Monobromobimane as an Affinity Label of the Xenobiotic Binding Site of Rat Glutathione S-Transferase 3-3*

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Monobromobimane (mBBr), besides being a substrate in the presence of glutathione, inactivates rat liver glutathione S-transferase 3-3 at pH 7.5 and 25 °C as assayed using 1-chloro-2,4-dinitrobenzene (CDNB). The rate of inactivation is enhanced about 5-fold by S-methylglutathione. Substrate analogs bromosulfophthalein and 2,4-dinitrophenol decrease the rate of inactivation at least 20-fold. Upon incubation for 60 min with 0.25 mM mBBr and S-methylglutathione, the enzyme loses 91% of its activity toward CDNB and incorporates 2.14 mol of reagent/mol of subunit, whereas incubation under the same conditions but with added protactein 2,4-dinitrophenol yields an enzyme that is catalytically active and contains only 0.89 mol of reagent/mol of subunit. mBBr-modified enzyme is fluorescent, and fluorescence energy transfer occurs between intrinsic tryptophan and covalently bound bimane in modified enzyme. Both Tyr115 and Cys114 are modified, but Tyr115 is the initial reaction target and its modification correlates with loss of activity toward CDNB. The fact that the activity toward mBBr is retained by the enzyme after modification suggests that rat isozyme 3-3 has two binding sites for mBBr.

Glutathione S-transferases constitute a family of enzymes that catalyze the nucelophilic attack by the thiol of glutathione on a variety of structurally diverse endogenous and xenobiotic substrates (1–6). Glutathione S-transferases are classified into five classes, one microsomal and four cytosolic (1–6). Glutathione that catalyzes the nucleophilic attack by the thiol of glutathione inactivation is enhanced about 5-fold by mBBr decrease the rate of inactivation at least 20-fold. Upon incubation for 60 min with 0.25 mM mBBr and S-methylglutathione, the enzyme loses 91% of its activity toward CDNB and incorporates 2.14 mol of reagent/mol of subunit, whereas incubation under the same conditions but with added proteinactein 2,4-dinitrophenol yields an enzyme that is catalytically active and contains only 0.89 mol of reagent/mol of subunit. mBBr-modified enzyme is fluorescent, and fluorescence energy transfer occurs between intrinsic tryptophan and covalently bound bimane in modified enzyme. Both Tyr115 and Cys114 are modified, but Tyr115 is the initial reaction target and its modification correlates with loss of activity toward CDNB. The fact that the activity toward mBBr is retained by the enzyme after modification suggests that rat isozyme 3-3 has two binding sites for mBBr.

Glutathione S-transferases, al- though activities of individual isozymes were not distinguished (21). In this paper, we report that mBBr, besides being a substrate of rat liver glutathione S-transferase 3-3, also acts as an affinity label of this isozyme in the presence of a glutathione analog, S-methylglutathione. A series of experiments are described that are designed to determine the amino acid and functional targets of mBBr within glutathione S-transferase 3-3. We report that covalent modification with mBBr causes substantial loss of enzymatic activity toward 1-chloro-2,4-dinitrobenzene (CDNB) with little or no effect toward another substrate, suggesting the existence of more than one binding site for xenobi otic substrates in the 3-3 isozyme. A preliminary version of this work was presented at the Eighth Symposium of the Protein Society (22).

EXPERIMENTAL PROCEDURES

Materials—Frozen Sprague-Dawley rat livers were purchased from Pel Freez Biologicals. Tyrosine, cysteine hydrochloride, glutathione, S-hexylglutathione, S-methylglutathione, 5-(nitrobenzyl)glutathione, S-hexylglutathione-Sepharose, bromosulfophthalein, 2,4-dinitrophenol, dinitrophenol.

