Structural and Functional Aspects of Rat Microsomal Glutathione Transferase

THE ROLES OF CYSTEINE 49, ARGinine 107, LYSINE 67, HISTidine, AND TYROSINE RESIDUES*

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Rolf Weinander‡§, Lena Ekström‡, Claes Andersson‡, Haider Raza†, Tomas Bergman†, and Ralf Morgenstern†***

From the Institute of Environmental Medicine, Division of Biochemical Toxicology, Karolinska Institutet, Box 210, S-171 77 Stockholm, Sweden and the Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-171 77 Stockholm, Sweden

Rat liver microsomal glutathione transferase is rapidly inactivated upon treatment with the arginine-selective reagent phenylglyoxal or the lysine-selective 1,3,5-trinitrobenzene sulfonate. Glutathione sulfonate, an inhibitor of the enzyme, gives nearly complete protection against inactivation and prevents modification, indicating that these residues form part of or reside close to the active site. Sequence analysis of peptides from peptic and tryptic digests of [7-14C]phenylglyoxal- and 1,3,5-trinitrobenzenesulfonate-treated microsomal glutathione transferase indicated arginine 107 and lysine 67 as the sites of modification. A set of mutant forms of microsomal glutathione transferase was constructed by site-directed mutagenesis and heterologously expressed in *Escherichia coli* BL21(DE3). Arginine 107 was exchanged for alanine and lysine residues. The alanine mutant (R107A) exhibited an activity and inhibition profile similar to that of the wild type enzyme but displayed a decreased thermostability. Thus, arginine 107 does not appear to participate in catalysis or substrate binding; instead, an important structural role is suggested for this residue. Lysine 67 was mutated to alanine and arginine with no effect on activity. All three histidines were replaced by glutamine, and the resulting mutant proteins had activities comparable with that of the wild type. It can thus be concluded that the chemical modification experiments indicating that arginine 107, lysine 67, and one of the histidines partake in catalysis can be disproved. However, protection from modification by a competitive inhibitor indicates that these residues could be close to the glutathione binding site. All tyrosine to phenylalanine substitutions resulted in mutants with activities similar to that of the wild type. Interestingly, the exchange of tyrosine 137 appears to result in activation of the enzyme. Thus, the microsomal glutathione transferase must display an alternate stabilization of the thiolate anion of glutathione other than through interaction with the phenolic hydroxyl group of a tyrosine residue. Substitution of cysteine 49 with alanine resulted in a semiactivated mutant enzyme with enzymatic properties partly resembling the activated form of microsomal glutathione transferase. The function of this mutant was not altered upon reaction with N-ethylmaleimide, and cysteine 49 is thus demonstrated as the site of modification that results in activation of microsomal glutathione transferase.

Microsomal glutathione transferase (1) is a membrane-bound member of the glutathione transferase family of enzymes (2). Glutathione transferases (GSTs) are a group of phase II detoxification enzymes that catalyze the conjugation of glutathione to a variety of molecules bearing different electrophilic centers, which are all hydrophobic (3). Thus, the GSTs aid in the detoxification of numerous carcinogenic, toxic, and pharmacologically active substances (3). The transferases exist as multiple cytosolic isomers with molecular mass values in the 24–28-kDa region (4–6) and a membrane-bound microsomal GST. Rat microsomal GST has a molecular mass of 17.3 kDa with an amino acid sequence analyzed both at the protein and cDNA level (7, 8). Microsomal GST is present at the highest levels in the liver and has been purified from rat (9), mouse (10), and human (11). These species express closely related enzymes. In contrast to the cytosolic GSTs, which are dimeric proteins, the microsomal transferase is a trimer that contains one single cysteine residue per subunit (7, 12). The microsomal GST is activated in *vitro* by various treatments such as covalent modification of cysteine 49 by sulphydryl reagents (13), limited proteolysis (14), heating (15), and reactive oxygen species (16). Activation by sulphydryl reagents or proteolysis has not been observed with cytosolic GSTs.

Three-dimensional structures of cytosolic GSTs from the Alpha (17), Mu (18), Pi (19), Theta (20), and Sigma (21) classes have been characterized. The projection structure of the membrane-bound, structurally distinct microsomal GST has been solved at 4-Å resolution (12). A previous investigation utilizing chemical modification with selective reagents indicated the importance of arginine, lysine, and histidine residues for the catalytic function of rat microsomal GST (22). In many cytosolic GSTs studied, a tyrosine residue has been identified as having a critical role in catalysis by stabilizing the nucleophilic thiolate anion of the enzyme-bound GSH molecule. This activation

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§ Supported by the Swedish Society for Medical Research.

