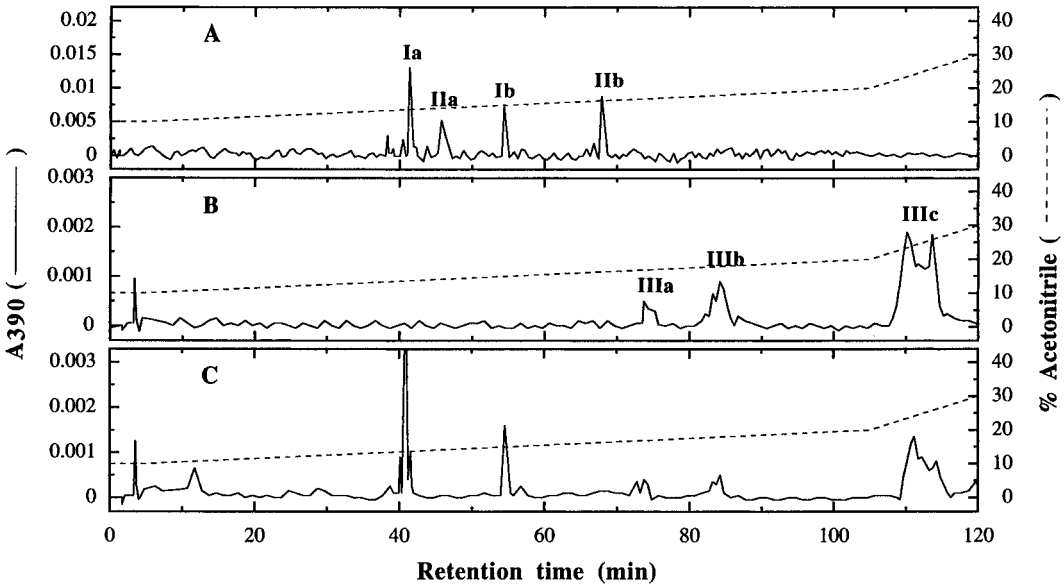

FIGURE 3: HPLC separation of proteolytic digests of bimane-GST 1-1, fluorescein-GST 1-1, and mB-NFM-GST 1-1. The modified enzyme was digested and separated using a C_{18} column and an acetonitrile gradient containing 0.1% trifluoroacetic acid as described under Experimental Procedures. (A) A_{390nm} profile of a digest of mB-GST 1-1, which is prepared by modification of GST 1-1 by 4 mM mBBr at pH 7.5 and 25 °C for 60 min. Fractions corresponding to peaks Ia, IIa, Ib, and IIb are fluorescent when exciting at 395 nm and measuring emission at 480 nm, characteristic of a bimane derivative. (B) A_{390nm} profile of a digest of NFM-GST 1-1, which is prepared by reacting GST 1-1 with 0.2 mM NFM at pH 7.5 and 25 °C for 30 min. Fractions corresponding to peaks IIIa, IIIb, and IIIc are fluorescent when exciting at 440 nm and measuring emission at 530 nm, characteristic of a fluorescein derivative at pH 2. (C) A_{390nm} profiles of a digest of mB-NFM-GST 1-1, which is prepared by modification of NFM-GST 1-1 by 4 mM mBBr at pH 7.5 and 25 °C for 60 min. Ia and Ib have the characteristic fluorescence of a bimane derivative (excitation at 395 nm and emission at 480 nm), and IIIa, IIIb, and IIIc have the characteristic fluorescence of a fluorescein derivative at pH 2 (excitation at 440 nm and emission at 530 nm).

roacetic acid as illustrated in Figures 2 and 3. The modified peptides were followed using the characteristic UV absorption and fluorescence properties of bimane and fluorescein. Both bimane and fluorescein were monitored at 390 nm for absorption. The fluorescence of bimane was also monitored by exciting at 395 nm and measuring emission at 480 nm; the fluorescence of fluorescein was monitored by exciting at 440 nm and measuring emission at 530 nm. It is noteworthy that the fluorescence excitation and absorption spectra of fluorescein are pH dependent: at pH 7.5 the excitation and absorption maxima are centered around 495 nm and the emission maximum around 530 nm, but at pH 2 the excitation and absorption maxima are blue shifted to 440 nm while there is no change in the emission maximum. This pH-dependent change in spectral properties is known for all fluorescein derivatives, and this characteristic has been used to measure intracellular pH (Babcock, 1983; Graber et al., 1986).