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The abbreviations used are: mBBr, monobromobimane; CDNB, 1-chloro-2,4-dinitrobenzene; HPLC, high performance liquid chromatography; mB-Tyr, O-mB-tyrosine; mB-Cys, S-mB-cysteine; mB-SG, S-mB-glutathione; PTH, phenylthiohydantoin; DPT, N,N'-diphenylthiourea; DMF, N,N-dimethylformamide.
Sephadex G-50, N-ethylmaleimide, and N-tosyl-l-phenylalanyl chlo-
roniumethylenetetra-acid were all obtained from Sigma. CDNB was
purchased from Aldrich, and guanidine HCl and urea were from
ICN Biochemicals, Inc. mBBr was obtained from Molecular Probes, Inc.
Polybuffer exchanger PBE 118 and Pharmalyte® pH 8–10.5 were pur-
chased from Pharmacia Biotech Inc. Hydroylanapitate (Bio-Gel H7) and
Bio-Rad protein assay dye reagent kit were obtained from Bio-Rad.
Preparation of O-methyltyrosine (mB-Tyr) and S-methylcysteine (mB-
Cys)—mB-Tyr was prepared by a modified procedure of Wünsch et al.
(23, 24). Briefly, l-tyrosine (18.1 mg, 100 µmol) was dissolved in 2 n
NaOH (100 µl), and a solution of cupric sulfate (pentahydrate, 12.5 mg,
50 µmol) in water (50 µl) was added. The mixture was heated to 60°C,
cooked 1 h, cooled to room temperature, diluted with 350 µl H2O,
and brought to 3.0 M NaCl, pH 7.5, by the addition of 30 µl 0.5 M NaCl,
and made to 1 M NaCl. The reaction mixture was vigorously stirred at room temperature for 5 h and at 4°C overnight. The mixture was neutralized by 1 n HCl (26 µl) and diluted with water (500 µl). High performance liquid chromatographic
(HPLC) analysis showed mB-Tyr as the major product eluting at
22% acetonitrile in a 30-min gradient of 5–30% acetonitrile containing
0.1% trifluoroacetic acid on a C8 column. The product, mB-Tyr, was
isolated by HPLC using the same system. The UV absorption spectrum
shows wavelength maxima at 275 nm and 390 nm. The fluorescence spectra
exhibit an excitation maximum at 395 nm and an emission maximum at
400 nm; the fluorescence intensity of mB-Tyr is only about 19% of that of mB-SG at comparable concentrations.
The PTH derivative of mB-Tyr yields a distinct peak appearing
between DPT and PTH-Trp on an Applied Biosystems gas-phase protein
(peptide) sequence; the amount of mB-Tyr was estimated using
Tyr as a standard.

mB-Cys was prepared by reacting mBBr with 20-fold excess cysteine
at pH 8. Briefly, a 50 mM mBBr solution in DMF (2 µl) was added to a
1 mM cysteine solution in 50 mM ammonium bicarbonate at pH 8 (2 ml).
The mixture was stirred at room temperature for 5 h, and the product
was purified by HPLC using the same system as for mB-Tyr, with
m-Bys eluting at 14% acetonitrile. The UV absorption spectrum for mB-Cys is similar to that of mB-SG; however, the fluorescence intensity
of mB-Tyr is only about 19% of that of mB-SG at comparable concentrations.
The PTH derivative of mB-Cys is a distinct peak appearing between
DPT and PTH-Trp on an Applied Biosystems gas-phase protein
(peptide) sequence; the amount of mB-Cys was estimated using
Tyr as a standard.

Determination of the Stability of mBBr—The rate of decomposition of
mBBr was determined by measurement of the time dependence of
bromide release from the molecule. Bromide release was monitored by
measuring the free bromide present in solution using a modified pro-
cedure of Zall et al. (25, 26) in which bromide displaces thiocyanate from
mercury(II) thiocyanate and the liberated thiocyanate reacts with
triacetic acid to form a colored complex, which is then measured spectropho-
tometrically. A solution of mBBr (5.0 mM) was incubated in 100 mM
potassium phosphate containing 10% DMF at pH 7.5; it was found later that one column was sufficient to remove the unreacted reagent. The protein concentration in the filtrate was deter-
minated by the Bio-Rad protein assay, which is based on the dye-binding
reaction and not the amino-terminal sequence was deter-
ded using an ε1270 of 37,700 M⁻¹ cm⁻¹ (28). A M, of 26,500 subunit
was used in calculations (29). HPLC was used to assess the purity of the
final preparation using a 30-min gradient of 30–48% acetonitrile con-
taining 0.1% trifluoroacetic acid on a Vydac C8 column. Based on
the absorbance at 280 nm, the major protein peak constitutes more than
95% of the final preparation. The amino-terminal sequence was deter-
mained on a gas-phase protein (peptide) sequence analyzer to be Pro-
Met-Ile-Leu-Gly-Tyr-Arg-Asn-Val-Ara-Gly-Leu-Thr-His-Pro-Ile-Arg,
consistent with the known sequence of the 3–3 isozyme, which is dis-
tinguished at five of these positions from the amino acid sequence of
the 4–4 isozyme (29, 30).

