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Characterization of methylation of rat liver cytosolic glutathione S-transferases by using reverse-phase h.p.l.c. and chromatofocusing

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Glutathione S-transferase (GST) subunits in rat liver cytosol were separated by reverse-phase h.p.l.c.; five major proteins were isolated and identified as subunits 1, 2, 3, 4 and 8. F.p.l.c. chromatofocusing resolved the affinity-purified GST pool into nine different isoenzymes. The five basic (Alpha class) dimeric peaks of GST activity were 1–1, 1–2a, 1–2b, 2–2a and 2–2b. Reverse-phase h.p.l.c. analysis revealed that subunit 8 was also present in the protein peaks designated 1–1, 1–2a and 1–2b. The four neutral (Mu class) isoenzymes were 3–3, 3–4, 3–6 and 4–4. The GST pool was methylated *in vitro* before reverse-phase h.p.l.c. or f.p.l.c. chromatofocusing. Chromatofocusing indicated that the Mu class isoforms (3–3, 3–4 and 4–4) were the primary GSTs methylated, and h.p.l.c. analysis confirmed that subunits 3 and 4 were the major methyl-accepting GST subunits. The addition of calmodulin stimulated the methylation *in vitro* of GST isoenzymes 3–3, 3–4 and 4–4 by 3.0-, 7.5- and 9.9-fold respectively. Reverse-phase h.p.l.c. also indicated that only the methylation of GST subunits 3 and 4 was stimulated by calmodulin. Basic GST isoenzymes were minimally methylated and the methylation was not enhanced by calmodulin. Investigation of the time course of methylation of GST subunits 3 and 4 indicated that at incubation times less than 4 h the methylation of both Mu class subunits was stimulated by calmodulin, and that under such conditions subunit 4 was the preferred substrate. In contrast, there was essentially no calmodulin-stimulated methylation at incubation times of 4 or 6 h, and the methylation of subunit 3 was predominant. Kinetic parameters at 2 h of incubation were determined in the presence and in the absence of calmodulin. The addition of calmodulin doubled the $V_{max.}$ for methylation of both subunits 3 and 4 and decreased the K_m of subunit 4 for S-adenosyl-L-methionine 3.6-fold. Finally, methylation was substoichiometric and after 6 h of incubation ranged from 2.8 to 7.6% on a mole-to-mole basis for subunits 4 and 3 respectively.

INTRODUCTION

The glutathione S-transferases (GSTs, EC 2.5.1.18) are a multifunctional group of proteins involved in metabolic detoxication of a variety of electrophilic compounds (Mannervik & Danielson, 1988). These enzymes catalyse nucleophilic attack of the electrophile by the cysteine sulphur atom of GSH, resulting in conjugate formation. GSTs have also been shown to take part in intracellular binding and transport of xenobiotics and endogenous compounds (Listowsky *et al.*, 1988). In addition, GSTs appear to be involved in the synthetic pathways of prostaglandins and leukotrienes (Chang *et al.*, 1987*a*,*b*). GSTs are dimeric proteins consisting of two identical or closely related subunits from the same class (Alpha, Mu or Pi; Mannervik & Danielson, 1988). Rat liver contains at least 14 dimers made up of 11 different subunits (Mannervik & Danielson, 1988; Ketterer *et al.*, 1988; Kispert *et al.* 1989).

A previous report from this laboratory noted that a 29000-M_r protein in rat liver was methylated *in vitro* (Siegel & Wright, 1985). The methylation was stimulated by dialysis and by the addition of calmodulin. Subsequent isolation and characterization of the 29000-M_r protein identified the protein as a member of the GST family, and the inhibitor of GST methylation removed by dialysis was found to be GSH (Neal *et al.*, 1988). It had been previously demonstrated that some GSTs were good substrates for Ca^{2+}/phospholipid-dependent protein kinase (Pyerin *et al.*, 1987). These two reports were the first to show post-translational modification of the GSTs.

