Tissue distribution of enzymic methylation of glutathione *S*-transferase and its effects on catalytic activity

Methylation of glutathione *S*-transferase 11–11 inhibits conjugating activity towards 1-chloro-2,4-dinitrobenzene

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Glutathione *S*-transferases (GSTs) were isolated from rat liver, lung, heart, kidney, testis and brain by coupled affinity chromatography and subunits were resolved by reverse-phase h.p.l.c. The reverse-phase h.p.l.c. technique was improved from our previously published work [Johnson, Neal, Collins & Siegel (1990) *Biochem. J.* **270**, 483–489] by changing from a C₄ to a C₁₈ wide-pore reverse-phase column; this resulted in baseline or near-baseline resolution of all GST subunits. There were significant tissue-dependent differences in the expression of GST subunits and the level of GST subunits present was quantitatively determined for each of the tissues. The extent of methylation of GSTs *in vitro* and distribution of GST methyltransferase (GST-MT) was determined in cytosolic fractions from each of these tissues. Purified GST isoenzymes were methylated with partially purified liver GST-MT. Methylation of Mu class subunits 3 and 4, the preferred substrates of methylation in liver, was substoichiometric in all tissues. The extent of methylation of subunit 3 ranged from 0.13 % to 0.94 % and subunit 4 from 0.03 % to 0.60 %. Methylation of Alpha class subunits was either not detectable or 5–10-fold less than that of Mu class subunits 3 and 4. Pi class subunit 7 was methylated to a greater extent than the Alpha class subunits but less than Mu class isoenzymes. A notable exception to this low level of methylation was GST 11–11, found mainly in testis and brain. Methylation of subunit 11 reached 21.9 % (219 pmol of methyl group/nmol of subunit 11) when this isoenzyme was incubated with partially purified liver GST methyltransferase. Methylation of GST 11–11 was found to inhibit the conjugating activity of this isoenzyme towards 1-chloro-2,4-dinitrobenzene; the degree of inhibition of conjugating activity correlated with the extent of methylation of GST 11–11. GST-MT activity toward GST subunits 3, 4 and 11 was present in kidney and liver, detectable in lung and heart, but absent from brain and testis. Anion-exchange chromatography of GST-MTs from liver and kidney suggested the presence of four different forms of GST-MT (I–IV) and indicated that GST-MT isoenzymes III and IV were present at significantly lower concentrations in kidney than liver. The present paper shows that methylation is an enzyme-catalysed reaction that differs in substrate-specificity with respect to different GST isoenzymes, that expression of GST-MT is tissue-dependent and multiple forms of the enzyme are present in liver and kidney, and that methylation inhibits GST activity. These data suggest that GST activity may be post-translationally regulated by methylation.

INTRODUCTION

The glutathione *S*-transferases (GSTs; EC 2.5.1.18) are a multifunctional group of enzymes involved in metabolic detoxication of a variety of electrophilic compounds (Mannervik & Danielson, 1988). GSTs have also been shown to participate in intracellular binding and transport of hydrophobic compounds (Listowsky *et al.*, 1988), and to catalyse key steps in the synthesis of prostaglandins and leukotrienes (Chang *et al.*, 1990). GSTs are dimeric proteins consisting of two identical or closely related subunits from the same class (Alpha, Mu, Pi or Theta; Mannervik & Danielson, 1988; Meyer *et al.*, 1991). Rat tissues contain at least 18 dimers made up of 13 different subunits (Mannervik & Danielson, 1988; Ketterer *et al.*, 1988; Kispert *et al.*, 1989; Hayes *et al.*, 1990a,b; Hiratsuka *et al.*, 1990; Tsuchida & Sato, 1990; Meyer *et al.*, 1991). GST activity is regulated at the transcriptional level by a variety of inducing compounds, including phenobarbital (Pickett *et al.*, 1984; Ding *et al.*, 1986; Di Simplicio *et al.*, 1989), 3-methylcholanthrene (Pickett *et al.*,

1984; Rushmore *et al.*, 1990), propylthiouracil (Lee *et al.*, 1986) and antioxidants (Di Simplicio *et al.*, 1989; Benson *et al.*, 1989). GST isoenzyme profiles are also altered in carcinogenesis and multi-drug-resistance, often with an appearance of Pi class GST (Satoh *et al.*, 1985; Cowan *et al.*, 1986; Moscow *et al.*, 1989). Post-translational regulation of GST is suggested by reports that GSTs are activated by active oxygen species (Murata *et al.*, 1990) and that Alpha class GSTs are substrates for protein kinase C (Pyerin *et al.*, 1987). Phosphorylation of GST 1–1 results in decreased affinity for bilirubin, indicating a functional significance for this post-translational modification (Taniguchi & Pyerin, 1989).

A previous report from this laboratory described the calmodulin-stimulated enzymic methylation of GSTs in rat liver cytosolic fractions (Neal *et al.*, 1988). More recently, GST methylation was shown to be substoichiometric and restricted to the Mu class GSTs (Johnson *et al.*, 1990). Precedence for substoichiometric yet functionally significant protein methylation is found in eukaryotic protein methylation of carboxy groups of

Abbreviations used: GST, glutathione *S*-transferase; GST-MT, GST methyltransferase; DTT, dithiothreitol.

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D-aspartic acid or L-isopartac acid residues (Aswad, 1984; Clarke, 1985, 1988). Other possible explanations for substoichiometric methylation of GSTs are: (1) that GSTs are methylated to near-stoichiometry *in vivo*, which would decrease the number of available sites remaining for methylation *in vitro*; (2) that the tertiary structure of GSTs presents limited access of methylation sites to the methyltransferase; or (3) that a highly purified methyltransferase, specific for GSTs, will be required to demonstrate stoichiometric methylation of GSTs. Des(methyl)-calmodulin, for example, can be methylated *in vitro* to stoichiometry (1 mol of trimethyl-lysine residue/mol) only by a highly purified and specific methyltransferase (Rowe *et al.*, 1986). The enzyme that methylates GSTs has not been characterized, and its tissue distribution has not been determined. One purpose of the present investigation was to determine the tissue distribution of GST methyltransferase (GST-MT). We predict that, if prior methylation *in vivo* was responsible for the substoichiometric methylation of GSTs, the extent of GST methylation *in vitro* would inversely correlate with the tissue distribution of GST-MT. This implies that GST isoenzymes isolated from tissues with low GST-MT activity would be better substrates for methylation *in vitro* than corresponding isoenzymes isolated from tissues with low or no GST-MT activity. A second aim of this investigation was to characterize more completely the GST-isoenzyme-specificity of GST-MT. Finally, the present study was designed to evaluate the effects of methylation on the catalytic activity of GST. In the course of this investigation, an improved chromatographic procedure for the analysis of GST subunits was developed. This method was used to determine the distribution of GST isoenzymes in rat brain, heart, kidney, liver, lung and testis.