¶ Supported by the Wenner-Gren foundation. Present address: Dept. of Biochemistry, Faculty of Medicine and Health sciences, U. A. E. University, P.O. Box 17686, Al Ain, United Arab Emirates.

1 The abbreviations used are: GST, glutathione S-transferase; NEM, N-ethylmaleimide; PG, phenylglyoxal; TNBS, 1,3,5-trinitrobenzenesulfonate; PCR, polymerase chain reaction; HPLC, high pressure liquid chromatography; CDNB, 1-chloro-2,4-dinitrobenzene.
of the thiol is suggested to occur by the phenolic hydroxyl group of the tyrosine residue (or serine hydroxyl in Theta class enzymes) interacting with the sulfur of GSH (23–26). Clearly, it is of interest to investigate a possible similar function of a tyrosine residue in the microsomal GST as well as the role of cysteine for activation. The development of heterologous expression and purification of recombinant microsomal GST from *Escherichia coli* has opened new possibilities to examine the structure and function of this enzyme by site-directed mutagenesis.

In this study, we have investigated whether amino acids previously implicated in catalysis or activation could be verified or excluded. Some of the results have been communicated in a preliminary form (27, 28).

**EXPERIMENTAL PROCEDURES**

**Materials**—Phenyglyoxyal and 1,3,5-trinitrobenzenesulfonate (TNBS) were obtained from Sigma. [3H,35S]dATP were manufactured by New England Nuclear. Glutathione was removed from the purified enzyme as described by Weinander *et al.* (30) or by ion exchange chromatography. For the latter procedure, the enzyme was diluted in 10 volumes of 10 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, 1% Triton X-100, 20% glycerol (v/v), and 1 mM GSH (buffer A) and applied to a CM-Sepharose cation exchange column (5 m × 1 cm). When maximal activity was reached (within 5 min), the reaction was terminated by the addition of 0.2 M KCl in buffer A. Removal of GSH was checked as described (30).

**Enzyme Assays**—Enzyme activities with 1-chloro-2,4-dinitrobenzene (CDNB; 0.5 mM) were assayed at 340 nm in 0.1 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, 1% Triton X-100, 20% glycerol (v/v), and 1 mM GSH (buffer A) and applied to a CM-Sepharose cation exchange column equilibrated with the same buffer. The column was washed with 5 volumes of buffer A devoid of GSH, and the glutathione-free enzyme was eluted by the addition of 0.2 M KCl in buffer A. Removal of GSH was checked as described (30).

**Activation of Microsomal Glutathione Transferase**—Activation of the purified enzyme with 2–5 mM NEM was performed at 4 °C in 20 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, 1% Triton X-100, 20% glycerol (v/v), and 1 mM GSH, and 0.1 M KCl. When maximal activity was reached (within 5 min), the reaction was terminated by the addition of GSH to give a final concentration of the free thiol of approximately 1 mM. The activity of the enzyme was activated by the addition of 0.5 mM NEM. Carry-over of NEM to the assay had only marginal effects on the GSH concentration (<0.5%).

**Modification of Arginine Residues**—Purified glutathione-free microsomal GST (about 2 mg/ml) in buffer A was treated with 0.5 mM NEM and mixed with an equal amount of 0.2 mM potassium phosphate, pH 8.0. The modification reaction was then started by the addition of 5 mM phenylglyoxyal (PG) dissolved in 50% ethanol. The incubation contained 2.5% (v/v) ethanol (final concentration), which did not influence control activity. Reactions were performed at room temperature in the absence and presence of 2 mM glutathione sulfonate, respectively. Aliquots were withdrawn at time points indicated in Fig. 1.

**Stoichiometry of Modification of Lysine Residues**—Approximately 5 nmol of glutathione-free, NEM-treated microsomal glutathione transferase was incubated with 14C-labeled PG (1100 cpnm/μl) in a volume of 200 μl of 50% buffer A, 50% 0.2 mM potassium phosphate, pH 8.0, at room temperature in the absence and presence of 2 mM glutathione sulfonate, 30-μl aliquots of the reaction mixture were withdrawn after 0, 0.5, 1, 2, 5, 10, 20, and 30 min and precipitated in 10% trichloroacetic acid. The precipitated enzyme was pelleted and washed three times with 1 ml of 10% trichloroacetic acid. Pellets were dissolved in 0.1 ml of formic acid, and the amounts of PG incorporated were determined by scintillation counting. In parallel incubations, 5 mM PG was added at room temperature in the absence and presence of 2 mM glutathione sulfonate, and the activity versus time was determined.