The role of protein methylation in higher organisms remains unknown. However, numerous proteins have been shown to be post-translationally methylated, including calmodulin (Siegel *et al.*, 1990; Vincent & Siegel, 1987; Murtaugh *et al.*, 1986), histones (Paik & Kim, 1970), apoprotein cytochrome *c* (Park *et al.*, 1987), lamin B (Chelsky *et al.*, 1989) and the Ha-*ras*-gene product (Clark *et al.*, 1988). It was determined that unmethylated calmodulin activated NAD^+ kinase, a calmodulin-regulated enzyme, to a greater extent than did N-methylated calmodulin (Roberts *et al.*, 1986). N-Methylation was also known 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). Finally, there is evidence implying that C-terminal methylation of the *ras*-gene product may be a signal for stable interaction with membranes (Deschenes *et al.*, 1989). Mutant *ras* proteins lacking the methylation site were not stably associated with the membrane (Deschenes & Broach, 1987). These data suggest that methylation plays a significant part in the regulation of diverse activities of some proteins.

The observation that GSTs were post-translationally methylated is a novel finding and the phenomenon may be involved in some aspect of regulation of GST. The present investigation was

Abbreviation used: GST, glutathione S-transferase.

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designed to evaluate GST methylation further by determining which of the GST isoenzymes and subunits are methylated, the effects of calmodulin on methylation and the stoichiometry of methylation.

MATERIALS AND METHODS

Materials

S-Adenosyl-L-[Me-^{3}H]methionine (80 Ci/mmol) was purchased from the NEN Division of duPont. S-Hexylglutathione and epoxy-activated Sepharose 6B were purchased from Sigma Chemical Co. and coupled as previously described (Mannervik & Guthenberg, 1981). DEAE-cellulose (DE-52) was from Whatman. Ultrafiltration units and filters were from Amicon. F.p.l.c. equipment, Polybuffer 96 and the Mono P HR 5/20 column were from Pharmacia. Acetonitrile was from Burdick and Jackson, and trifluoroacetic acid was from Aldrich Chemical Company. System Gold from Beckman Instruments was used for h.p.l.c. analysis. Male Sprague–Dawley rats were purchased from Harlan Sprague–Dawley, Madison, WI, U.S.A. All other materials were products of Sigma Chemical Co.

Isolation of GSTs

Hepatic liver cytosol was prepared by ultracentrifugation (100000 *g*) of liver homogenized in 10 mM-Tris/HCl buffer, pH 7.4, containing 0.25 M-sucrose and 1.0 mM-dithiothreitol. All purification steps were carried out at 4 °C. The cytosol was dialysed overnight against 10 mM-Tris/HCl buffer, pH 7.8, containing 1.0 mM-dithiothreitol, and GSTs were isolated as described previously (Ålin *et al.*, 1985). Protein concentrations were determined by the method of Bradford (1976), with bovine γ-globulin as standard. GST preparations were stored at −80 °C.

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

H.p.l.c. conditions were similar to the method of Ostlund Farrants *et al.* (1987). 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 C_4 wide-pore reverse-phase column (0.4 cm × 5.0 cm) with a linear gradient from 50 % (v/v) solvent B (35 % acetonitrile) to 62.5 % (v/v) solvent B (43.8 % acetonitrile) over 50 min. The flow rate was 1 ml/min, and the 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, 1986). M_r standards were phosphorylase *b* (M_r 92500), BSA (M_r 66200), ovalbumin (M_r 45000), carbonic anhydrase (M_r 31000), soya-bean trypsin inhibitor (M_r 21500) and lysozyme (M_r 14400). Most of the subunits could be identified by SDS/PAGE; however, subunits 3 and 4, which co-migrated on gels, were distinguished from one another by *N*-terminal sequencing (results not shown).

The amount of protein was quantified 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 (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 this method the ϵ_{214} values for subunits 1, 2, 3, 4 and 8 were 29.5×10^4, 31.1×10^4, 43.1×10^4, 44.1×10^4 and 44.1×10^4 $M^{-1} \cdot cm^{-1}$ respectively. These values were similar to those reported by Ostlund Farrants *et al.* (1987).

Separation of GST dimers by f.p.l.c.