MATERIALS AND METHODS

Materials

S-Adenosyl-L-[Me-³H]methionine (80 Ci/mmol) was purchased from the NEN Division of duPont. S-Hexylglutathione, GSH and epoxy-activated Sepharose 6B were purchased from Sigma Chemical Co. and coupled as previously described (Mannervik & Guthenberg, 1981; Simons & Vander Jagt, 1981). DEAE-cellulose (DE-52) was from Whatman. F.p.l.c. equipment, Mono Q HR 5/5 and S-Sepharose were from Pharmacia. Ultrafiltration units, filters and Centricon-10 microconcentrators were from Amicon. Acetonitrile was from Burdick and Jackson and trifluoroacetic acid was from Aldrich Chemical Co. System Gold from Beckman Instruments was used for h.p.l.c. analysis. Male Holtzman rats (Harlan Sprague-Dawley, Madison, WI, U.S.A.) were reared in our breeding colony. All other materials were products of Sigma Chemical Co.

Isolation of GSTs

Tissue cytosols were prepared by ultracentrifugation (100000 g) of tissues homogenized in 10 mM-Tris/HCl buffer, pH 7.8, containing 0.25 M-sucrose and 1.0 mM-dithiothreitol (DTT). All purification steps were carried out at 4 °C. Cytosolic fractions were dialysed overnight against 10 mM-Tris/HCl buffer, pH 7.8, containing 1.0 mM-DTT (buffer A), and GSTs were isolated by coupled affinity chromatography. Cytosolic fractions were first loaded on to a S-hexylglutathione-Sepharose affinity column and the 'flow-through' was immediately loaded on to a GSH-Sepharose affinity column according to the method of Hayes (1988). Both columns (1.6 cm × 10 cm) were equilibrated with buffer A containing 0.2 M-NaCl (buffer B) and eluted with buffer B containing 5 mM-S-hexylglutathione. Preliminary experiments indicated that GSTs bound to either S-hexylglutathione-Sepharose or GSH-Sepharose could be eluted with either buffer

B containing 5 mM-S-hexylglutathione or with 50 mM-Tris/HCl buffer, pH 9.6, containing 1.0 mM-DTT and 10 mM-GSH. This was determined by comparison of the h.p.l.c. profiles of the isolated GSTs (results not shown). GST 11-11 was purified from the rat testis GST pool by f.p.l.c. anion-exchange chromatography on Mono Q (Kispert *et al.*, 1989). The purity of the GST 11-11 preparations was confirmed by SDS/PAGE and reverse-phase h.p.l.c. Protein concentrations were determined by the method of Bradford (1976), with bovine γ -globulin as standard. GST preparations were concentrated by ultrafiltration in 10 mM-Tris/HCl buffer, pH 7.8, containing 1.0 mM-DTT and stored at -80 °C.

Separation of GST subunits by h.p.l.c.

H.p.l.c. conditions were modified from previously published methods (Johnson *et al.*, 1990). Solvent A was 0.1% (v/v) trifluoroacetic acid and solvent B was 70% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. GST subunits were resolved on a Vydac 30 nm C₁₈ (4.6 mm × 25 cm) wide-pore reverse-phase column with a linear gradient from 55% (v/v) solvent B (38.5% acetonitrile) to 75% (v/v) solvent B (52.5% acetonitrile) over 85 min. The flow rate was 1 ml/min and u.v. absorbance at 214 and 280 nm was monitored. There was no evidence of memory between samples, and recovery ranged from 90 to 100%. Individual peaks were collected and concentrated with a Speed Vac Concentrator (Savant) before SDS/PAGE (Hayes & Mantle, 1986b). GST subunits were identified by SDS/PAGE and by comparison with previously published h.p.l.c. profiles (Ostlund Farrants *et al.*, 1987; Ketterer *et al.*, 1988; Kispert *et al.*, 1989; Meyer *et al.*, 1989; Hiratsuka *et al.*, 1990; Hayes *et al.*, 1990b; Johnson *et al.*, 1990).

The amount of each GST subunit was determined from the peak area at 214 nm, by using the molar absorption coefficients (ϵ) for the individual subunits. Each ϵ_{214} was estimated from the ϵ_{280} , calculated from the known subunit complement of tyrosine and tryptophan (Suguoka *et al.*, 1985; Abramovitz & Listowsky, 1987; Mannervik & Danielson, 1988; Ålin *et al.*, 1989), by multiplying ϵ_{280} by the ratio of the absorbance at 214 nm to the absorbance at 280 nm (Ostlund Farrants *et al.*, 1987). By using this method, the ϵ_{214} for subunits 1, 1', 2, 3, 4, 6, 7 and 8 were 29.5×10^4 , 29.5×10^4 , 31.1×10^4 , 43.1×10^4 , 44.1×10^4 , 39.4×10^4 , 30.2×10^4 and 30.5×10^4 M⁻¹·cm⁻¹ respectively. The Mu class subunits 9 and 11 have not been completely sequenced, but, since their absorbance ratios (A_{214}/A_{280}) are very similar to that of Mu subunits 3, 4 and 6, the ϵ_{214} values of these subunits (3, 4 and 6) were averaged and this value (42.2×10^4 M⁻¹·cm⁻¹) was used to estimate the quantity of subunit 9 and 11.

Partial purification of GST-MT

Liver cytosolic fractions were prepared by ultracentrifugation (100000 g) from tissue homogenized in 25 mM-Tris/HCl buffer, pH 8.2, containing 0.25 M-sucrose, 2.0 mM-2-mercaptoethanol and 2.0 mM-EDTA. GST-MT was partially purified by a 40–60%-saturation (NH₄)₂SO₄ precipitation. The 40–60%-saturation (NH₄)₂SO₄ pellet was dissolved in 25 mM-Tris/HCl buffer, pH 8.2, containing 2.0 mM-2-mercaptoethanol and 2.0 mM-EDTA (buffer C), dialysed against this buffer overnight and then chromatographed on DE-52 DEAE-cellulose (2.5 cm × 20 cm) equilibrated with buffer C at a flow of 1.5 ml/min. All purification steps were carried out at 4 °C. Most of the GST-MT activity (> 90%) was eluted with buffer C containing 75 mM-NaCl, concentrated by ultrafiltration in 10 mM-Tris/HCl buffer, pH 7.8, containing 1.0 mM-DTT, and stored at -80 °C. GST-MT activity (pmol of methyl group incorporated/nmol of subunit per mg of protein) of the cytosolic fraction (22.7), 40–60%-saturation (NH₄)₂SO₄ pellet (80.1) and 75 mM-NaCl elution (250) towards

Fig. 1. Reverse-phase h.p.l.c. of rat cytosolic GSTs isolated from brain, heart, kidney, liver, lung and testis

Affinity-purified rat cytosolic GSTs (100 μ g) from (a) brain, (b) heart, (c) kidney, (d) liver, (e) lung and (f) testis in 10 mM-Tris/HCl buffer, pH 7.8, containing 1.0 mM-DTT were subjected to analysis by reverse-phase h.p.l.c. A gradient from 55% (v/v) solvent B [70% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid] to 75% (v/v) solvent B over 85 min (-----) separated the GST subunits. The absorbance at 214 nm was monitored (—). GST subunits were numbered as previously described by Jakoby *et al.* (1984), Mannervik & Danielson (1988), Ketterer *et al.* (1988) and Hayes *et al.* (1990a).

subunit 11 indicated that there was a 11.0-fold purification of the enzyme. This DE-75 GST-MT preparation was used to methylate GST pools isolated from the different tissues.