The HPLC pattern of bimane-GST 1-1 (mB-GST 1-1), prepared by reacting GST 1-1 with 4 mM mBBr but without

Table 4: Representative Amino Acid Sequences of NFM-Modified Peptides Present for the NFM-Inactivated Enzyme[a]

cycle no.	amino acid (pmol) peptide IIIa	peptide IIIb	peptide IIIc (early)[b]	peptide IIIc (late)[b]
1	Val(49)	Val(86)	Leu(159)	Leu(343)
2	Ile(38)	Ile(80)	Val(183)	Val(221)
3	**NFM-Cys**	**NFM-Cys**	Ile(143)	Ile(234)
4	Pro(35)	Pro(53)	**NFM-Cys**	**NFM-Cys**
5	Pro(64)	Pro(52)	Pro(93)	Pro(176)
6	Asp(38)	Asp(57)	Pro(166)	Pro(170)
7	Gln(27)	Gln(41)	Asp(106)	Asp(125)
8	Lys(29)	Lys(31)	Gln(130)	Gln(144)
9	Glu(39)[c]	Glu(47)	Lys(74)	Lys(82)
10		Ala(38)	Glu(125)	Glu(69)[d]
11		Lys(15)	Ala(98)	Ala(22)
12		Thr(15)[c]	Lys(128)	Lys(12)
13			Thr(106)	Thr(10)
14			Ala(61)[c]	Ala(6)[c]

[a] Rat liver GST 1-1 (1.2 mg/mL) was incubated for 30 min with 0.2 mM NFM at pH 7.5 and 25 °C. The modified enzyme was isolated and digested with thermolysin. The peptides were separated by HPLC on a C_{18} column with a 0.1% trifluoroacetic acid/acetonitrile/H_2O system (Figure 3B). 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 IIIc (early) and peptide IIIc (late) refer to the early and late fractions of peak IIIc. [c] Peptide ends. [d] Peptide may end here.

NEM or NFM pretreatment, is shown in Figure 2A,B together with that of mB-NEM-GST 1-1 (Figure 2C). We have determined in an earlier paper that peptides Ia and Ib are peptides containing mBBr-modified Cys^{17} and peptides IIa and IIb are derived from a different region containing mBBr-modified Cys^{111} (Hu et al., 1996). As shown in Figure 2C, reaction of GST 1-1 with NEM, prior to mBBr modification, eliminated peptides IIa and IIb while retaining peptides Ia and Ib. These results indicate that NEM reaction occurs specifically at Cys^{111}, thus blocking further reaction of this residue with mBBr, and that Cys^{17} is still free after NEM treatment and is available for subsequent modification by mBBr.

The HPLC patterns resulting from thermolysin digestion of fluorescein-GST 1-1 and mB-NFM-GST 1-1 are shown in Figure 3B,C, respectively, together with that of bimane-GST 1-1 (Figure 3A). Peptides IIIa, IIIb, and IIIc are all derived from the peptide region containing an NFM-modified Cys111 (Table 4). The mB-NFM-GST 1-1 digest was found to contain peptides Ia and Ib and peptides IIIa, IIIb, and IIIc, indicating that NFM, like NEM, targets specifically the cysteine residue at position 111.

Comparison of Kinetic Properties of Native and NEM- and NFM-Modified Enzymes. The catalytic properties of NEM- and NFM-modified enzymes were investigated using CDNB and glutathione as substrates. Table 5 shows the kinetic properties of NEM- and NFM-GST 1-1 as compared to those of native GST 1-1. Although NEM and NFM target the same Cys^{111} in GST 1-1, the resulting modified enzymes exhibit different catalytic activities. NEM modification has little effect on k_{cat} and the apparent K_m values, while the bulkier NFM dramatically increases the K_m's and decreases the k_{cat}, with the effect on CDNB affinity being greater than on glutathione. These results indicate that the size of the chemical label is an important factor in affecting the residual activity of the modified enzyme.