Enzymic Assays—Unless otherwise indicated, enzymatic activity
was measured using a Gilford model 240 spectrophotometer by moni-
toring the formation of the conjugate of CDNB (1 mM) and glutathione
(2.5 mM) at 340 nm (Δε = 9.6 mmol cm⁻¹) in 0.1 M potassium phosphate
buffer, pH 6.5, at 25°C according to the method of Habig et al. (31). All
measurements were corrected for the spontaneous nonenzymatic rate
of the formation of the conjugate of glutathione and CDNB.

Enzymatic Assays—Unless otherwise indicated, enzymatic activity
for mBBr was determined using an Applied Biosystems gas-phase protein
(peptide) sequence analyzer to be Pro-Met-Ile-Leu-Gly-Tyr-Arg-Asn-
Val-Ara-Gly-Leu-Thr-His-Pro-Ile-Arg, consistent with the known sequence of the 3–3 isozyme, which is dis-
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tinguished at five of these positions from the amino acid sequence of
the 4–4 isozyme (29, 30).
Affinity Labeling of Glutathione S-Transferase

Preparation of Modified Glutathione S-Transferase—Glutathione S-transferase 3–3 (0.3 mg/ml) was incubated for the indicated time with 0.25 mM mBBr in the presence of 5 mM S-methylglutathione, with or without the addition of 10 mM 2,4-dinitrophenol 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 $\epsilon_{390} = 5360 \text{ M}^{-1} \cdot \text{cm}^{-1}$, which is the characteristic absorptivity for the bimane moiety in model compounds such as mB-SG (20). Similar results were obtained when measurements were performed under nondenaturing and denaturing conditions.

Preparation of Proteolytic Digest of Modified Glutathione S-Transferase—Glutathione S-transferase 3–3 (0.3 mg/ml) was incubated for the indicated time with 0.25 mM mBBr in the presence of 5 mM S-methylglutathione with or without the addition of 10 mM 2,4-dinitrophenol under standard reaction conditions. Excess reagent was removed by gel filtration as described above. The thiol groups of free cysteine residues in the enzyme were blocked by reaction with N-ethylmaleimide (10 mM) for 5 min under nondenaturing conditions at pH 7.5 and 25°C and for an additional 30 min under denaturing conditions in 9 M urea at pH 7.5 and 25°C. The solution was then dialyzed against 6 liters 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 $\mu\text{l}$) by incubation at 37°C for 2 h, after which 750 $\mu\text{l}$ 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 a concentration of 2 M. The modified glutathione S-transferase was digested at 37°C with two additions of N-tosyl-L-phenylalanine chloromethyl-ketone-treated trypsin (2.5% w/w) at 1-h intervals. After trypsin digestion, the digest was lyophilized and stored at −20°C.

Separation of Modified Peptides by HPLC—The tryptic peptides were separated by HPLC on a Varian 5000 LC equipped with a Vydac C18 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 15% solvent B at 55 min followed by a linear gradient to 27% solvent B at 75 min, 40% solvent B at 205 min, and 95% solvent B at 210 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 isotratic 10% solvent B at 5 min followed by a linear gradient to 100% solvent B for a total of 149 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 (S-(N-ethylsuccinimidomaleic)cysteine) was identified by a doublet migrating on the HPLC column of the sequencer between the PTH derivatives of Pro and Met (34), mB-Cys by a distinct peak appearing between PTH derivatives of Tyr and Pro, and mB-Tyr by a characteristic peak appearing between PTH derivatives of Pro and Met. The amount of mB-Cys and mB-Tyr in picomoles was estimated using PTH derivatives of Met and Tyr, respectively, as standards.

Preparation of Modified Glutathione S-Transferase for Fluorescence and Kinetic Studies—Glutathione S-transferase 3–3 (0.3 mg/ml) was incubated for 60 min with 0.25 mM mBBr in the presence of 5 mM S-methylglutathione under standard reaction conditions. Excess reagents were removed by gel filtration and the protein concentration determined by the Bio-Rad dye-binding method. The modified enzyme was frozen quickly and stored at −80°C.

Measurement of Fluorescence Spectroscopy—Steady-state fluorescence spectroscopy was measured on a Perkin-Elmer MPF-3 fluorescence spectrophotometer equipped with a Beckman model 13 chart recorder. The samples were excited at 280 nm, and a bandwidth of 5 nm and the emission spectra were monitored at a bandwidth of 10 nm from 300 to 600 nm, a range that includes emission from both tryptophan and bimane fluorophores. The spectra were uncorrected.