The method of Ålin *et al.* (1985) was used, with slight modifications. The f.p.l.c. pumps and buffers were refrigerated and the temperature was maintained at 10 °C. Before injection all samples were adjusted to pH 10.6 by addition of 1 M-NaOH. The flow rate was 0.75 ml/min, and elutions of the first and second pH gradients were each performed with 80 ml of buffer. Approx. 5 mg of GST protein gave optimum resolution of the different isoenzymes. The quantity of protein in each isoenzyme peak from the f.p.l.c. was estimated from the peak area. The area of each peak was divided by the total area and from this ratio the protein content of each peak was estimated as a fraction of total loaded protein. Fractions were collected and assayed for GST activity with 1-chloro-2,4-dinitrobenzene as substrate (Habig & Jakoby, 1981). Peaks of GST activity were pooled, dialysed overnight against 10 mM-Tris/HCl buffer, pH 7.8, containing 1.0 mM-dithiothreitol, concentrated and stored at −80 °C. H.p.l.c. analysis was performed to determine which subunits were present in each of the pooled dimer peaks.

Methylation *in vitro*

Incubation mixtures of 150 μl contained 100 μg of partially purified GST methyltransferase (Neal *et al.*, 1988), 25 μg of affinity-purified GSTs, 12.5 μCi of S-adenosyl-L-[Me-^{3}H]-methionine (80 Ci/mmol; 6.9 μM), 100 mM-Tris/HCl buffer, pH 8.0, 1.0 mM -dithiothreitol, 2.5 mM-$MnCl_2$ and 100 μg of calmodulin. All incubations were at 37 °C, and S-adenosyl-L-methionine concentrations were altered by adding unlabelled S-adenosyl-L-methionine. If the amount of GSTs was increased, then the other components of the methylation reaction were increased proportionally. After incubation, methylated GSTs were re-isolated by S-hexylglutathione–Sepharose affinity chromatography and analysed by either h.p.l.c. or f.p.l.c. Fractions (1 ml) were collected and the radioactivity incorporated into the GSTs was determined by liquid-scintillation spectrometry.

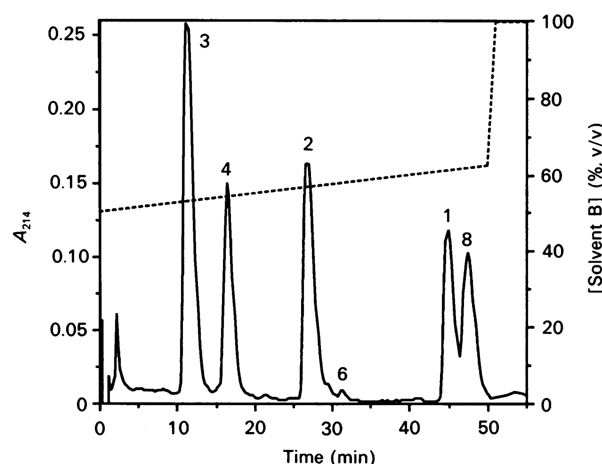


Fig. 1. Reverse-phase h.p.l.c. of rat liver cytosolic GSTs eluted from S-hexylglutathione–Sepharose

Approx. 300 μg of S-hexylglutathione–Sepharose-affinity-column-purified rat liver GSTs in 10 mM-Tris/HCl buffer, pH 7.8, containing 0.2 mM-dithiothreitol were subjected to analysis by reverse-phase h.p.l.c. A gradient from 50 % solvent B (70 % acetonitrile) to 62.5 % solvent B over 50 min (------) separated the GST subunits (see the Materials and methods section). The absorbance at 214 nm was monitored (——). GST subunits were numbered as previously described by Jakoby *et al.* (1984), Mannervik & Danielson (1988) and Ketterer *et al.* (1988).