**Proteolytic Digestion, Peptide Separation, and Identification of Reagent-modified Enzyme**—300 nmol of microsomal GST (glutathione-free and NEM-treated) was reacted with 20 μCi of [14C]PG in a volume of 4 ml of the buffer described above in the absence and presence of 2 mM glutathione sulfonate, respectively. The final concentration of PG in the mixture was 5 mM. Reactions were carried out at room temperature for 15 min. After centrifugation, 10 μl of the resulting supernatant was used for GST activity to ensure that inactivation of enzyme was complete. De- and free PG was removed from the protein by cation exchange chromatography on a Sep Pak Accell Plus CM column (Waters) that had been equilibrated with buffer A. The column was washed with 5 mM potassium phosphate followed by water. The enzyme was eluted with 4 × 1 ml of formic acid, and the two fractions with the highest protein content (as determined by absorption at 280 nm) were pooled and dried under N2. Thereafter, the enzyme was dissolved in 100 μl of formic acid and digested by trypsin (0.25 mg/ml) in a volume of 2 ml of 5% formic acid at 37 °C overnight. The resulting peptide mixtures were dried with N2 and dissolved in 0.5 ml of formic acid.

**Peptides** were purified by reverse-phase HPLC on a C-18 column (5 μm, 4.6 × 250 mm). The solvent system consisted of 0.1% trifluoroacetic acid/acetonitrile with a linear gradient of 0–50% acetonitrile over 10 min followed by 50–50% acetonitrile over 110 min and, thereafter, 50–80% acetonitrile over 10 min. Elution was monitored at 214 nm, and the flow rate was 1 ml/min. Peak fractions were collected manually, and the radioactivity in each fraction was determined by scintillation counting of a 25-μl aliquot. Fractions containing the peak radioactivity were rechromatographed on the same column using the same solvent system. Fractions containing the radioactive peptides from this second round of chromatography were sequenced as described below. 50% of the obtained amino acid phenylthiohydantoin derivatives were subjected to amino acid identification, and 50% were taken for scintillation counting.

**Modification of Lysine Residues**—Purified glutathione-free, NEM-treated microsomal GST (about 2 mg/ml) was reacted with the same amount of 0.2 mM potassium phosphate, pH 7.0. The reaction was started by the addition of 0.5 mM TNBS dissolved in 0.2 mM potassium phosphate, pH 7.0. Incubation was performed at room temperature in the absence/presence of 2 mM glutathione sulfonate, and aliquots were withdrawn for assay at the time points indicated in Fig. 1. To ensure that protection of the enzyme activity by glutathione sulfonate was not a result of reaction of the inhibitor and TNBS, the reaction rate of 0.5 mM TNBS and 2 mM glutathione sulfonate was determined at 345 nm, assuming a molar extinction coefficient for the reaction product of 1.1 × 104 M−1 cm−1 (33). In all experiments, this rate was very low, and the possibility that glutathione sulfonate gave protection by reacting with and depleting TNBS could thus be excluded.

**Stoichiometry of Modification**—Purified glutathione-free, NEM-treated microsomal GST was reacted with 0.5 mM TNBS in a volume of 100 μl of 50% buffer A, 50% 0.2 mM potassium phosphate, pH 7.0, in the absence and presence of 2 mM glutathione sulfonate at room temperature. 20-μl aliquots of the reaction mixture were withdrawn after 0, 0.5, 1, 5, 10, 20, and 30 min, denatured, and dissolved in 5% formic acid. The number of trinitrophenylated amino acid residues in the microsomal GST was reacted with 0.5 mM TNBS was determined from the absorbance at 345 nm using a molar extinction coefficient of 1.1 × 104 M−1 cm−1 (33).
temperature. A 10-μl aliquot of the respective mixture was withdrawn for assay by the inactivation/protetion. Reactions were terminated by the addition of 0.5 M L-lysine, and the mixtures were dialyzed against 0.4 M Tris-HCl, pH 8.0, 2 mM EDTA, 5 M urea until all Triton X-100 was removed from the solutions as determined from the decrease in absorbance at 275 nm. Thereafter, the mixtures were dialyzed against H2O for 48 h. The protein was digested by trypsin (1:50, w/v) in 50 mM ammonium bicarbonate at 57 °C for 5 h. Peptide mixtures were dried with N2 and dissolved in 0.5 ml of formic acid.

Peptides were purified by reverse-phase HPLC using the same system as described above with a linear gradient of 0–80% acetonitrile over 45 min. Elution was monitored at 214 and 345 nm. Peak fractions were collected manually, and the fraction containing the peak absorbance at 345 nm was subjected to sequence analysis.