Molecular Modeling—Modeling was conducted using the program Insight II from Biosym Technologies on a Silicon Graphics workstation. The molecular model of mBBr was built and energy minimized using the Insight II program. The atomic coordinates for the rat 3–3 isozyme were obtained from the Brookhaven Protein Data Bank.

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data bank (9). The mBBr molecule was positioned manually into the xenobiotic binding site of rat isoenzyme subunit 3 by sequentially rotating and translating it along the x, y, and z axes, and the intermolecular energy in terms of both van der Waals’ and electrostatic interactions and the interatomic distance between the phenolic oxygen atom of Tyr115 and the bromine-bearing carbon atom of mBBr were continuously adjusted to conformations with reasonable distances and potential energies constituting possible productive interactions for chemical modification of the tyrosyl hydroxyl group. mBBr-modified models were submitted to the Discover® program from Biosym for extensive energy minimization using steepest descent and conjugate gradient methods to relieve residual van der Waals’ overlaps and to optimize the structures.

RESULTS

mBBr as a Substrate for Rat Liver Glutathione S-Transferase

The formation of the glutathione conjugate with mBBr is catalyzed by glutathione S-transferase 3–3, and the catalysis follows Michaelis-Menten kinetics (Fig. 1). A Lineweaver-Burk plot of 1/v versus 1/[mBBr] gives a K_{m} of 0.54 mM and a V_{max} of 3.4 mmol/min/mg of protein at pH 6.5 and 25°C. For comparison, rat glutathione S-transferase 3–3 has a K_{m} of 60 mM and a V_{max} of 66 mmol/min/mg of protein when using CDNB as the substrate (31).

Inactivation of Rat Glutathione S-Transferase 3–3 with mBBr—Incubation of rat glutathione S-transferase 3–3 with 2 mM mBBr at pH 7.5 and 25°C results in a time-dependent inactivation of the enzyme (Fig. 2). 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 enzyme exhibits a linear dependence on the concentration of mBBr (Fig. 2, inset), with a second order rate constant of 0.026 min^{-1}M^{-1}.

Effect of Substrate Analogs on the Rate of Inactivation of Glutathione S-Transferase by mBBr—The results shown in Table I summarize the effect of substrate analogs on the reaction rate of 2.0 mM mBBr with rat liver glutathione S-transferase 3–3. The addition of 5 mM S-methylglutathione causes a 5-fold increase in the rate constant of inactivation over the mBBr concentration range tested (0.15–2.0 mM). Other S-alkylglutathione derivatives such as S-hexylglutathione and S-(p-nitrobenzyl)glutathione, as well as xenobiotic substrate analogs like bromosulfophthalein and 2,4-dinitrophenol, alone afford limited protection against mBBr inactivation of glutathione S-transferase 3–3.

Concentration Dependence of the Rate Constant for mBBr-Inactivation of Glutathione S-Transferase 3–3 in the Presence of S-Methylglutathione—Since S-methylglutathione enhances the rate of inactivation of glutathione S-transferase 3–3 by mBBr, we were able to use lower reagent concentrations when inactivation was carried out in the presence of 5 mM S-methylglutathione. Glutathione S-transferase 3–3 was incubated with various concentrations of mBBr (0.15–2.0 mM) to determine the dependence of the rate of inactivation on the reagent concentration. As shown in Fig. 3, the rate constant of inactivation, k_{obs}, appears to exhibit a nonlinear dependence on the reagent concentrations. At higher concentrations of mBBr, k_{obs} could not be measured with confidence. The observed rate constant, k_{obs}, for inactivation at various mBBr concentrations were fitted to equation k_{obs} = (k_{max}·[mBBr])/(K_{I} + [mBBr]), where k_{max} is the maximum rate of inactivation at saturating concentrations of the reagent and K_{I} = (k_{-1} + k_{max})/k_{1} represents the reagent concentration that results in half of the maximal inactivation rate (35). This nonlinear curve fitting gave k_{max} = 0.56 min^{-1} and K_{I} = 1.73 mM, with k_{max}/K_{I} = 0.32 min^{-1}M^{-1}.