To characterize GST-MT further, DE-75 GST-MT was dialysed against 25 mM-sodium acetate buffer, pH 4.5, containing 1.0 mM-DTT (buffer D), and cation-exchange chromatography was done on a fast-flow S-Sepharose column (3.0 cm $\times$ 20 cm) equilibrated with buffer D at a flow of 2.5 ml/min. Most of the GST-MT activity (> 90%) was eluted with buffer D containing 200 mM-NaCl, concentrated by ultrafiltration in 10 mM-Tris/HCl buffer, pH 7.8, containing 1.0 mM-DTT and stored at -80°C . The SS-200 GST-MT (703) had 2.80-fold higher specific activity than DE-75 GST-MT (250) towards subunit 11 and this indicated

that there was a 30.8-fold purification of the enzyme from the cytosolic fraction. This SS-200 GST-MT was used to methylate GST 11–11 in studies correlating methylation with effects on GST activity.

Finally, to determine if there were different isoenzymes of GST-MT, liver and kidney cytosolic fractions were prepared as described above, but, instead of being eluted with buffer C containing 75 mM-NaCl, GST-MTs were eluted from the DE-52 DEAE-cellulose with a 400 ml linear gradient of 0–100 mM-NaCl in buffer C. GST-MT activity was measured with GST 11–11 as substrate and the absorbance at 280 nm was monitored. Peaks of GST-MT activity were pooled, concentrated and stored at -80°C .

Methylation of GST pools *in vitro*

Incubation mixtures contained 100 μ g of DE-75 GST-MT, 25 μ g of affinity-purified GSTs, 12.5 μ Ci of *S*-adenosyl-L-[*Me*-³H]methionine (80 Ci/mmol), 100 mM-Tris/HCl buffer, pH 8.0, 1.0 mM-DTT, 2.5 mM-MnCl₂ and 40 μ M-calmodulin in a final volume of 150 μ l. The concentration of *S*-adenosyl-L-methionine was adjusted to 50 μ M by adding non-radioactive *S*-adenosyl-L-methionine. All mixtures were incubated for 2 h at 37 °C, and methylated GSTs were re-isolated by coupled affinity chromatography and analysed by reverse-phase h.p.l.c. Fractions (1 ml) were collected, and the radioactivity incorporated into the GSTs was determined by liquid-scintillation spectrometry. This procedure was used in all subsequent experiments.

Methylation of GST 11–11 *in vitro* and the effect on GST activity

Incubation mixtures containing 0, 0.10, 0.25, 0.50 and 1.0 mg of SS-200 GST-MT, 10 μ g of purified GST 11–11, 12.5 μ Ci of *S*-adenosyl-L-[*Me*-³H]methionine (80 Ci/mmol), 100 mM-Tris/HCl buffer, pH 8.0, 1.0 mM-DTT and 2.5 mM-MnCl₂ in a final volume of 150 μ l. The concentration of *S*-adenosyl-L-methionine was adjusted to 200 μ M by adding non-radioactive *S*-adenosyl-L-methionine. Appropriate unmethylated controls were prepared by omitting *S*-adenosyl-L-methionine from the incubation mixture. All mixtures were incubated for 2 h at 37 °C, and methylated GST 11–11 was re-isolated by GSH–Sepharose affinity chromatography, eluted with 50 mM-Tris/HCl buffer, pH 9.6, containing 1.0 mM-DTT and 10 mM-GSH, and concentrated in Centricon-10 microconcentrators. GST activity was assayed with 1-chloro-2,4-dinitrobenzene as substrate (Habig & Jakoby, 1981). The remaining methylated sample and corresponding unmethylated controls were analysed by reverse-phase h.p.l.c.

GST-MT tissue distribution

Incubation mixtures for the assay of GST-MT activity contained 500 μ g of dialysed tissue cytosolic fractions devoid of endogenous GSTs (coupled affinity column 'flow-through'), 25 μ g of purified liver GSTs (predominantly subunits 3 and 4) or 10 μ g of purified GST 11–11, 12.5 μ Ci of *S*-adenosyl-L-[*Me*-³H]methionine (80 Ci/mmol), 100 mM-Tris/HCl buffer, pH 8.0, 1.0 mM-DTT, 2.5 mM-MnCl₂ and 40 μ M-calmodulin in a final volume of 150 μ l. The concentration of *S*-adenosyl-L-methionine was adjusted to 50 μ M by adding non-radioactive *S*-adenosyl-L-methionine. Samples were incubated for 2 h at 37 °C, and methylated GSTs were re-isolated by coupled affinity chromatography (for 3 and 4) or GSH–Sepharose affinity chromatography (for 11) and analysed by reverse-phase h.p.l.c.

Methylation *in vitro* of GST subunits by GST-MT isoenzymes

Incubation mixtures contained 100 μ g of the partially purified GST-MT isoenzymes, 25 μ g of purified liver GSTs (predominantly subunits 3 and 4) or 10 μ g of purified GST 11–11, 12.5 μ Ci of *S*-adenosyl-L-[*Me*-³H]methionine (80 Ci/mmol), 100 mM-Tris/HCl buffer, pH 8.0, 1.0 mM-DTT, 2.5 mM-MnCl₂ and 40 μ M-calmodulin in a final volume of 150 μ l. The concentration of *S*-adenosyl-L-methionine was adjusted to 50 μ M by adding non-radioactive *S*-adenosyl-L-methionine. Samples were incubated for 2 h at 37 °C, and methylated GSTs were re-isolated by coupled affinity chromatography (for 3 and 4) or GSH–Sepharose affinity chromatography (for 11) and analysed by reverse-phase h.p.l.c.

Statistical analysis

All groups of data were evaluated by analysis of variance and the individual means compared by the Fisher PLSD test

($P < 0.05$). Student's *t* test was used for analysis of paired sets of data ($P < 0.05$).