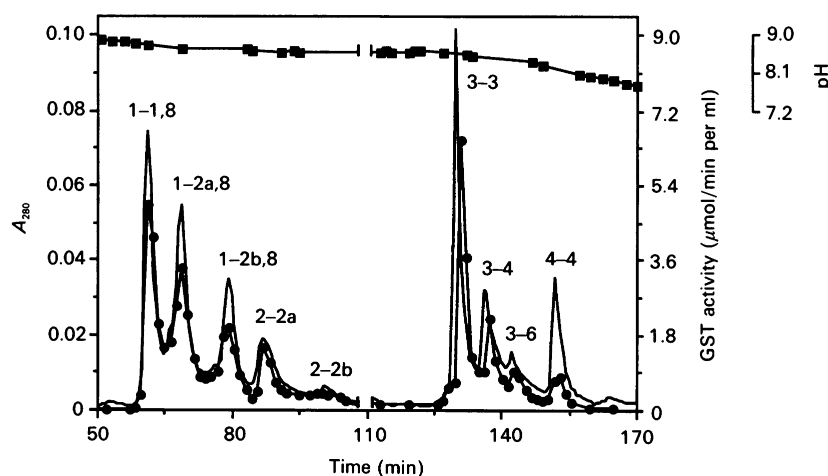


Fig. 2. F.p.l.c. chromatofocusing of rat liver cytosolic GSTs eluted from *S*-hexylglutathione–Sepharose

GSTs (5 mg) eluted from the *S*-hexylglutathione–Sepharose affinity column were adjusted to pH 10.6 by addition of 1 M-NaOH before loading of the sample on the chromatofocusing column. The entire f.p.l.c. system including buffers was refrigerated and the temperature was maintained at 10 °C. The Mono P column was equilibrated with 100 ml of 25 mM-triethylamine, pH 10.6, overnight at a flow rate of 0.1 ml/min; the flow rate was increased to 0.75 ml/min 1 h before the sample was loaded on to the column. Basic GST dimers were eluted with 80 ml of 0.59% (v/v) Pharmalytes, pH 9.0, and the neutral isoenzymes were eluted with 80 ml of 0.5% Pharmalytes/2.5% Polybuffer 96, pH 7.5. The pH (■) and absorbance at 280 nm (——) were monitored. Fractions were collected and the GST activity was measured by assaying conjugation of 1-chloro-2,4-dinitrobenzene with GSH (●). For subunit numbering see Fig. 1 legend.

The subunit methylation data were evaluated by analysis of variance and the individual means were compared by the Fisher PLSD test ($P < 0.05$). Michaelis–Menten kinetic constants K_m and $V_{max.}$ were determined by using the computer program Enz-Fitter. Student's *t* test was used for analysis of paired sets of data ($P < 0.05$).

RESULTS

H.p.l.c. of GSTs

The separation of rat liver GST subunits by h.p.l.c. is represented in Fig. 1. The order of elution of the subunits was 3, 4, 2, 6, 1 and 8, with corresponding retention times of 11.4, 16.5, 26.8, 31.3, 44.9 and 47.5 min respectively. All subunits were cleanly separated except for a slight overlap of subunits 1 and 8. Electrophoretic mobility aided in the identification of protein peaks from the h.p.l.c. (Hayes & Mantle, 1986). Assignment of the first and second peaks as subunits 3 and 4 (both with M_r 26900) was made by *N*-terminal sequencing (results not shown).

F.p.l.c. of GSTs

The separation of GST isoenzymes by f.p.l.c. yielded nine different absorbance (280 nm) and corresponding GST activity peaks (Fig. 2). The Alpha class dimers were resolved into at least five different isoforms, namely 1–1, 1–2a, 1–2b, 2–2a and 2–2b eluted at pH 8.74, 8.72, 8.68, 8.66 and 8.63 respectively. H.p.l.c. analysis demonstrated that subunit 8 was also present in the first three peaks (Fig. 3). Peak 1–1 contained approximately equal quantities of subunit 1 and subunit 8 (Fig. 3*a*). There was a greater amount of subunit 1 than subunit 8 in 1–2a, whereas the opposite was seen for GST 1–2b (Figs. 3*b* and 3*c*). In both cases the quantity of subunit 2 was equal to the sum of the protein content of subunits 1 and 8. There was no observable difference in the h.p.l.c. patterns of 2–2a and 2–2b (results not shown).

The Mu class dimers were separated into the four forms 3–3, 3–4, 3–6 and 4–4. These data were in agreement with the results obtained previously (Ålin *et al.*, 1985). The pH values of elution for GST dimers were pH 8.60 for 3–3, pH 8.46 for 3–4, pH 8.38 for 3–6 and pH 8.15 for 4–4.