Table 5: Apparent Kinetic Constants of Rat Liver GST 1-1, NEM-GST 1-1, and NFM-GST 1-1

enzyme/ substrate	$K_{m\ app}$ (μM)	$V_{max\ app}$ [μmol/(min mg)]	$k_{cat\ app}$[a] (s^{-1})	$(k_{cat}/K_m)_{app} \times 10^{-6}$ ($M^{-1}\ s^{-1}$)
GST 1-1				
GSH	120	78	33	0.28
CDNB	450	80	34	0.076
NEM-GST 1-1				
GSH	140	65	28	0.20
CDNB	500	70	30	0.060
NFM-GST 1-1				
GSH	450	7	3.0	0.0067
CDNB	>3000			

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

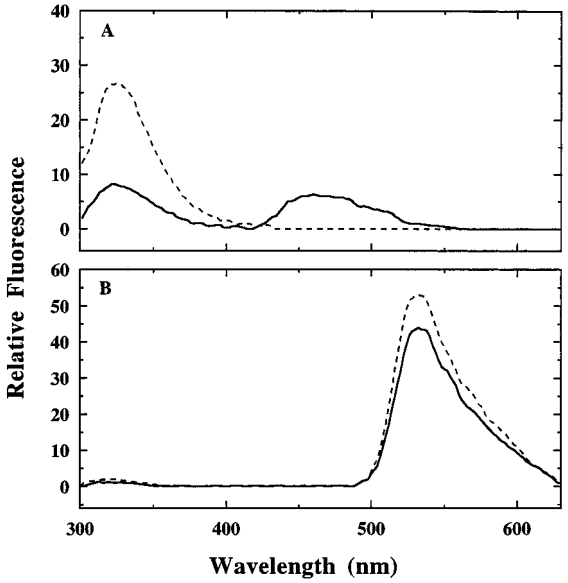

FIGURE 4: Corrected fluorescence emission spectra of modified enzymes under native conditions in 0.1 M potassium phosphate buffer containing 1 mM EDTA, pH 7.5 at 25 °C, when excited at 295 nm. Panel A: NEM-GST 1-1 at 30 μg/mL (- - -) and mB-NEM-GST 1-1 at 15 μg/mL (—). Panel B: NFM-GST 1-1 at 7.6 μg/mL (- - -) and mB-NFM-GST 1-1 at 8.7 μg/mL (—).

Fluorescence Spectral Properties of Modified GST 1-1. The fluorescence emission spectra of modified enzymes are shown in Figure 4. The emission maximum of Trp^{20} in NEM-GST 1-1 is around 325 nm, about 25 nm lower than free tryptophan in solution. The emission maxima of bimane and fluorescein in mB-NEM-GST 1-1 and NFM-GST 1-1 are 460 and 530 nm, respectively. The absence of bimane emission in mB-NFM-GST 1-1 suggests that there is substantial fluorescence energy transfer between bimane and fluorescein in mB-NFM-GST 1-1.

Calculation of R_0's. The overlap between the emission spectrum of the donor, either Trp^{20} or bimane at Cys^{17}, and the absorption spectrum of the acceptor, bimane at Cys^{17} or fluorescein at Cys^{111}, is shown in Figure 5. The respective overlap integrals were calculated to be 4.6×10^{-15} $cm^3\ M^{-1}$ for the donor−acceptor pair of Trp^{20} and bimane at Cys^{17}, 1.6×10^{-14} $cm^3\ M^{-1}$ for the donor−acceptor pair of Trp^{20} and fluorescein at Cys^{111}, and 2.1×10^{-13} $cm^3\ M^{-1}$ for the donor−acceptor pair of bimane at Cys^{17} and fluorescein at Cys^{111}. The quantum yields for donor fluorophores in the absence of an acceptor, i.e., Trp^{20} in NEM-GST 1-1 and bimane at Cys^{17} in mB-NEM-GST 1-1, were determined to be 0.11 and 0.18, respectively, using quinine sulfate in 0.1