RESULTS

Tissue distribution of GST subunits

To determine the tissue distribution of GST subunits, GSTs were isolated from brain, heart, kidney, lung, liver and testis by coupled affinity chromatography and subunits were resolved by reverse-phase h.p.l.c. (Fig. 1). The reverse-phase h.p.l.c. technique was improved from our previously published work (Johnson *et al.*, 1990) by changing from a C₄ to a C₁₈ wide-pore reverse-phase column; this resulted in essentially baseline resolution of all GST subunits. Quantitative differences between the GST subunit tissue profiles in Fig. 1 are described in Table 1. In brain and heart, and to lesser extent in lung and testis, an initial doublet (7–8 min) was observed. The first peak of this doublet had very low electrophoretic mobility $M_r < 14400$, suggesting that this protein was probably not a member of the GST family, and the second had an electrophoretic mobility corresponding to glyoxalase I (Hayes, 1988). In liver and testis, both Mu class subunits 3 and 4 were major GST subunits, but in kidney these subunits were present at concentrations just above the limit of detection. In brain, subunit 3, but not subunit 4, was expressed, whereas the opposite was seen in lung and heart. Extrahepatic tissues expressed other Mu class subunits (6, 9 and 11) not substantially seen in liver. In testis subunits 6, 9 and 11 were present, in brain subunits 6 and 11 were expressed, and in heart subunit 6 was seen (Table 1 and Fig. 1). Small quantities of subunit 6 were also seen in lung and liver, and very low concentrations of subunit 11 were also present in lung and heart. All tissues had Alpha class subunits 2 and 8 to various degrees; however, subunits 1 and 1' were mainly limited to the kidney and liver, with a small amount of subunit 1 seen in testis. None of the adult tissues had detectable concentrations of Alpha class subunit 10 (Y1). In brain, kidney, lung and heart the Pi class subunit 7, not present in liver, was a major portion of the total GST subunits present. Theta class subunits 5, 12 (Meyer *et al.*, 1991) and Yrs (Hiratsuka *et al.*, 1990) do not bind to the affinity matrices and the tissue distribution of these subunits was not

Table 1. Tissue distribution of GST subunits from brain, heart, kidney, liver, lung and testis

Relative concentrations of GST subunits present in each tissue were derived from the reverse-phase h.p.l.c. profiles of purified GST pools isolated from the tissues (Fig. 1). Values represent the percentage of subunit present with respect to the total GST subunits in a given tissue. GST subunits were named as previously described by Jakoby *et al.* (1984), Mannervik & Danielson (1988), Ketterer *et al.* (1988) and Hayes *et al.* (1990a). –, The GST subunit indicated was < 1.0 % (1.0 μ g) of the total GST subunits in a given tissue.

GST subunit	Liver	Kidney	Lung	Heart	Testis	Brain
1 (Ya ₁)	10.6	26.4	–	–	1.8	–
1' (Ya ₂)	3.8	2.0	–	–	–	–
2 (Yc)	19.2	33.8	22.8	6.3	17.8	19.1
3 (Yb ₁)	26.3	1.3	4.4	3.6	12.5	17.6
4 (Yb ₂)	34.6	4.0	18.9	41.3	20.6	2.7
6 (Yb ₃ , Yn ₁)	2.2	–	1.6	11.3	10.9	17.4
7 (Yf, Yp)	–	23.0	38.4	22.6	4.8	25.9
8 (Yk)	3.4	9.5	12.5	12.7	3.7	4.9
9 (Yn ₂)	–	–	–	–	11.1	–
10 (Y1)	–	–	–	–	–	–
11 (Yo)	–	–	1.4	2.2	16.6	12.4

Table 2. Methylation of GSTs from different rat tissues with partially purified rat liver GST-MT

GSTs were methylated with 0.1 mg of GST-MT as described in the Materials and methods section. The subunits were separated by h.p.l.c. and values are the means $\pm$ s.e.m. ($n = 3$) expressed as pmol of methyl group incorporated/nmol of subunit per mg of protein. N.D., methylation of the subunit indicated was not detectable; —, the subunit indicated was not present.

GST subunit...		Extent of methylation (pmol of methyl group/nmol per mg of protein)									
		1	1'	2	3	4	6	7	8	9	11
Liver	Calmodulin absent	N.D.	N.D.	5.69 $\pm$ 3.24	25.4 $\pm$ 2.39	18.2 $\pm$ 2.02	N.D.	—	N.D.	—	—
	Calmodulin present	N.D.	N.D.	6.10 $\pm$ 1.13	46.3 $\pm$ 2.09*	33.7 $\pm$ 1.64*	N.D.	—	N.D.	—	—
	Increase (fold)			1.07	1.82	1.85					
Kidney	Calmodulin absent	1.88 $\pm$ 0.08	N.D.	7.41 $\pm$ 0.14	45.8 $\pm$ 4.92	17.1 $\pm$ 0.60	—	12.8 $\pm$ 0.70	2.29 $\pm$ 0.43	—	—
	Calmodulin present	3.45 $\pm$ 0.69	N.D.	7.77 $\pm$ 1.37	70.7 $\pm$ 4.47*	25.9 $\pm$ 3.53*	—	16.1 $\pm$ 0.63	3.24 $\pm$ 0.76	—	—
	Increase (fold)	1.84		1.05	1.54	1.51		1.26	1.41		
Lung	Calmodulin absent	—	—	9.71 $\pm$ 1.31	57.9 $\pm$ 3.88	16.9 $\pm$ 0.44	N.D.	25.9 $\pm$ 1.21	7.41 $\pm$ 0.07	—	306 $\pm$ 9.30
	Calmodulin present	—	—	9.32 $\pm$ 1.32	94.3 $\pm$ 1.15*	23.8 $\pm$ 0.67*	N.D.	27.9 $\pm$ 1.67	6.83 $\pm$ 0.90	—	246 $\pm$ 13.2*
	Increase (fold)			0.96	1.63	1.41		1.08	0.92		0.80
Heart	Calmodulin absent	—	—	N.D.	N.D.	3.45 $\pm$ 0.16	N.D.	2.91 $\pm$ 0.09	N.D.	—	112 $\pm$ 2.94
	Calmodulin present	—	—	N.D.	N.D.	5.33 $\pm$ 0.25*	N.D.	3.26 $\pm$ 0.12	N.D.	—	93.5 $\pm$ 2.04*
	Increase (fold)					1.54		1.12			0.83
Testis	Calmodulin absent	N.D.	—	2.12 $\pm$ 0.52	12.9 $\pm$ 0.67	5.95 $\pm$ 1.05	5.97 $\pm$ 0.32	13.6 $\pm$ 2.76	N.D.	N.D.	438 $\pm$ 6.44
	Calmodulin present	N.D.	—	4.21 $\pm$ 0.67	23.6 $\pm$ 1.83*	9.72 $\pm$ 0.51*	7.57 $\pm$ 0.59	18.2 $\pm$ 1.79*	N.D.	N.D.	378 $\pm$ 20.7*
	Increase (fold)			1.99	1.83	1.63	1.27	1.34			0.86
Brain	Calmodulin absent	—	—	5.85 $\pm$ 0.48	30.0 $\pm$ 0.92	42.1 $\pm$ 1.60	3.84 $\pm$ 0.47	11.6 $\pm$ 0.13	N.D.	—	272 $\pm$ 22.0
	Calmodulin present	—	—	6.76 $\pm$ 2.38	61.7 $\pm$ 4.78*	60.3 $\pm$ 2.99*	6.03 $\pm$ 0.61*	20.3 $\pm$ 2.70*	N.D.	—	298 $\pm$ 18.6
	Increase (fold)			1.16	2.06	1.43	1.57	1.75			1.10

* Significantly different from the corresponding value in the absence of calmodulin ($P < 0.05$).