Effect of Xenobiotic Substrate Analogs on the Rate of Inactivation in the Presence of S-Methylglutathione—Table II shows the effect of adding a xenobiotic substrate analog, like bromosulfophthalein or 2,4-dinitrophenol, on the rate constant of
inactivation of glutathione S-transferase 3–3 by 0.25 mM mBBr in the presence of S-methylglutathione. Such additions are found to give effective protection against inactivation; incubation with 400 \( \mu \)M bromosulfophthalein reduces the observed rate of inactivation by 20-fold while the addition of 10 mM 2,4-dinitrophenol reduces the rate by more than 100-fold. Both bromosulfophthalein and 2,4-dinitrophenol, under the same conditions, have no effect on the half-life of the reagent, which is 37.3 h as determined by release of bromide ion from the bimane molecule. These results indicate that reaction with mBBr in the presence of S-methylglutathione occurs at the xenobiotic substrate binding site.

Incorporation of mBBr by Glutathione S-Transferase 3–3—Glutathione S-transferase 3–3 was incubated with 0.25 mM mBBr in the presence of S-methylglutathione with or without the added protectant, 2,4-dinitrophenol, after which the modified enzymes were isolated and the incorporation of bimane was measured from the characteristic absorbance of bimane moiety at 390 nm. The time-dependent incorporation of mBBr into glutathione S-transferase 3–3 and corresponding loss of activity in the absence of the added protectant are shown in Table III. Incubation of glutathione S-transferase 3–3 with 0.25 mM mBBr in the presence of S-methylglutathione for 60 min affords a modified enzyme that is 91% inactivated and contains 2.14 mol of reagent/mol of subunit. Incubation under the same conditions, but with the added protectant, 2,4-dinitrophenol, yields an enzyme that is catalytically active and contains only 0.89 mol of reagent/mol of subunit, suggesting that reaction occurs at a limited number of sites in glutathione S-transferase, which includes the catalytic site, but that some additional reactions occur at amino acid(s) not essential for enzymatic activity.

Isolation of Tryptic Peptides from Modified Glutathione S-Transferase—Glutathione S-transferase (0.3 mg/ml) was inactivated for 60 min by 0.25 mM mBBr in the presence of 5 mM S-methylglutathione at pH 7.5 and 25°C. The resulting modified enzyme with 9% residual activity was isolated, incubated with \( \text{N} \)-ethylmaleimide, dialyzed, and digested with trypsin.
Representative sequences of modified peptides present for the inactive enzyme

Rat liver glutathione S-transferase 3–3 (0.3 mg/ml) was incubated for 60 min with 0.25 mM mBBr in the presence of 5 mM S-methylglutathione at pH 7.5 and 25 °C. The modified enzyme was isolated, treated with N-ethylmaleimide, and digested with trypsin. The peptides were separated by HPLC on a C18 column with 0.1% trifluoroacetic acid/acetonitrile/H2O system (Fig. 4, A and B). These sequences are representative and were not all derived from the same tryptic digest. Thus, the amounts of peptides do not represent the relative magnitude of the peaks shown in Fig. 4 B.

Table IV

| Cycle | Peptide I | Amino acid (pmol) | Peptide II | Amino acid (pmol) | Peptide III | Amino acid (pmol) | Peptide IV | Amino acid (pmol) |
|-------|-----------|------------------|------------|------------------|------------|------------------|------------|------------------|
| 1     | Met108    | (168)            | mB-Cys     | (166)            | Met108     | (206)            | mB-Cys     | (166)            |
| 2     | Gin (162) |                   | Gln (143)  |                   | Gin (161)  |                   | Gln (168)  |                   |
| 3     | Leu (174) |                   | Ile (203)  |                   | Leu (161)  |                   | Leu (185)  |                   |
| 4     | Pro (175) |                   | Ile (153)  |                   | Ile (150)  |                   | Ile (200)  |                   |
| 5     | Met (157) |                   | Met (126)  |                   | Met (142)  |                   | Met (190)  |                   |
| 6     | Leu (137) |                   | Leu (92)   |                   | Leu (123)  |                   | Leu (165)  |                   |
| 7     | mB-Cys    | (91)             | NEM-Cys    | (80)             | mB-Cys     | (78)             |           |                  |
| 8     | mB-Tyr    | (90)             | mB-Tyr     | (70)             |           |                  |           |                  |
| 9     | Asn (94)  |                   | Asn (93)   |                   | Asn (99)   |                   | Asn (53)   |                   |
| 10    | Pro (58)  |                   | Pro (61)   |                   | Pro (74)   |                   | Pro (95)   |                   |
| 11    | Asp (54)  |                   | Asp (47)   |                   | Asp (64)   |                   | Asp (54)   |                   |
| 12    | Phe (68)  |                   | Phe (54)   |                   | Phe (59)   |                   | Phe (102)  |                   |
| 13    | Glu (50)  |                   | Glu (34)   |                   | Glu (53)   |                   | Glu (56)   |                   |
| 14    | Lys121(30)|                   | Lys121(15)|                   | Lys121(18)|                   | Lys121(58)|                   |

a The amount of mB-Cys in pmol was estimated using the PTH derivative of Met as the standard.
b The amount of mB-Tyr in pmol was estimated using the PTH derivative of Tyr as the standard.