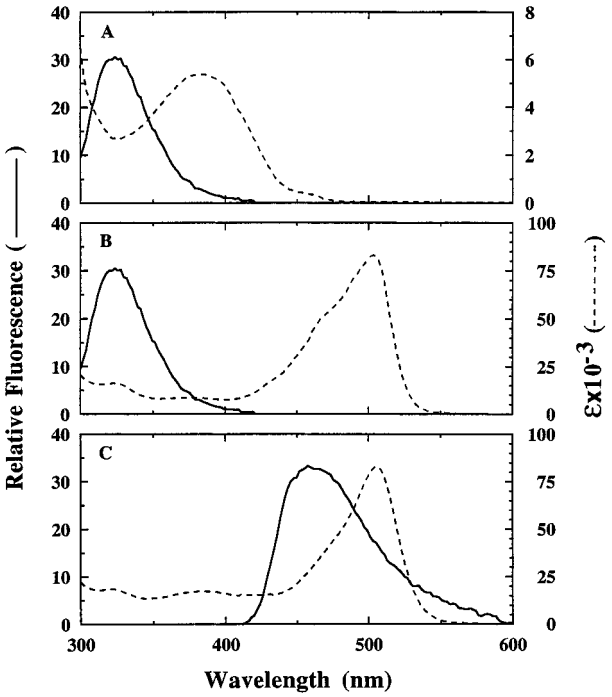

FIGURE 5: Spectral overlap between the corrected emission spectrum of the donor (—) and the absorbance spectrum of the acceptor (- - -) in 0.1 M potassium phosphate buffer containing 1 mM EDTA, pH 7.5 at 25 °C. The concentrations of the modified enzymes are between 0.3 and 1 μM for fluorescence and between 20 and 60 μM for absorption measurements. Panel A: Spectral overlap between the corrected emission spectrum of donor Trp^{20} (exciting at 295 nm) in NEM-GST 1-1 and the absorbance spectrum of acceptor bimane at Cys^{17} in mB-NEM-GST 1-1. Panel B: Spectral overlap between the corrected emission spectrum of donor Trp^{20} (exciting at 295 nm) in NEM-GST 1-1 and the absorbance spectrum of acceptor fluorescein at Cys^{111} in NFM-GST 1-1. NEM-GST 1-1 has no significant absorption above 300 nm. Panel C: Spectral overlap between the corrected emission spectrum of donor bimane at Cys^{17} (exciting at 390 nm) in mB-NEM-GST 1-1 and the absorbance spectrum of acceptor fluorescein at Cys^{111} in mB-NFM-GST 1-1.

Table 6: Energy Transfer between a Donor and an Acceptor in Modified GST 1-1[a]

donor−acceptor pair	$J \times 10^{14}$ (cm^3 M^{-1})	ϕ_D[b]	R_0 (Å)	E	R (Å)
Trp^{20} and bimane at Cys^{17}	0.46	0.11	21	0.58	20
Trp^{20} and fluorescein at Cys^{111}	1.6	0.11	25	0.86	19
bimane at Cys^{17} and fluorescein at Cys^{111}	21	0.18	42	>0.98	<22

[a] The overlap integral J was calculated using eq 6. The Förster critical distances (R_0) were calculated using eq 5 assuming a κ^2-factor of $^2/_3$ and an n of 1.4. Energy transfer efficiency (E) was determined by steady-state quenching using eq 7 and corrected for the less than 1 mol incorporation of acceptor per subunit. Distances (R) were calculated using eq 4 between Trp^{20} and bimane at Cys^{17} in mB-NEM-GST 1-1, between Trp^{20} and fluorescein at Cys^{111} in NFM-GST 1-1, and between bimane at Cys^{17} and fluorescein at Cys^{111} in mB-NFM-GST 1.1. Protein concentrations were from 0.3 to 1 μM. [b] Quantum yields for donor only ($\phi = 0.70$ as the standard). An alternative value of 0.55 exists in the literature for the quantum yield of the quinine sulfate standard. If this value is used, both the R_0 and R distances calculated are only aobut 1 Å lower.

N H_2SO_4 as the standard (see footnote *b* in Table 6). If the fluorophores rotate isotropically and rapidly relative to their fluorescence lifetimes, κ^2 assumes a value of $^2/_3$, and the resulting Förster critical distances (R_0) calculated from these measurements are 21 Å for the pair of Trp^{20} and bimane at Cys^{17}, 25 Å for the pair of Trp^{20} and fluorescein at Cys^{111}, and 42 Å for the pair of bimane at Cys^{17} and fluorescein at Cys^{111} (Table 6).

Determination of Efficiency of Fluorescence Resonance Energy Transfer. Analysis of donor steady-state quenching (300−430 nm) exciting at 295 nm yielded an efficiency of fluorescence energy transfer of 0.58 between Trp^{20} and bimane at Cys^{17} and of 0.86 between Trp^{20} and fluorescein at Cys^{111} after correction for less than 1 mol of incorporation of acceptor per subunit. The fluorescence energy transfer between bimane at Cys^{17} and fluorescein at Cys^{111} was found to be nearly quantitative (>0.98), as no significant fluorescence between 400 and 500 nm was observed when exciting at 390 nm, the excitation maximum of bimane. The distances between Trp^{20}, bimane at Cys^{17}, and fluorescein at Cys^{111} were calculated as described in Experimental Procedures and are listed in Table 6.