Table 3. Effect of methylation of GST 11-11 on the activity of this isoenzyme towards 1-chloro-2,4-dinitrobenzene

GST 11-11 was methylated as described in the Materials and methods section. GST activity was measured by assaying conjugation of 1-chloro-2,4-dinitrobenzene with GSH. GST activity was determined for both methylated and corresponding unmethylated control preparations and values are the means $\pm$ S.E.M. ($n = 4$) expressed as μmol of 1-chloro-2,4-dinitrobenzene conjugated/min per nmol of GST dimer. The extent of methylation and quantity of subunit were determined by h.p.l.c. and values are the means $\pm$ S.E.M. ($n = 4$) expressed as pmol of methyl group incorporated/nmol of subunit 11.

Sample	GST activity ($\mu\text{mol}/\text{min per nmol}$)		Extent of methylation (pmol of methyl group/nmol of subunit)		
	GST activity	Inhibition (%)	(pmol/nmol)	(% of subunits methylated)	(% of dimers methylated)
0.00 mg of GST-MT					
S-Adenosyl-L-methionine absent	1.95 $\pm$ 0.07	—	—	—	—
S-Adenosyl-L-methionine present	2.00 $\pm$ 0.09	0.00	0.00	0.00	0.00
0.10 mg of GST-MT					
S-Adenosyl-L-methionine absent	2.04 $\pm$ 0.42	—	—	—	—
S-Adenosyl-L-methionine present	1.98 $\pm$ 0.37	3.09	70.3 $\pm$ 3.95	7.03	14.1
0.25 mg of GST-MT					
S-Adenosyl-L-methionine absent	1.83 $\pm$ 0.14	—	—	—	—
S-Adenosyl-L-methionine present	1.46 $\pm$ 0.08*†	20.2	108 $\pm$ 6.94	10.8	21.6
0.50 mg of GST-MT					
S-Adenosyl-L-methionine absent	1.90 $\pm$ 0.10	—	—	—	—
S-Adenosyl-L-methionine present	1.30 $\pm$ 0.06*†	31.6	173 $\pm$ 13.4	17.3	35.6
1.0 mg of GST-MT					
S-Adenosyl-L-methionine absent	2.22 $\pm$ 0.08	—	—	—	—
S-Adenosyl-L-methionine present	1.28 $\pm$ 0.12*†	42.3	219 $\pm$ 10.8	21.9	43.8

* Significantly different from the corresponding sample in the absence of S-adenosyl-L-methionine ($P < 0.05$).

† Significantly different GST activity compared with the corresponding sample containing 0.00 mg of GST-MT ($P < 0.05$).

Table 4. Tissue distribution of rat GST-MT

GSTs were methylated with 0.5 mg of tissue cytosol devoid of endogenous GSTs (passed through both S-hexylglutathione-Sepharose and GSH-Sepharose) as described in the Materials and methods section. The subunits were separated by h.p.l.c. and values are the means $\pm$ S.E.M. ($n = 3$) expressed as pmol of methyl group incorporated/nmol of subunit per mg of protein. N.D., GST-MT activity was not detectable.

GST subunit...	Extent of methylation (pmol of methyl group/nmol per mg of protein)		
	3	4	11
Liver cytosol			
Calmodulin absent	0.99 $\pm$ 0.08	1.22 $\pm$ 0.12	22.7 $\pm$ 0.61
Calmodulin present	1.36 $\pm$ 0.14*	2.54 $\pm$ 0.04*	20.9 $\pm$ 0.22
Increase (fold)	1.38	2.09	0.92
Kidney cytosol			
Calmodulin absent	3.10 $\pm$ 0.52	1.81 $\pm$ 0.05	13.6 $\pm$ 0.93
Calmodulin present	5.00 $\pm$ 0.42*	3.64 $\pm$ 0.15*	11.9 $\pm$ 0.29
Increase (fold)	1.61	2.01	0.88
Lung cytosol			
Calmodulin absent	0.47 $\pm$ 0.06	0.54 $\pm$ 0.05	3.50 $\pm$ 0.17
Calmodulin present	0.62 $\pm$ 0.13	1.19 $\pm$ 0.10*	—
Increase (fold)	1.32	2.19	—
Heart cytosol			
Calmodulin absent	N.D.	N.D.	2.30 $\pm$ 0.19
Calmodulin present	N.D.	N.D.	—
Increase (fold)	—	—	—
Testis cytosol			
Calmodulin absent	N.D.	N.D.	N.D.
Calmodulin present	N.D.	N.D.	N.D.
Increase (fold)	—	—	—
Brain cytosol			
Calmodulin absent	N.D.	N.D.	N.D.
Calmodulin present	N.D.	N.D.	N.D.
Increase (fold)	—	—	—

* Significantly different from the corresponding control value without the addition of calmodulin ($P < 0.05$).

determined. Protein peaks identified by '?' may be uncharacterized GST subunits (Fig. 1). The largest of these unknown proteins appeared to be specific to the heart (Fig. 1b). This protein was not completely separated from subunit 4 and was eluted before subunit 9 (Fig. 1e).

Methylation *in vitro* of GSTs isolated from different rat tissues

To determine if GSTs common to liver and extrahepatic tissues can be methylated *in vitro* to the same extent, and if GSTs expressed in extrahepatic tissues, but not in liver, are substrates for GST-MT, GST pools from each tissue were methylated *in vitro* with *S*-adenosyl-L-[Me-³H]methionine and re-isolated by coupled chromatography, and GST subunits were separated by h.p.l.c. for determination of radioactivity (Table 2). Mu class GST subunits 3 and 4 were found to be the preferred subunits for methylation, and Alpha class subunits were minimally methylated. Calmodulin stimulation was Mu-class-specific for liver GSTs (Table 2). Analysis of variance indicated there were statistically significant tissue differences between the extent of subunit 3 and 4 methylation, but the extent of methylation of subunits 3 and 4 was not inversely related to the tissue distribution of GST-MT (Table 4). GSTs isolated from tissues with low GST-MT activity were not better substrates for methylation *in vitro*.

In all cases the methylation of Alpha class subunits (1, 1', 2 and 8) was less than that of subunits 3 and 4 (Table 2). Subunit 7 was methylated more than the Alpha class subunits but less than subunit 3 and 4, and the effect of calmodulin on subunit 7 methylation was variable. Two other Mu class subunits (6 and 9) were minimally methylated to an extent comparable with that of Alpha class GST subunits. Subunit 11 was methylated to a greater extent than other subunits and on a mole-to-mole basis reached a maximum of 4.4% in testis (Table 2). This level of stoichiometry exceeded that of subunits 3 and 4 by 10–20-fold. In addition, calmodulin had no stimulatory effect on the methylation of subunit 11 and actually inhibited the methylation of subunit 11 in testis, heart and lung.