Table 1. F.p.l.c. chromatofocusing of methylated rat liver GSTs

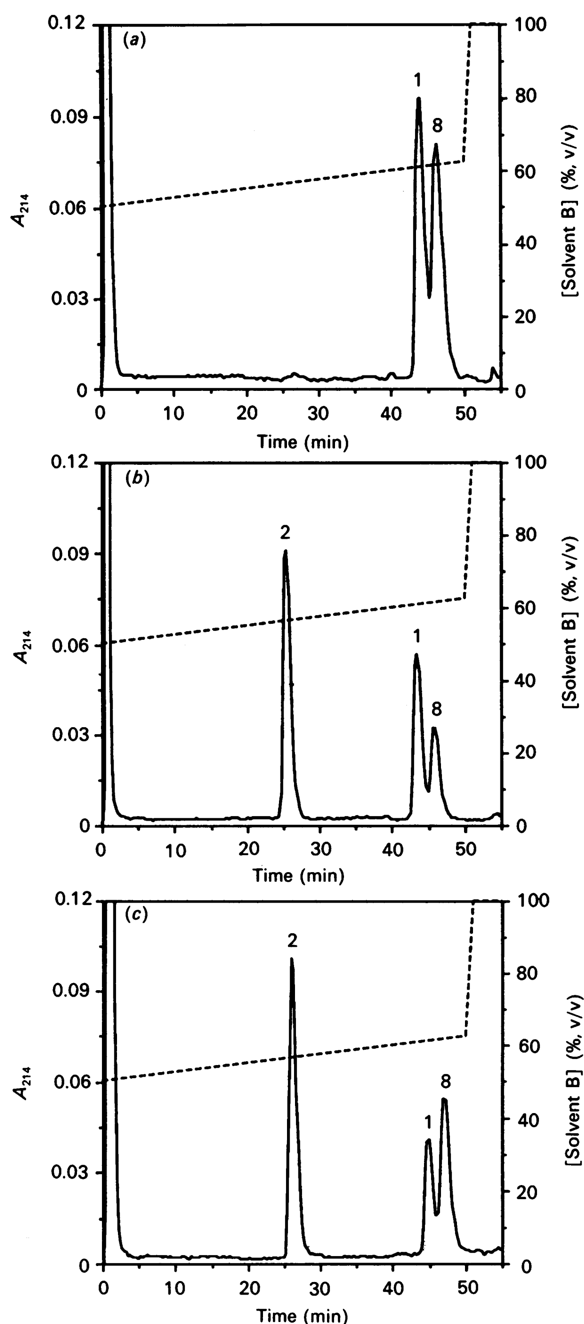
The GST pool eluted from the *S*-hexylglutathione–Sepharose affinity column was methylated for 1 h as described in the Materials and methods section. The dimers were separated by f.p.l.c. and values are expressed as fmol of methyl group incorporated/nmol of subunit.

	Extent of methylation (fmol of methyl group/nmol)		
GST dimer	Calmodulin absent	Calmodulin present	Increase (fold)
1–1,8	2.00	5.00	2.52
1–2a,8	0.90	1.20	1.36
1–2b,8	1.30	3.20	2.50
2–2a	0.90	1.50	1.75
2–2b	0.20	0.40	2.00
3–3	17.7	58.0	3.29
3–4	15.9	119	7.49
4–4	39.7	393	9.90

F.p.l.c. and h.p.l.c. of methylated GSTs

Samples were methylated in the absence and in the presence of calmodulin and then separated by f.p.l.c. (Table 1). All of the isoenzymes were methylated, but there was an obvious preference for the Mu class isoenzymes 3–3, 3–4 and 4–4. Similarly, the stimulatory effect of calmodulin on methylation was greatest for 3–3, 3–4 and 4–4. In the absence of calmodulin Mu class isoenzymes 3–3, 3–4 and 4–4 were methylated approx. 17–40-fold more, and with addition of calmodulin 23–160-fold more, than the Alpha class dimers (Table 1).