Fluorescence Polarization Measurements. The mobility of the fluorescent probes in or attached to GST 1-1 was estimated by measuring the steady-state polarization of fluorescence. Results are shown in Table 7. For comparison, the polarization of fluorophores attached to glutathione or just tryptophan itself in solution was also measured. The P values of these freely rotating fluorophores are 1−2 orders of magnitude lower than of probes attached to GST 1-1, indicating that fluorophores in GST 1-1 are relatively less mobile, as expected for fluorescent probes attached to large proteins.

Table 7: Fluorescence Polarization of Fluorophores in Modified GST 1-1 and Model Compounds[a]

sample	λ_{ex} (nm)	λ_{em} (nm)	P
Trp^{20} in NEM-GST 1-1	295	330	0.30
bimane at Cys^{17} in mB-NEM-GST 1-1	390	460	0.28
fluorescein at Cys^{111} in NFM-GST 1-1	500	530	0.32
tryptophan	295	350	0.0051
glutathione modified by mBBr	390	480	0.0056
glutathione modified by NFM	490	530	0.027

[a] Modified enzymes were prepared as described under Experimental Procedures. Modified glutatione samples were prepared by reacting 20 μM solutions of mBBr and NFM with a 25-fold excess glutathione at pH 7.5 and 25 °C overnight. Tryptophan, glutathione modified by mBBr, and NFM were measured at 20, 2, and 1 μM, respectively. Modified enzyme samples are at 0.6 μM.

DISCUSSION

Monobromobimane (mBBr) acts as a substrate and an affinity label of rat liver GST 1-1, and sites of modification corresponding to loss of enzyme activity were identified as Cys^{17} and Cys^{111} (Hu et al., 1996). In seeking to selectively introduce a fluorescent label at one of the two cysteines, we found that the maleimide-based sulfhydryl-selective reagent, *N*-ethylmaleimide (NEM), reacts preferentially at Cys^{111}. This lead us to believe and we have demonstrated that fluorescein 5-maleimide (NFM), a reagent bearing the same reactive maleimide group, targets the same Cys^{111} residue in GST 1-1. This provides us with a means of introducing a fluorescent probe specifically at this position. After modification of the enzyme with either NEM or NFM, the enzyme can still be modified at Cys^{17} by mBBr. Since there are spectral overlaps between the emission spectrum of the only intrinsic tryptophan residue and the absorption spectra of both bimane and fluorescein and between the emission spectrum