Effect of methylation of GST 11–11 on GST activity

Since GST 11–11 could be methylated to a greater extent than other GST isoenzymes, this enzyme was used to determine the effects of methylation on GST activity. GST 11–11 was isolated from a rat testis GST pool and methylated with partially purified hepatic GST-MT to a level of stoichiometry of 21.9% with respect to GST subunit and 43.8% with respect to GST dimer on a mole-to-mole basis (Table 3). Methylation of GST 11–11 was found to inhibit the conjugating activity of this isoenzyme towards 1-chloro-2,4-dinitrobenzene, and the degree of inhibition of conjugating activity correlated with the extent of methylation of GST 11–11 (Table 3). GST activity towards 1-chloro-2,4-dinitrobenzene was inhibited by 0, 3.09, 20.2, 31.6 and 42.3% when GST 11–11 was methylated 0, 14.1, 21.6, 35.6 and 43.8% respectively. The values expressed per dimer assumed that only one subunit per dimer was methylated.

Tissue distribution of GST-MT

These experiments were done to establish the best source of GST-MT for subsequent purification of this enzyme. The tissue distribution of GST-MT was determined by methylation of liver GST subunits 3, 4 and 11 with tissue cytosolic fractions devoid of endogenous GSTs (passed through both *S*-hexylglutathione-Sepharose and GSH-Sepharose). Kidney possessed the greatest GST-MT activity, followed by liver, lung and heart (Table 4). No GST-MT activity was detected in the cytosolic fractions of either brain or testis. The data indicated that,

Fig. 2. Anion-exchange chromatography of liver and kidney GST-MTs

Rat GST-MTs were partially purified from (a) liver and (b) kidney cytosolic fractions by a 40–60% saturation $(\text{NH}_4)_2\text{SO}_4$ precipitation followed by anion-exchange chromatography on DE-52 DEAE-cellulose. The 40–60% saturation $(\text{NH}_4)_2\text{SO}_4$ pellet was dissolved in 25 mM-Tris/HCl buffer, pH 8.2, containing 2.0 mM-2-mercaptoethanol and 2.0 mM-EDTA, dialysed against this buffer overnight and chromatographed on DE-52 DEAE-cellulose. GST-MT was eluted from the DE-52 DEAE-cellulose with a 400 ml linear gradient of 0–100 mM-NaCl (-----). GST-MT activity (d.p.m.) was measured with GST 11–11 as substrate (●) and the absorbance at 280 nm was monitored (—). Peaks of GST-MT activity were pooled and concentrated for subsequent analysis.

although the highest concentration of GST-MT is present in the kidney, owing to its greater mass the liver was still the most practical source of GST-MT.

Partial purification of GST-MT isoenzymes

To determine if multiple forms of GST-MT were present in rat tissues, GST-MTs were partially purified from liver and kidney cytosolic fractions by a 40–60% saturation $(\text{NH}_4)_2\text{SO}_4$ precipitation followed by gradient elution from DE-52 DEAE-cellulose. The results of the chromatography of liver (Fig. 2a) and kidney (Fig. 2b) cytosolic fractions indicated the presence of four different forms of GST-MT (I–IV) in both tissues. GST-MT isoenzymes III and IV appeared to be present at significantly lower concentrations in kidney than in liver. GST-MTI was eluted before the start of the gradient, and GST-MTs II, III and IV were eluted at 20 mM-, 35 mM- and 65 mM-NaCl respectively (Fig. 2). No GST-MT activity was eluted with subsequent 200 mM- and 1000 mM-NaCl washes of the DE-52 DEAE-cellulose (results not shown). The fractions containing GST-MT activity were pooled and concentrated for further analysis.

Table 5. Methylation of GSTs by liver GST-MT isoenzymes I, II, III, IV

GSTs were methylated with 0.1 mg of GST-MT as described in the Materials and methods section. The subunits were separated by h.p.l.c. and values are the means $\pm$ S.E.M. ($n = 3$) expressed as pmol of methyl group incorporated/nmol of subunit per mg of protein.

GST-MT isoenzyme...	Extent of methylation (pmol of methyl group/nmol per mg of protein)			
	I	II	III	IV
Subunit 3				
Calmodulin absent	6.90 $\pm$ 0.31†	13.5 $\pm$ 1.29†	7.68 $\pm$ 0.53†	9.01 $\pm$ 0.08†
Calmodulin present	14.2 $\pm$ 1.22*†	18.9 $\pm$ 2.53*†	14.1 $\pm$ 2.27*	13.7 $\pm$ 1.03*
Increase (fold)	2.05 $\pm$ 0.09‡	1.40 $\pm$ 0.06§	1.82 $\pm$ 0.18‡	1.52 $\pm$ 0.10§
Subunit 4				
Calmodulin absent	5.52 $\pm$ 0.53†	8.68 $\pm$ 0.48†	5.39 $\pm$ 0.61†	6.09 $\pm$ 0.25†
Calmodulin present	17.1 $\pm$ 1.23*†	15.6 $\pm$ 1.06*†	14.8 $\pm$ 0.98*	15.8 $\pm$ 1.19*
Increase (fold)	3.11 $\pm$ 0.08‡	1.79 $\pm$ 0.04‡	2.78 $\pm$ 0.14	2.62 $\pm$ 0.29
Ratio of subunit 3/4				
Calmodulin absent	1.26 $\pm$ 0.07	1.54 $\pm$ 0.07	1.44 $\pm$ 0.08	1.48 $\pm$ 0.05
Calmodulin present	0.83 $\pm$ 0.01*¶	1.20 $\pm$ 0.09*‡	0.94 $\pm$ 0.09*¶	0.89 $\pm$ 0.14*¶
Subunit 11				
Calmodulin absent	141 $\pm$ 4.84†	291 $\pm$ 12.7†	188 $\pm$ 1.68†	202 $\pm$ 4.52†

* Significantly different from control values without added calmodulin ($P < 0.05$).

† Significantly different from corresponding values for all other subunits ($P < 0.05$).

‡ Significantly different from corresponding values for all other GST-MT isoenzymes ($P < 0.05$).

§ Significantly different from corresponding values for GST-MT isoenzymes I and III ($P < 0.05$).

|| Significantly different from corresponding values for GST-MT isoenzymes I and II ($P < 0.05$).

¶ Significantly different from corresponding values for GST-MT isoenzyme II ($P < 0.05$).