The digest was subjected to HPLC separation using a C18 column and an acetonitrile gradient in 0.1% trifluoroacetic acid as illustrated in Fig. 4, A and B. Five peptide regions showing the characteristic bimane absorbance at 390 nm (Fig. 4B) are labeled as I, Ia, II, III, and IV.

Fig. 4C shows the HPLC pattern of a tryptic digest, as monitored at 390 nm, of catalytically active, modified glutathione S-transferase prepared by incubation of the rat liver enzyme for 60 min with 0.25 mM mBBr in the presence of 5 mM S-methylglutathione and 10 mM 2,4-dinitrophenol. The digest of this active enzyme (Fig. 4C) contains no significant amount of peptides corresponding to peaks I–IV of the inactive enzyme digest (Fig. 4B); the measured reagent incorporation of 0.89 mol/mol of subunit was distributed in small amounts at different nonspecific sites as shown using the more sensitive measurement of fluorescence associated with each peptide (data not shown). These results indicate that peaks I, Ia, II, III, and IV contain modified peptides, whose appearance correlates with inactivation.

The development of these modified peptide peaks was monitored as a function of time of reaction and corresponding degree of inactivation. The enzyme was 35% inactivated after incubation for 8 min with 0.25 mM mBBr in the presence of 5 mM S-methylglutathione and 10 mM 2,4-dinitrophenol. The digest of this active enzyme (Fig. 4C) contains no significant amount of peptides corresponding to peaks I–IV of the inactive enzyme digest (Fig. 4B); the measured reagent incorporation of 0.89 mol/mol of subunit was distributed in small amounts at different nonspecific sites as shown using the more sensitive measurement of fluorescence associated with each peptide (data not shown). These results indicate that peaks I, Ia, II, III, and IV contain modified peptides, whose appearance correlates with inactivation.

The amount of mB-Cys in pmol was estimated using the PTH derivative of Met as the standard.
The relative amount of this mutated form is batch-dependent (data not shown). Such a mutation at an equivalent position of the rat glutathione S-transferase μ class subunit Yb was reported previously by others (37, 38) and resulted in an enzyme that was shown to be difficult to separate from the other μ class isozymes (38).

Fluorescence Properties of mB-modified Glutathione S-Transferase Under Nondenaturing and Denaturing Conditions—Modified enzyme exhibits excitation maxima at 280 and 395 nm and emission maxima at 330 and 460 nm under nondenaturing conditions at pH 7.5 and 25 °C. The 330-nm emission is characteristic of tryptophan residues in a hydrophobic environment as is observed for rat glutathione S-transferase 1-1 (39). The 460-nm emission is the result of the incorporated bimane moiety (20). Under denaturing conditions in 4 M guanidine HCl, pH 7.5, and 25 °C, the modified enzyme exhibits a 20-nm red shift in both emission maxima when excited at 280 nm (Fig. 5), consistent with the fact that the fluorophores are exposed to a polar environment after denaturation. There is substantial spectral overlap between the emission spectrum of the native enzyme and excitation spectrum of bimane-modified enzyme (data not shown), which makes fluorescence energy transfer measurement between the two fluorophores possible, with a calculated Förster critical distance of 20 Å, using in the calculation values of 1.4 for the refractive index (m) between the two fluorophores and of ½ for the orientation factor (g) (40). The relative increase in tryptophan emission (350/330 nm) and decrease in bimane emission (480/460 nm) after denaturation, when excited at 280 nm (Fig. 5), suggest that there is significant energy transfer between the tryptophan and bimane fluorophores in the modified enzyme under nondenaturing conditions. In contrast, if the samples are excited at the bimane excitation maximum of 395 nm, the modified enzyme shows the same 20-nm red shift in bimane emission (460-480 nm) but with a slight increase in intensity (about 10%) after denaturation in 4 M guanidine HCl, pH 7.5, and 25 °C (data not shown). The emission maximum for bimane-modified enzyme after denaturation (480 nm) is the same as that for mB-Tyr and mB-Cys in aqueous solutions, models for the modified enzyme, and the same as that for mB-SG in aqueous solutions (20).