Amino Acid Sequence Analysis—The amino acid sequence of the peptides was determined by automated Edman degradation using an Applied Biosystems 470 A instrument. Amino acid phenylthiohydantoin-derivatives were analyzed by reverse-phase HPLC as described (34).

Site-directed Mutagenesis—The plasmid pSP197LTrmQT, in which cDNA coding for wild type rat microsomal GST had been ligated (35), was used as a template for site-directed mutagenesis utilizing PCR. Oligonucleotide primers used to construct mutants are shown in Table I. To construct K67A and K67R, the PCRs were performed with the primer rU1, which contains the sense sequence of the 5′-end of the coding region of the microsomal GST cDNA and the respective antisense primer directing the Lys67 mutations. The PCR products were digested with NdeI and MluI, gel-purified, and ligated into the vector pSP197LTrmQT that had been cut with NdeI and MluI and gel-purified.

The Arg92G7T mutations and H105Q were constructed in a PCR with the respective antisense mutagenic primer against rU1. Products were digested with NdeI and HhaI, gel-purified, and ligated in a mixture with the vector pSP197LTrmQT that had been cut with NdeI and HindIII and a DNA segment coding for the C-terminal part of wild type rat microsomal GST, which had been cut with HhaI and HindIII. In order to introduce the mutations C49A, H75Q, H116Q, Y115F, Y120F, the PCRs were made with the respective mutagenic primer and the antisense primer rU1, which contains the 3′-end of the coding region. The resulting PCR products were gel-purified and thereafter used as mutagenic “megaprimers” in a second PCR against rU1. Y92F and Y153F were also constructed with the “megapriming” technique. In this case, the antisense mutagenic primers were used in PCRs together with the sense primer rU1, and the products were subsequently used as megaprimers against rL in the second PCR.

All products from this second PCR were digested with NdeI and HindIII, gel-purified, and ligated into the expression vector pSP197LTLI and transformed into competent E. coli JM-109 or XL-1 Blue as described (36), positive clones were identified, and the plasmids were isolated. The inserts were sequenced by the chain termination method of Sanger et al. (37), and plasmids containing the desired mutations were transformed into E. coli BL 21 (DE 3) (that contained the plasmid pLysSL (38)). Glycogen stocks were prepared and stored frozen at −70 °C for subsequent use as starting material for the expression experiments.

Expression of Microsomal GST in E. coli BL 21 (DE 3) and Isolation of Membranes—A small aliquot (1–2 μl) of bacterial glycerol stock was grown in 1.5 ml of 2X YT overnight at 37 °C. The culture was diluted 1:100 in TB (territ broth) and grown until the A600 was 0.4–1.2. At this point, the temperature was switched to 30 °C, and expression was induced by the addition of 0.4 mM isopropyl-1-thiogalactopyranoside, followed by another 4 h shaking (250 rpm) of the culture. All steps were performed in the presence of ampicillin (75 μg/ml) and chloramphenicol (10 μg/ml). Thereafter, cells were pelleted and resuspended in TSEG buffer (15 mM Tris-HCl, pH 8.0, 0.25 M sucrose, 0.1 mM EDTA, 1 mM GSH). The cells were lysed by sonication using four 30-s pulses from a MSE Soniprep 150 sonifier at 40–60% of maximum power. Cell debris was removed by centrifugation at 5000 × g for 10 min. The supernatant was then centrifuged at 250,000 × g for 60 min, and the membrane pellets were suspended in 10 mM potassium phosphate, pH 7.0, 20% glycerol, 0.1 mM EDTA, 1 mM GSH.