Methylation *in vitro* of GST subunits by specific GST-MT isoenzymes

To determine if there were differences in activity between the four GST-MT isoenzymes, the substrate specificity of each GST-MT isoenzyme was determined for GST subunits 3, 4 and 11 (Table 5). The effect of calmodulin on each GST-MT was also determined. Calmodulin significantly increased the methylation of subunit 3 and 4 by all GST-MT isoenzymes and significantly decreased the ratio of subunit 3 to subunit 4 methylation. This decrease in the ratio of subunit 3 to subunit 4 methylation revealed a tendency for calmodulin to make subunit 4 rather than subunit 3 a preferred methylation substrate (Table 5). Statistical analysis of methylation data showed a rank order for calmodulin stimulation of GST-MT isoenzymes of $I > III > II = IV$ for the fold increase in methylation of subunit 3 and $I > III = IV > II$ for the fold increase in methylation of subunit 4 by addition of calmodulin.

Recovery of methylated GST 11–11 by GSH-Sepharose affinity chromatography

It has been demonstrated that methylation of GSTs is inhibited by physiological concentrations of GSH (Neal *et al.*, 1988). This suggested that methylation may interfere with the binding of GSH by GST. Since methylated GSTs were re-isolated from incubation mixtures by GSH-Sepharose affinity chromatography, there may be preferential loss of methylated GSTs in the affinity-column 'flow-through' with only a minor fraction of methylated GSTs binding to the affinity matrix. To determine if methylation affected the binding of GSTs to the affinity column, SDS/PAGE and fluorography were performed as previously described (Neal *et al.*, 1988) on the complete incubation mixture, the affinity-column 'flow-through' and GSH elution (Fig. 3). Half of the incubation mixture was prepared for SDS/PAGE, and GST 11–11 was purified from the remaining portion of the sample. The Coomassie Brilliant Blue-stained gel (Fig. 3a) and corresponding fluorogram (Fig. 3b) of liver GST-MT isoenzymes

I, II, III and IV are depicted. Methylation of GST 11–11 with liver GST-MT isoenzymes I, II, III and IV reflected the specific-activity values shown in Table 5. The only substrate for GST-MT present in the incubations was GST 11–11 (Fig. 3). In all cases, no significant amount of GST 11–11 protein or radio-labelled GST 11–11 was present in the affinity-column 'flow-through' (Fig. 3, lanes 2, 5, 8 and 11). There did appear to be some loss of 'flow-through' protein and GST 11–11 protein eluted from the affinity column compared with the complete incubation mixture. This is probably due to loss of some protein during concentration in Centricon-10 microconcentrators of the 'flow-through' and elution, but not the complete incubation mixture.

DISCUSSION

Differences in the tissue distribution and expression of GST isoenzymes have been documented (Tu *et al.*, 1983; Hayes & Mantle, 1986a,b; Ostlund Farrants *et al.*, 1987; Abramovitz & Listowsky, 1987, 1988; Hayes, 1988; Ketterer *et al.*, 1988; Kispert *et al.*, 1989; Meyer *et al.*, 1989; Tsuchida & Sato, 1990; Hiratsuka *et al.*, 1990). Early work characterizing GSTs was done mainly by comparison of SDS/PAGE and immunoblotting (Tu *et al.*, 1983; Hayes & Mantle, 1986a,b). However, there were and still are problems with cross-reactivity of GST antibodies and quantification by immunoblotting. Similarly, the co-migration of GST subunits 3, 4, 6, 9 and 11, which differ by less than M_r 500 on SDS/PAGE, causes difficulties in identification and quantification by SDS/PAGE. Development of an h.p.l.c. technique by Ostlund Farrants *et al.* (1987) significantly improved the ability to identify and quantify GST proteins, and has been used extensively (Ketterer *et al.*, 1988; Kispert *et al.*, 1989; Meyer *et al.*, 1989; Hiratsuka *et al.*, 1990; Johnson *et al.*, 1990; Hayes *et al.*, 1990b; Meyer *et al.*, 1991). We have modified the h.p.l.c. technique so that all known subunits can be separated with essentially baseline resolution. GST subunits in rat cytosolic fractions isolated from brain, heart, kidney, liver, lung and testis

Fig. 3. Methylation of GST 11-11 with liver GST-MT isoenzymes I, II, III and IV: recovery of methylated GST from GSH-Sepharose affinity column

GST 11-11 was methylated as described in the Materials and methods section. At the end of the incubation, half of the sample mixture was removed and prepared for SDS/PAGE. The remaining sample was loaded on to a GSH-Sepharose affinity column. Both the 'flow-through' and GSH elution were collected and concentrated in Centricon-10 microconcentrators before SDS/PAGE. The Coomassie Brilliant Blue-stained gel (a) and corresponding fluorogram (b) of liver GST-MT isoenzymes I (lanes 1-3), II (lanes 4-6), III (lanes 7-9) and IV (lanes 10-12) are depicted. Each set of three lanes contain the complete incubation mixture (lanes 1, 4, 7 and 10), affinity-column 'flow-through' (lanes 2, 5, 8 and 11) and GSH elution (lanes 3, 6, 9 and 12) for the indicated GST-MT isoenzyme. The time of exposure for fluorography was 7 days.

were separated by reverse-phase h.p.l.c., revealing dramatically different patterns in GST subunit expression (Table 1 and Fig. 1). The data presented here correlate well with previously published data determined by immunoblotting and h.p.l.c. profiles of GSTs purified from the same tissues (Hayes & Mantle, 1986a; Ostlund Farrants *et al.*, 1987; Ketterer *et al.*, 1988; Kispert *et al.*, 1989; Meyer *et al.*, 1989; Hiratsuka *et al.*, 1990). For example, Kispert *et al.* (1989) determined that the percentage of subunit 11 present with respect to all subunits in testis, brain, heart (cardiac muscle), lung, kidney and liver to be 16.5, 12.1, 2.5, 1.2, 1.0 and 0.8% respectively. In the same tissues, our data were 16.6, 12.3, 2.2,

1.4, < 1.0 and < 1.0% respectively. The present investigation establishes quantitative values of GST subunit expression in several rat tissues, data not previously available. These values can then be used to determine the tissue- and/or subunit-specific effects of various compounds known to alter GST activity on changes in the patterns of subunit expression. Benson *et al.* (1989) have already utilized such techniques to show sex-specific differences in GST expression and subunit-specific induction of GSTs by two anticarcinogenic compounds, 2(3)-t-butyl-4-hydroxyanisole and bisethylxanthogen, in mice. In addition, Hayes *et al.* (1990b) demonstrated the preferential induction of subunit 1' (Ya₂) in rat livers bearing aflatoxin-induced pre-neoplastic nodules. Furthermore, h.p.l.c. can be used to identify and characterize new subunits (Kispert *et al.*, 1989; Meyer *et al.*, 1989; Hiratsuka *et al.*, 1990; Hayes *et al.*, 1990b; Meyer *et al.*, 1991). Our data suggest the presence of a major unidentified rat heart subunit (Fig. 1b).