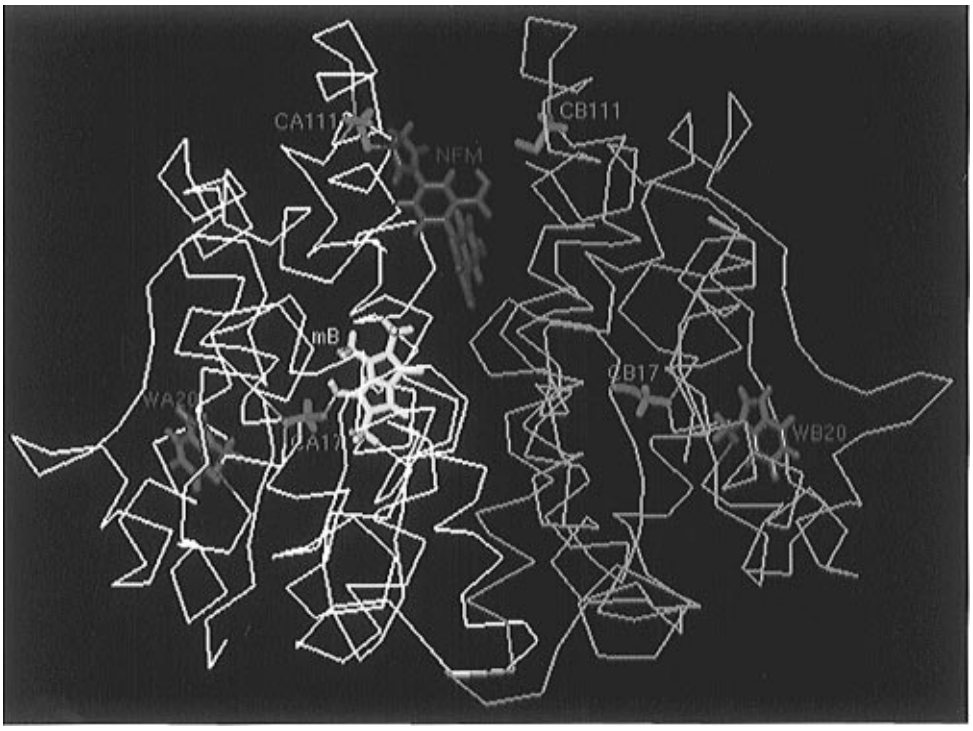

FIGURE 6: Molecular model of modified GST 1-1 showing one possible orientation of bimane linked to Cys^{17} and of fluorescein to Cys^{111} of subunit A (white) and an unmodified subunit B (orange) as a control. Bimane is shown in yellow, fluorescein is in red, cysteines are in green, and the intrinsic tryptophan is in pink.

of bimane and the absorption spectrum of fluorescein, fluorescence resonance energy transfer between these probes can provide information about the solution structure properties of GST 1-1 (Fairclough & Cantor, 1978). This approach has been used to measure distances between ligand binding sites in other systems including chloroplast coupling factor 1, isocitrate dehydrogenase, glutamate dehydrogenase, the cAMP-dependent protein kinase catalytic subunit, and glutamine synthetase (Cantley & Hammes, 1975; Bailey & Colman, 1987; Jacobson & Colman, 1984; Lark & Colman, 1990; First et al., 1989; Maurizi et al., 1986).

The effects of modification by these labels on enzymatic activity of glutathione *S*-transferase were measured, and results clearly indicate a dependence on the size of the label. When the enzyme is modified by the smaller reagent, NEM, it retains 92% of its original activity. Modification by NFM, in which a bulky fluorescein replaces the ethyl group in NEM, reduces the enzymatic activity to 15% even though the reaction occurs at the same Cys^{111} residue. The presence of 2,4-dinitrophenol, or a glutathione derivative including *S*-methylglutathione, decreases the rate of NFM inactivation by about 5-fold. It is interesting that *S*-methylglutathione (the smallest glutathione analogue) protects the enzyme against inactivation by NFM but slightly enhances the rate of inactivation of GST 1-1 by mBBr, which targets Cys^{111} as well as Cys^{17} (Hu et al., 1996). This difference in the effect of *S*-methylglutathione on the inactivation rate observed with the two reagents suggests that, in contrast to mBBr, the bulkier reagent NFM not only occupies the xenobiotic binding site but also projects into (or at least closer to) the glutathione binding site. This orientation effectively interferes with access of glutathione and/or xenobiotic substrates to the active site.

Whether the enzyme is modified by mBBr at both Cys^{17} and Cys^{111} (Hu et al., 1996) or just at Cys^{17} (Table 1) or by NFM at Cys^{111} (Table 2), the best protectant has consistently been 17β-estradiol 3,17-disulfate, which reduces the rate constant of inactivation by about 12−20-fold. These results suggest that Cys^{17} and Cys^{111} are near or at a steroid binding site, which may overlap with the classically defined xenobiotic binding site(s). The fact that Δ^5-androstene-3,17-dione does not significantly protect the inactivation of GST 1-1 by either mBBr or NFM, while 17β-estradiol 3,17-disulfate does, suggests that the reaction characteristics at Cys^{17} and Cys^{111} provide a way of differentiating between the Δ^5-androstene-3,17-dione substrate site and the nonsubstrate steroid site. The crystal structure of a GST from the parasitic worm *Schistosoma japonica* with an antischistosomal drug praziquantel bound indicates that occupation of a binding site close to the dimer interface, other than the classically defined substrate binding site, can be responsible for inhibition of GST functions by praziquantel (McTigue et al., 1995).

Fluorescence spectral characteristics of modified and control GST 1-1 were also investigated. Trp^{20} in native GST 1-1 emits at 325 nm, about 25 nm blue shifted from the 350 nm for the free tryptophan in solution. The emission maximum for bimane in the modified enzyme mB-NEM-GST 1-1 is blue shifted from 480 nm for free bimane in solution to 460 nm. However, the fluorescence spectra of NFM-modified GST 1-1 samples (NFM-GST 1-1 and mB-NFM-GST 1-1) show an emission maximum around 530 nm, which is red shifted about 10 nm from that of NFM-modified glutathione. Denaturation of the enzymes in 8 M guanidine hydrochloride results in red shifts for Trp^{20} and bimane at Cys^{17} and a blue shift for fluorescein at Cys^{111} (data not shown). These results suggest that Trp^{20} and bimane attached to Cys^{17} are located in a relatively hydrophobic environment in the native enzyme while fluorescein attached to Cys^{111} is likely to be in a rather polar environment (Duszynski et al., 1988; Hu & Colman, 1995; Wang et al., 1993). A molecular

model of a modified enzyme (Figure 6) shows that fluorescein attached Cys^{111} in subunit A is surrounded by charged residues such as Arg^{14}, Asp^{100}, and Glu^{103} from both subunits (A and B) and a Lys^{119} from subunit B.