Expression and Partial Purification of Recombinant Microsomal GST Mutants—Overnight cultures were prepared as described above and diluted into 3 liters of TB containing ampicillin (75 μg/ml) and chloramphenicol (10 μg/ml) in a 5-liter flask placed in a thermostated water bath. The expression of mutant microsomal GST was induced, and the culture was grown under the same conditions as described above except that oxygenation was provided by air bubbling instead of shaking. After the cells were pelleted and resuspended, lysozyme was added to a final concentration of 0.2 mg/ml, and the mixture was gently stirred for 30 min at 4 °C. The resulting spheroplasts were pelleted (8000 × g, 10 min), resuspended in TSEG buffer, and lysed by sonication as described above. Magnesium chloride was added to a final concentration of 6 mM, and DNA and RNA were hydrolyzed by DNase I (4 μg/ml) and RNase A (4 μg/ml) for an additional 30 min at 4 °C with gentle stirring. Membranes were prepared as described above and solubilized by the addition of an equal volume of 10 mM potassium phosphate, pH 7.0, 20% glycerol, 0.1 mM EDTA, 1 mM GSH, and 10% Triton X-100. Insoluble particles were removed by centrifugation (100,000 × g, 20 min). Partial purification of recombinant mutant microsomal GST was performed as follows. Solubilized membranes were adsorbed to 30 g of hydroxysapite (Bio-Gel HTP, Bio-Rad) equilibrated with buffer A for 15 min. Hydroxyapatite elution was performed by a batch procedure where the hydroxyapatite was pelleted by a low speed centrifugation pulse and washed with 4 volumes of buffer A, followed by 1 volume of 50 mM potassium phosphate in buffer A. Recombinant mutant enzyme elution was performed with 0.4 mM potassium phosphate in buffer A and desalted by dialysis for 20 h against 30 volumes of buffer A. Further purification was performed by ion exchange chromatography on a 5-ml HiTrap SP column (Pharmacia, Sweden) equilibrated with buffer A. The enzyme was eluted by a linear gradient of 1 M KCl in buffer A, and 1-mL fractions were collected at a flow rate of 2.5 ml/min. Microsomal GST content of the fractions was
determined by measurement of GST activity. Solubilization and purification was performed at 4 °C. Fractions with peak activity were analyzed by gel electrophoresis and Coomassie Blue staining. Since it was evident that a completely pure protein had not been obtained, the amounts of microsomal GST in peak fractions were determined by Western blots as described below.

**Gel Electrophoresis of Proteins**—Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to Laemmli (39) in 15% polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue R-250. Western blots and immunodetection with specific antisera toward the rat liver enzyme were performed as described (40). Polyclonal antibodies raised toward a synthetic peptide composed of amino acid residues 41–52 of rat microsomal GST were used for the detection of mutants, since the antisera directed toward full-length protein reacted differently with the mutants. For the detection of C49A, polyclonal antisera directed toward full-length microsomal GST where the antibodies reacting with this specific domain had been removed by the addition of an excess of the peptide described above were used. Purified rat microsomal GST was used as standard. Rainbow marker molecular weight standards were from Amersham.

**Protein Determination**—Protein was determined by the method of Peterson (41) with bovine serum albumin as standard.

**RESULTS**

**Inactivation by Phenylglyoxal**—Microsomal GST is rapidly inactivated by 5 mM PG at pH 8.0 (Fig. 1a). Inclusion of the substrate analogue and inhibitor glutathione sulfonate provided almost complete protection against inactivation (Fig. 1a). The stoichiometry of inactivation was examined by calculating the incorporation of [7-14C]PG into protected and unprotected enzyme. 1.7 mol of PG is incorporated per mol of unprotected enzyme, while 0.8 mol of PG is incorporated per mol of protected enzyme (Fig. 2a). Microsomal GST was reacted with [14C]PG in the presence and absence of glutathione sulfonate and digested by pepsin. The digests were separated on a C-18 reverse-phase column. Two major radioactive peaks that were preferentially labeled in the absence of glutathione sulfonate appeared in the HPLC chromatogram (not shown). Sequence determination of one of these peaks identified Arg107 as X-Ile-Phe-Val-Gly, where the first amino acid phenylthiohydantoin-derivative released had high radioactivity and eluted at a retention time that did not correspond with any amino acid standard phenylthiohydantoin-derivative (12.8 min compared with 11.8 min for phenylthiohydantoin-arginine). This sequence is identical to that from Arg107 to Gly111 of microsomal GST. The other peak consisted of a mixture of peptides where no residue could be unambiguously identified. These observations therefore suggest Arg107 to be the essential residue modified by PG.

**Site-directed Mutagenesis of Arg 107**—To further investigate the role of Arg107, the arginine was replaced with alanine and lysine residues by site-directed mutagenesis. The mutant enzymes were expressed in *E. coli* BL21(DE3), and recombinant

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**Fig. 1**. a, inactivation of microsomal glutathione transferase by phenylglyoxal (5 mM), in the absence (△) and presence of 2 mM glutathione sulfonate (▲). b, inactivation of microsomal glutathione transferase by 1,3,5-trinitrobenzenesulfonate (0.5 mM), in the absence (○) and presence of 2 mM glutathione sulfonate (●). Experimental details are described under “Methods.”