Comparison of Kinetic Properties of mB-modified Glutathione S-Transferase—The catalytic properties of mB-modified and control enzymes were investigated using as substrates CDNB, bromosulfophthalein, and mBBr. In the rate measurements of Figs. 2 and 3, the reaction of glutathione S-transferase with mBBr was monitored only with CDNB. Modification of glutathione S-transferase by 0.25 mM mBBr for 60 min yields an enzyme with only 6–9% residual activity for both CDNB and bromosulfophthalein but, surprisingly, retains full activity for mBBr when assayed with 30 μM mBBr and 600 μM glutathione at pH 6.5.

The apparent K<sub>m</sub> and V<sub>max</sub> values of modified and control enzymes for mBBr and glutathione were determined in order to further evaluate the effect of modification. As shown in Table V, the modification affects both the apparent K<sub>m</sub> and V<sub>max</sub>, with the effect on K<sub>m</sub> being much greater than that on V<sub>max</sub>, and the effect on mBBr being greater than that on glutathione. For mBBr, the K<sub>m</sub> was increased by 50-fold, and V<sub>max</sub> was also increased by about 3-fold. Overall, the catalytic efficiency of the modified enzyme decreased by about 15-fold as judged by the apparent k<sub>cat</sub>K<sub>m</sub> for glutathione. For glutathione, the K<sub>m</sub> was increased by about 8-fold and V<sub>max</sub> by about 2.3-fold, resulting in an overall decrease in the catalytic efficiency by 3.6-fold.

**DISCUSSION**

The results of this paper demonstrate that mBBr reacts predominantly with 2 amino acid residues of glutathione S-transferase 3-3: Tyr<sup>115</sup> and Cys<sup>114</sup>. However, modification occurs initially at Tyr<sup>115</sup>, paralleling the loss of enzymatic activity toward CDNB. Cys<sup>114</sup> modification appears to be slow and secondary, and it is not clear whether modification of only this residue is sufficient to cause inactivation. A schematic representation of the sequence of reaction of mBBr with glutathione S-transferase is shown in Scheme I.

\[
\begin{align*}
E & \xrightarrow{k_1} E^{Y\text{mB}} \\
\downarrow k_2 & \quad \downarrow k_4 \\
E^{\text{Cys-mB}} & \xrightarrow{k_3} E^{\text{Y\text{mB}-mB}} \\
\end{align*}
\]

**Scheme I**

From the time-dependent development of modified peptides, we conclude that the rate of reaction of mBBr at Tyr<sup>115</sup> of the native enzyme, k<sub>1</sub>, is greater than the reaction rate at Cys<sup>114</sup> of the native enzyme, k<sub>2</sub>. The accumulation of peptide II and the slow appearance of peptide I (and Ia) at the early period of reaction indicate that k<sub>1</sub> is greater than k<sub>3</sub>. The slow appearance and the existence of significant amount of peptide III peak at 60 min indicates that modification of Cys<sup>114</sup> slows down further modification at Tyr<sup>115</sup>, i.e. k<sub>3</sub> > k<sub>4</sub>. Therefore, the relative order of the rate constants for modification is k<sub>1</sub> > k<sub>2</sub> > k<sub>3</sub> and k<sub>1</sub> > k<sub>4</sub>. Cys<sup>86</sup> is not appreciably modified by mBBr in our studies, although it does react with S-(4-bromo-2,3-dioxobutyl)glutathione (16). This difference could be due to the more hydrophobic character of mBBr, which makes it more likely to react within the interior of proteins.

From the crystal structure represented in Fig. 6, Tyr<sup>115</sup> is positioned in a conformation to react with mBBr docked at the active site, which would explain why this residue is modified first and the rate of reaction is greater. Fig. 6 also suggests that Cys<sup>114</sup> is located in the vicinity of the active site; but the sulfur atom of this cysteinyI residue is pointed away from the substrate binding cavity, making it inaccessible to the active site-bound reagent. As suggested in the affinity labeling studies of
glutathione S-transferase 3–3 using 2-(S-glutathionyl)-3,5,6-trichloro-1,4-benzoquinone (18), a conformation different from the one shown in Fig. 6 may exist in solution, in which the cysteinyl side chain is more exposed to the active site-bound reagent.