Three sets of donor−acceptor pairs were used in this study; they are Trp^{20} vs bimane at Cys^{17}, Trp^{20} vs fluorescein at Cys^{111}, and bimane at Cys^{17} vs fluorescein at Cys^{111}. The fluorescence resonance energy transfer between the donor and acceptor in each pair was determined by comparing the fluorescence quantum yield of a modified enzyme containing both the donor and acceptor with that of a modified enzyme in the absence of an acceptor. Several assumptions have been made in calculating the fluorescence energy transfer distances in Table 6. The quantity with the greatest uncertainty is the dipole orientation factor κ^2, as has been discussed (Cantley & Hammes, 1975; Matsumoto & Hammes, 1975; Bell et al., 1989; First et al., 1989). In this case, for example, a value of $^2/_3$ was used for the orientation factor, κ^2, in eq 5, which is often assumed if the donor and acceptor are relatively free to rotate within the lifetime of fluorescent probes. Fluorescence polarization of Trp^{20} is 0.3, very close to the maximum limiting polarization, suggesting there is little mobility for the Trp^{20} in the enzyme (Valeur & Weber, 1977). However, the fluorescence polarizations of bimane at Cys^{17} and fluorescein at Cys^{111} are in the order of 0.28−0.32, which are higher than those of the free probes in solution (Table 7) but lower than the limiting polarization, which is 0.5 (Cantley & Hammes, 1975). This depolarization indicates that some rotation of the chromophores occurs even though the probes are less flexible than those free in solution. Therefore, this assumption will give a reasonable estimate of the distances between probes within the enzyme as discussed in detail by Haas et al. (1978) and by Wu and Brand (1992). Indeed, Wu and Brand (1992) have emphasized that many authors have found that average distances can be obtained without large uncertainty and that, particularly in cases involving extrinsic probes, the distances obtained from resonance energy transfer measurements are very close to those determined by X-ray crystallography.

Our molecular modeling studies indicate that the fluorescein moiety must lie in the cleft between the two subunits to meet the distance requirements calculated from our fluorescence energy transfer measurements: 19 Å between Trp^{20} and fluorescein at Cys^{111} and <22 Å between bimane at Cys^{17} and fluorescein at Cys^{111}. One possible model is shown in Figure 6. The distance between Trp^{20} and bimane at Cys^{17} is 20 Å, which places bimane in the opposite direction, with regard to the peptide backbone, of the chromophore in Trp^{20}. This orientation suggests that mBBr must approach from the dimer interface to reach its target residue Cys^{17}. In our modeling studies, the distance between Trp^{20} and bimane at Cys^{17} within one subunit is about half of that between subunits, while other donor−acceptor distances are about equal for the pair of Trp^{20} and fluorescein at Cys^{111} and of bimane at Cys^{17} and fluorescein at Cys^{111}. Therefore, the contribution of the acceptor in the other subunit to fluorescence energy transfer in the pair of Trp^{20} and bimane at Cys^{17} is minimal, whereas in the other two pairs, the contribution is the average of within and between subunits. The resonance energy transfer measurements for the enzyme in solution yield distances which are consistent with the crystal structure.

In summary, preferential modification of Cys^{111} in rat liver GST 1-1 was achieved by reacting the enzyme with sulfhydryl-selective maleimides. Fluorescence spectral analysis of enzymes modified by NFM at Cys^{111} and bimane at Cys^{17} suggests that Trp^{20} and bimane at Cys^{17} are located in a relative hydrophobic environment while fluorescein at Cys^{111} is located in a polar environment. Resonance energy transfer and molecular modeling studies indicate that fluorescein attached to Cys^{111} lies in the pocket between the two subunits, where it is surrounded by polar, charged residues, and that mBBr reaction at Cys^{17} could only be accomplished by approaching from the dimer interface.

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