**Fig. 2**. a, modification of arginine residues of microsomal glutathione transferase as determined by the incorporation of [7-14C]phenylglyoxal in unprotected and glutathione sulfonate-protected enzyme. b, modification of lysine residues of microsomal glutathione transferase as determined by the incorporation of 1,3,5-trinitrobenzenesulfonate in unprotected and glutathione sulfonate-protected enzyme. Experimental details are described under “Methods.”
Enzyme activity of site-directed mutants of microsomal GST with CDNB as electrophilic substrate

| Mutation | Specific activity with CDNB $\mu$mol min$^{-1}$ mg$^{-1}$ |
|----------|---------------------------------------------------------|
| Wild type | 3.5                                                     |
| K67A     | 5.0                                                     |
| K67R     | 2.5                                                     |
| R107A    | ND*                                                     |
| R107K    | 2.2                                                     |
| H75Q     | 1.2                                                     |
| H105Q    | 1.5                                                     |
| H116Q    | 0.7                                                     |
| C49A     | 6.4                                                     |
| Y92F     | 3.8                                                     |
| Y115F    | 2.6                                                     |
| Y120F    | 1.7                                                     |
| Y137F    | 1.3                                                     |
| Y145F    | 2.6                                                     |
| Y153F    | 2.4                                                     |

* ND, Not detected. No activity above background could be observed.

Activity was determined in bacterial membranes as described under "Methods," and the values shown are from a typical experiment.

The amounts expressed of this mutant were considerably lower, although the majority was recovered in the membrane fraction and no increase in formation of inclusion bodies could be observed. R107K, on the other hand, where the arginine is replaced by lysine, exhibits a behavior indistinguishable to the wild type enzyme. An electrostatic interaction, involving the side chain of lysine, exhibits a behavior indistinguishable from that observed for R107A (Figs. 3 and 4).

Partial Purification of R107A—Solubilization of bacterial membranes with Triton X-100 followed by hydroxyapatite and ion exchange chromatography yielded a partially pure mutant enzyme. The elution profile was similar to wild type microsomal GST, but the yield was lower, and a completely pure protein could not be recovered. Quite surprisingly, it then became apparent that R107A actually exhibits an activity very similar to that of the wild type enzyme. Specific activities with CDNB were $4.2 \pm 0.6$ $\mu$mol min$^{-1}$ mg$^{-1}$ for the unactivated and $24 \pm 6$ $\mu$mol min$^{-1}$ mg$^{-1}$ for the NEM-treated enzyme, respectively (means $\pm$ S.D.). Like the wild type microsomal GST, R107A is also active toward N-acetyl-l-cysteine with a specific activity of $1.5$ $\mu$mol min$^{-1}$ mg$^{-1}$ for the unactivated mutant. Also, inhibition characteristics of R107A were identical to those of the wild type control and consistent with earlier reports of inhibition of microsomal GST (not shown). The difficulties in determining the enzyme activity of R107A in membrane fractions are probably a result of the low amounts expressed perhaps combined with a lower stability. In fact, these observations could be explained by a dramatic decrease in thermostability of R107A, since gradual and irreversible loss of enzyme activity results when R107A is simply left to stand at room temperature. The wild type microsomal GST is virtually unaffected (Fig. 3). When wild type enzyme was reacted with PG on ice, no significant decrease of activity was observed, not even when the incubation time was prolonged to 2 h to compensate for the presumed lower rate of reaction at 0 °C (Fig. 4).

As soon as the mixture was shifted to room temperature (24 °C), the enzyme was inactivated in a time course indistinguishable from that observed for R107A (Figs. 3 and 4). R107A thus exhibits a behavior much like the PG-modified microsomal GST, and Arg$^{107}$ most likely is essential for conformational stability. It is highly interesting to note that the PG-modified (30) and mutated (R107A) enzyme forms (the latter after thermal denaturation (Fig. 3)) both retain $\leq 50\%$ of the activity toward N-acetyl-l-cysteine. A conformational change rather than complete denaturation is thus indicated.

**Inactivation by TNBS**—Incubation of microsomal GST with 0.5 mM TNBS resulted in rapid, near complete loss of activity, and glutathione sulfonate provided very good protection (Fig. 1b). The stoichiometry of trinitrophenylation of the microsomal GST was measured by the absorbance increase at 345 nm. Assuming that the modified amino acid residue is 2,4,6-trinitrophenyl-lysine, two lysine residues were found to be modified upon inactivation. Glutathione sulfonate protected one of these residues (Fig. 2b).