Alternatively, the sulfhydryl group of Cys114 may react with mBBr from the outside of the protein molecule. In the structure shown in Fig. 6, this sulfhydryl group is imbedded in a hydrophobic environment −7–8 Å from the surface, surrounded by the hydrophobic side chains of Trp214, Phe119, Phe169, Phe208, and Leu110. Close examination of the structure reveals that a bimane molecule can be placed in a position to react with the sulfhydryl group of Cys114, requiring minimal perturbation of conformations of residues in this region. This is consistent with the results of energy minimization of a model (not shown) of glutathione S-transferase 3–3 with covalently bound bimanes at both Tyr115 and Cys114, which leads only to movements of less than 2 Å in atoms of residues of this region. Movements in side chain atoms of Trp214, Phe119, and Phe169 are the most apparent; these residues are distant from the active site.

Fig. 5 suggests that the bimane moiety in the modified enzyme may serve as a fluorescence resonance energy acceptor in the determination of distances between sites in glutathione S-transferases. Under non-denaturating conditions, there is substantial reduction of the intrinsic tryptophan fluorescence by the covalently bound bimane in the active site. In rat liver glutathione S-transferase 3–3, there are 4 tryptophan residues/subunit, three of which (Trp7, Trp45, Trp214) are in the range of 8–14 Å to the bound bimane moiety in the same subunit (Trp7, 8.2 Å; Trp45, 12.4 Å; Trp214, 13.8 Å), while the fourth tryptophan as well as tryptophan residues from the adjacent subunit are more than 25 Å away from the bound bimane moiety in an energy minimized mB-modified glutathione S-transferase 3–3 model. In addition, the efficiency of fluorescence energy transfer is inversely related to the sixth power of the distance between the two fluorophores (40). It is, therefore, considered that tryptophan residues at positions 7, 45, and 214 make the dominant contributions to the transfer of fluorescence energy to the bimane fluorophore under non-denaturating conditions. Studies are presently in progress to use this property to investigate solution conformational properties of the enzyme.

The data presented in this paper indicate that glutathione S-transferase 3–3 may have two different binding sites for mBBr, one that is identical or overlaps with the CDNB site and a second or alternate site that is independent of the CDNB site. Glutathione S-transferase 3–3 incubated with mBBr in the presence of S-methylglutathione yields a modified enzyme that exhibits only about 6–9% residual activity toward CDNB and bromosulfophthalein but retains full activity toward mBBr, indicating that the site shared with CDNB and bromosulfophthalein is covalently modified by mBBr, while the other independent site remains available. Since the modified enzyme is still catalytically competent toward mBBr, both sites must be very close to the glutathione binding site. There may not be any accessible nucleophiles to react covalently with mBBr in the second site, which would explain why only one site is modified.
Our molecular modeling simulations suggest that after modification of Tyr\textsuperscript{115} by active site-bound mBBr and of Cys\textsuperscript{114} by mBBr approaching from the outside as discussed above, there is still sufficient room in the xenobiotic binding cavity to bind another molecule of mBBr. Evidence for the existence of a second xenobiotic binding site for other glutathione S-transferases has been obtained previously from equilibrium binding experiments (41, 42).

S-Methylglutathione enhances the rate of reaction between the enzyme and mBBr, probably through S-methylglutathione-induced conformational change, which facilitates the binding of mBBr to the enzyme. Fig. 3, inset, showing saturation kinetics for inactivation in the presence of S-methylglutathione, suggests that mBBr first binds to the enzyme before covalently modifying the enzyme and causing inactivation. Comparison of the second order rate constant for reaction of mBBr with the enzyme alone (0.026 min\textsuperscript{−1} m\textsuperscript{−1}) with the k\textsubscript{max}/K\textsubscript{i} measured in the presence of S-methylglutathione (0.32 min\textsuperscript{−1} m\textsuperscript{−1}) indicates a 12-fold enhancement of the reactivity of the target site in the presence of the glutathione derivative. The use of S-methylglutathione in our present study enables us to reduce the concentration of mBBr needed to obtain the desired inactivation, thus reducing nonspecific labeling of the enzyme.

In summary, mBBr modification of rat liver glutathione S-transferase 3-3 occurs initially at the active site residue Tyr\textsuperscript{115}, which leads to loss of catalytic activity toward CDNB and bromosulfophthalein. Prolonged treatment of the enzyme with mBBr also results in the modification of Cys\textsuperscript{114}, which is located in the vicinity of the active site. Glutathione S-transferase 3-3 is shown to have 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 also catalytically active after modification of the first site.

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