On the basis of the GST-MT tissue distribution, it could be hypothesized that GSTs may be methylated *in vivo* in liver and kidney, but not in testis and brain. If this is true, the extent of methylation *in vitro* of subunits 3 and 4 isolated from testis and brain would be much greater than that of the same subunits isolated from kidney and liver. The data presented in Table 2 do not support this hypothesis, but they do suggest that under normal physiological conditions the tissue concentrations of GSH, an inhibitor of GST methylation (Neal *et al.*, 1988), may be sufficient to suppress GST methylation *in vivo*. Methylation of GSTs may therefore take place only on depletion of GSH. A variety of conditions can result in decreased concentrations of GSH. Oxidative stress induced by a large number of toxic compounds and therapeutic agents, as well as metal ions and radiation, decrease tissue concentrations of GSH (Meister & Anderson, 1983; Meister, 1988). *In vivo*, depletion of GSH may be achieved experimentally by use of inhibitors that interfere with GSH synthesis. Buthionine sulfoximine inhibits γ -glutamylcysteine synthetase and significantly decreases GSH concentrations *in vivo* (Meister & Anderson, 1983). Depletion of rat liver GSH by injection of buthionine sulfoximine resulted in decreased concentrations of hepatic GSH and increased methylation of GSTs *in vitro* (Neal *et al.*, 1988). In addition to the depletion of GSH and enhanced methylation of GSTs, there was a significant decrease in the GST activity towards 1-chloro-2,4-dinitrobenzene in buthionine sulfoximine-treated animals compared with control saline-injected animals (Neal *et al.*, 1988). This decrease in GST activity was not attributable to a loss of GST protein, as determined by SDS/PAGE (results not shown). These data suggested that conditions that decrease the normal concentrations of GSH may allow for methylation of the GSTs *in vivo*, resulting in loss of GST activity without significant loss of GST protein.

Testis GST 11-11 was methylated by partially purified GST-MT isolated from liver. Methylation of GST 11-11 was found to inhibit the conjugating activity of this isoenzyme towards 1-chloro-2,4-dinitrobenzene (Table 3), indicating a correlation between inhibition of GST activity and GST methylation. If a single GSH-binding site and substrate-binding site were present on each subunit of a GST dimer and these were catalytically independent of one another, the inhibition of GST activity should correlate with the level of methylation expressed per subunit. In contrast, if subunit-subunit interactions were co-operative and this co-operative interaction is required for catalytic activity, the inhibition of GST activity should correlate with the level of methylation expressed per dimer. The data indicated that the degree of inhibition of conjugating activity correlated with the extent of methylation of GST 11-11 dimers, implying that methylation of one subunit of a dimer was sufficient for

inhibition of GST activity (Table 3). Experiments evaluating the effect of methylation of GST 11-11 on the kinetics of GST activity (K_m and V_{max} for GSH and 1-chloro-2,4-dinitrobenzene), as well as comparison of unmethylated and methylated GST 11-11 activity towards other characterized substrates [e.g. 1,4-dichloro-2-nitrobenzene, 4-hydroxynonenal, *trans*-stilbene oxide and 1,2-epoxy-3-(*p*-nitrophenoxy)propane] should aid in elucidating the effects of methylation on GST activity. If each subunit of a dimer was catalytically independent, the methylation of one subunit of a dimer GST should not prevent binding to GSH-Sepharose regardless of the position of methylation, since the unmethylated subunit could still bind. Methylation did not affect the binding of GST 11-11 to the GSH-Sepharose affinity column (Fig. 3). These data support the hypothesis that the methylated amino acid(s) of GST 11-11 is (are) present in the substrate-binding site and not in the GSH-binding site of the protein; and that the inhibition of methylation by physiological concentrations of GSH (Neal *et al.*, 1988) may be a result of steric protective effects induced by the binding of GSH to GST. Thus, even though GST 11-11 was isolated from testis and methylated with liver GST-MT, the methylation of GST 11-11 provides a model system in which to characterize the effect of methylation on GST activity (Table 3).

The role of protein methylation in higher organisms is not known. Numerous proteins have been shown to be post-translationally methylated, including calmodulin (Murtaugh *et al.*, 1986; Vincent & Siegel, 1987; Siegel *et al.*, 1990), histones (Paik & Kim, 1970), apoprotein cytochrome *c* (Park *et al.*, 1987), lamin B (Chelsky *et al.*, 1989), the Ha-*ras* gene product (Clarke *et al.*, 1988) and brain G protein γ -subunits (Yamane *et al.*, 1990). It was determined that unmethylated calmodulin activated NAD kinase, a calmodulin-regulated enzyme, to a greater extent than *N*-methylated calmodulin (Roberts *et al.*, 1986). *N*-Methylation was also shown to protect calmodulin from ATP/ubiquitin-dependent degradation (Gregori *et al.*, 1985). The methylation of lamin B during mitosis occurred before incorporation of the protein into the nuclear envelope at the completion of mitosis (Chelsky *et al.*, 1989). If lamin B methylation was slowed, then the assembly of the nuclear membrane was delayed. Methylation of apoprotein cytochrome *c* facilitated the import of the protein into the mitochondria by what appeared to be a receptor-mediated mechanism (Park *et al.*, 1987). There is evidence implying that C-terminal methylation of a cysteine in the *ras*-gene product may be a signal for stable interaction with membranes (Deschenes *et al.*, 1989). Mutant *ras*-gene-product proteins lacking the methylation site were not stably associated with the membrane (Deschenes & Broach, 1987). The same mechanism is postulated for the G protein γ -subunit, which interacts with the G protein β -subunit to form a complex that is membrane-associated (Yamane *et al.*, 1990). Both the G protein γ -subunit and *ras*-gene-product protein are also isoprenylated at the same C-terminal residue (Clarke *et al.*, 1988; Deschenes *et al.*, 1989; Yamane *et al.*, 1990). The stable nature of prenylation due to a thioether linkage implies that this type of modification may be permanent, whereas methylation may serve as a signal designating the site of subsequent prenylation (Yamane *et al.*, 1990). These various reports indicate that methylation plays a significant role in the regulation of the activities of a diverse group of proteins.

GSTs are involved in metabolic detoxication of a variety of electrophilic compounds (Mannervik & Danielson, 1988), intracellular binding and transport of hydrophobic compounds (Listowsky *et al.*, 1988) and catalysis of key steps in the synthesis of prostaglandins and leukotrienes (Chang *et al.*, 1990). GST isoenzyme profiles are altered in carcinogenesis and multi-drug-resistance, often with an appearance of Pi class GST (Sato *et*

al., 1985; Cowan *et al.*, 1986; Moscow *et al.*, 1989). Since the demonstration of GST methylation is a novel and relatively recent observation, we do not know where or if methylation is involved in altering endogenous GST functions. The techniques to separate methylated from unmethylated or quantify the fraction of GSTs methylated *in vivo* do not currently exist. However, the finding that GST 11-11 was methylated to near-stoichiometry has allowed us to determine the effect of methylation on GST activity *in vitro*. At the present time, we know methylation is an enzyme-catalysed reaction that differs in substrate-specificity with respect to the different GST isoenzymes, that expression of GST-MT is tissue-dependent and multiple forms of the enzyme are present in liver and kidney, and that methylation *in vitro* inhibits GST activity.

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