Tryptic digests of microsomal GST reacted with 0.5 mM TNBS in the presence and absence of glutathione sulfonate were analyzed by reverse-phase HPLC on a C-18 column. The elution of peptides containing trinitrophenylated residues was monitored directly by the absorbance at 345 nm. One distinct peak that had high absorbance at 345 nm appeared. When glutathione sulfonate was present in the modification reaction, this peak was almost undetectable. The labeled fraction was subjected to amino acid sequence analysis. Although not completely pure, one major peptide sequence could be clearly identified as Thr-Asp-Glu-X-Val-Glu-Arg. This sequence corre-
obtained. Cys49 is clearly demonstrated as the site of activation of wild type microsomal GST, and a semipurified protein was used. However, as is demonstrated here, and amply in the literature, in vitro mutagenesis shows that this is often not the case.

The arginine-selective reagent phenylglyoxal rapidly inactivated wild type enzyme. The arginine-selective reagent phenylglyoxal rapidly inactivates microsomal GST. Protection against inactivation is provided by the substrate analogue and competitive inhibitor glutathione sulfonate. About 2 mol of the radiolabeled reagent is incorporated in the unprotected enzyme, and the inhibitor prevents incorporation of approximately 1 mol of PG (Fig. 2). The stoichiometry of the reaction between PG and arginine residues in proteins has been suggested to be both 2:1 (42) and 1:1 (43), and thus it is difficult to determine whether one or two arginine residues are modified in the unprotected microsomal GST. However, since glutathione sulfonate prevents 50% of the incorporation while protecting from inactivation, it appears most likely that inactivation of microsomal GST by PG is the result of modification of one arginine residue. Peptide digests of PG-inactivated microsomal GST displayed very complicated patterns of small overlapping peptides making separation difficult. A substantially purified peptide contained the most extensively labeled residue, which was identified as Arg107. Exchange of Arg107 to alanine by site-directed mutagenesis gave a mutant protein that was expressed and recovered in the membrane of the bacterial host. Partially purified R107A displayed an activity and inhibition profile that is indistinguishable from the wild type enzyme. Clearly, Arg107 does not appear to directly participate in catalysis or substrate binding. Nevertheless, Arg107 might well be masked by bound glutathione sulfonate, indicating proximity to the active site, but the possibility of long range conformational stabilization cannot be excluded. The decreased thermostability of R107A indicates a structural role for this arginine. R107A is irreversibly inactivated at room temperature following a time course that closely resembles the inactivation of PG-modified wild type microsomal GST (Fig. 3). Reaction of wild type enzyme with PG also induces a thomsensitization that appears to be identical to that of R107A (Fig. 4). Both the thermoinactivated R107A and the PG-inactivated wild type are able to retain a substantial activity toward N-acetyl-L-cysteine, indicating a conformational change to a new, stable “low activity” form. The positive charge of the arginine side chain appears to be important, since replacement by lysine in R107K does not affect enzyme function. Therefore, it seems likely that an electrostatic interaction is important for maintaining the optimal conformation of the active site. The new low activity form might be amenable to structural analysis and could yield valuable insights into the structure-function relation of microsomal GST.

## DISCUSSION

Selective chemical reagents that covalently modify their targets can be useful in the screening of amino acid residues that are involved in catalysis and substrate binding. Inactivation of an enzyme by such reagents accompanied by protection against inactivation by competitive inhibitors for the enzyme is used as a criterion to assess whether modification is active site-directed. However, as is demonstrated here, and amply in the literature, in vitro mutagenesis shows that this is often not the case.

The arginine-selective reagent phenylglyoxal rapidly inactivate microsomal GST. Protection against inactivation is provided by the substrate analogue and competitive inhibitor glutathione sulfonate. About 2 mol of the radiolabeled reagent is incorporated in the unprotected enzyme, and the inhibitor prevents incorporation of approximately 1 mol of PG (Fig. 2). The stoichiometry of the reaction between PG and arginine residues in proteins has been suggested to be both 2:1 (42) and 1:1 (43), and thus it is difficult to determine whether one or two arginine residues are modified in the unprotected microsomal GST. However, since glutathione sulfonate prevents 50% of the incorporation while protecting from inactivation, it appears most likely that inactivation of microsomal GST by PG is the result of modification of one arginine residue. Peptide digests of PG-inactivated microsomal GST displayed very complicated patterns of small overlapping peptides making separation difficult. A substantially purified peptide contained the most extensively labeled residue, which was identified as Arg107. Exchange of Arg107 to alanine by site-directed mutagenesis gave a mutant protein that was expressed and recovered in the membrane of the bacterial host. Partially purified R107A displayed an activity and inhibition profile that is indistinguishable from the wild type enzyme. Clearly, Arg107 does not appear to directly participate in catalysis or substrate binding. Nevertheless, Arg107 might well be masked by bound glutathione sulfonate, indicating proximity to the active site, but the possibility of long range conformational stabilization cannot be excluded. The decreased thermostability of R107A indicates a structural role for this arginine. R107A is irreversibly inactivated at room temperature following a time course that closely resembles the inactivation of PG-modified wild type microsomal GST (Fig. 3). Reaction of wild type enzyme with PG also induces a thomsensitization that appears to be identical to that of R107A (Fig. 4). Both the thermoinactivated R107A and the PG-inactivated wild type are able to retain a substantial activity toward N-acetyl-L-cysteine, indicating a conformational change to a new, stable “low activity” form. The positive charge of the arginine side chain appears to be important, since replacement by lysine in R107K does not affect enzyme function. Therefore, it seems likely that an electrostatic interaction is important for maintaining the optimal conformation of the active site. The new low activity form might be amenable to structural analysis and could yield valuable insights into the structure-function relation of microsomal GST.

TNBS is a water-soluble lysine-selective reagent that is convenient for modification experiments because it allows direct monitoring of trinitrophenylation of lysine residues without the use of radioactivity. Since it can also react with cysteine thiols, the enzyme was pretreated with NEM to prevent modification of Cys49. Microsomal GST is very rapidly inactivated by trinitrophenylation. Inactivation, as well as modification of a lysine residue, is prevented by the inhibitor glutathione sulfonate. Tryptic digests of TNBS-inactivated microsomal GST yielded...
one extensively labeled peptide that, when glutathione sulfo-
ate was present in the reaction, appeared only as a minor
shoulder in the HPLC chromatogram. Amino acid sequence an-
alysis of this peptide strongly suggested Lys\textsuperscript{67} as the modified
residue, and the active site location of Lys\textsuperscript{67} appeared obvious.

Exchange of Lys\textsuperscript{67} to alanine and arginine by site-directed
mutagenesis, however, yielded a different result than expected
from the chemical modification experiments. Neither K67A nor
K67R showed any noticeable change in enzymatic activity as
compared with the wild type enzyme. Thus, it appears that
Lys\textsuperscript{67} does not have any direct function in substrate binding or
catalysis. It is possible, however, that Lys\textsuperscript{67} might be situated in
close proximity to the active site so that the introduction of the
relatively large and bulky trinitrophenyl group at the side
chain of Lys\textsuperscript{67} induces an unfavorable conformation of the
active site or simply blocks access. Exchange of this single
residue by site-directed mutagenesis is a more subtle change
that, in this case, had no effect on the function of the enzyme.

Chemical modification of microsomal GST also indicated the
involvement of a histidine residue in catalysis (22). Mutation of the
three histidine residues in microsomal GST did not yield any
major alterations of activity and function. No essential functions
are therefore assigned to histidine residues in microsomal GST.

When the tyrosine residues of microsomal GST\textsuperscript{2} were
exchanged for phenylalanines, we expected to find a mutant that
displayed a dramatically lowered turnover number, indicating
that a thiolate anion stabilizing function of a tyrosine hydroxyl
side chain of Lys\textsuperscript{67} induces an unfavorable conformation of the
active site or simply blocks access. Exchange of this single
residue by site-directed mutagenesis is a more subtle change
that, in this case, had no effect on the function of the enzyme.

Missense mutation of histidine residues is a more subtle change
that, in this case, had no effect on the function of the enzyme.

Methionine 137F was partially activated, making it a candidate
for structural analysis of changes accompanying activation.

Microsomal GST is activated by sulfhydryl reagents, and
Cys\textsuperscript{49} was therefore early implied as the target. One could
expect that removal of the thiol would yield a constitutionally
active or an unactivable enzyme. Mutation of Cys\textsuperscript{49} to ala-
ine yielded a semiacivated enzyme that had properties of both
the unactivated and activated microsomal GST (Table III).

Incubation of C49A with NEM did not have any effect on the
activity with any of the substrates tested. Cys\textsuperscript{49} is therefore
expected to find a mutant that displayed such behavior.

In conclusion, we have demonstrated an essential structural,
noncatalytic role of Arg\textsuperscript{107} and the importance of Cys\textsuperscript{49} as the
site of activation by sulfhydryl reagents. Lys\textsuperscript{67} and all histidine
residues are not essential for catalysis. Furthermore, microso-
mal GST does not have an active site tyrosine residue assisting
the activation of GSH like the majority of cytosolic GSTs. A
distinct catalytic mechanism for the microsomal GST is there-
fore suggested.

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\textsuperscript{2} Tyrosine 18 was not exchanged, since it is known that residues 1–41
can be proteolytically removed from the enzyme that remains active
(44).

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