GSTs were methylated before separation by reverse-phase h.p.l.c. Similarly to the f.p.l.c. data, there was a high degree of



specificity of methylation with respect to Mu class GST subunits 3 and 4 (Table 2). Furthermore, the effects of added calmodulin on methylation were also Mu-class-specific. Subunits 3 and 4 were equally good substrates in the absence of calmodulin, and addition of calmodulin significantly increased the methylation of both subunits 3 and 4, by 3.0-fold and 7.1-fold respectively (Table 2). Calmodulin had little effect on the methylation of subunits 1, 2 and 8 (Table 2).

Mono P purification of GST dimers before methylation resulted in increased methylation of the three Alpha class subunits by 2–5-fold regardless of the absence or the presence of calmodulin. Similarly to the GST pool, subunits 3 and 4 from isolated dimers were equally good substrates in the absence of calmodulin (Table 2). However, isolation dramatically diminished the stimulatory effects of calmodulin on methylation. In an attempt to explain the effects of f.p.l.c. purification on the methylation pattern of GST subunits, the GST pool was incubated with Pharmalytes for 24 h at 4 °C before methylation. Exposure to Pharmalytes resulted in loss of calmodulin-stimulated methylation of subunits 3 and 4, and increased the extent of methylation of the Alpha class subunits compared with untreated GSTs (results not shown). These results were comparable with the extents of subunit methylation seen with f.p.l.c.-purified GST isoenzymes. On the basis of these data, further experiments were focused exclusively on subunits 3 and 4 from the GST pool.

Time-dependent methylation of Mu class GSTs

Methylation reaction mixtures were incubated for 0–6 h in buffer containing 100 μM-*S*-adenosyl-L-methionine to ensure that the methyl donor utilized by GST methyltransferase was not limiting. The time course of methylation of subunits 3 and 4 in the absence and in the presence of calmodulin is depicted in Fig. 4. At incubation times of less than 4 h the extents of methylation of subunits 3 and 4 without added calmodulin were very similar; the addition of calmodulin to the incubation mixture significantly

Fig. 3. Comparison of GST isoenzymes 1–1, 1–2a and 1–2b by reverse-phase h.p.l.c.

Approx. 50 μg of f.p.l.c.-purified dimers (*a*) 1–1, (*b*) 1–2a and (*c*) 1–2b were subjected to analysis by reverse-phase h.p.l.c. A gradient from 50% solvent B (70% acetonitrile) to 62.5% solvent B over 50 min (------) separated the different GST subunits (see the Materials and methods section). The absorbance at 214 nm was monitored (——). For subunit numbering see Fig. 1 legend.

Table 2. GST subunit methylation before and after f.p.l.c. isolation of the different isoenzymes

GST dimers in the affinity column pool were isolated by f.p.l.c. The GST pool and purified dimers were methylated for 1 h as described in the Materials and methods section. The subunits were separated by h.p.l.c. and values are the means ± S.E.M. ($n \geq 3$) expressed as fmol of methyl group incorporated/nmol of subunit.

	Extent of methylation (fmol of methyl group/nmol)				
Sample	1	2	3	4	8
GST pool					
Calmodulin absent	4.00 ± 0.10	5.20 ± 1.70	59.1 ± 15.4	74.1 ± 22.8	4.90 ± 0.70
Calmodulin present	7.10 ± 1.60	7.70 ± 2.20	178 ± 55.4*	525 ± 179*	7.90 ± 1.40
Increase (fold)...	1.77	1.48	3.01	7.08	1.61
Purified dimers					
Calmodulin absent	10.2 ± 5.20	25.7 ± 11.7	81.2 ± 31.5	40.7 ± 14.3	17.0 ± 6.40
Calmodulin present	12.3 ± 5.90	23.5 ± 9.80	116 ± 36.7	58.8 ± 13.2†	16.9 ± 6.80
Increase (fold)...	1.20	0.91	1.43	1.44	0.99

* Significantly different from the corresponding value in the absence of calmodulin ($P < 0.05$).
† Significantly different from the extent of subunit methylation in the GST pool ($P < 0.05$).

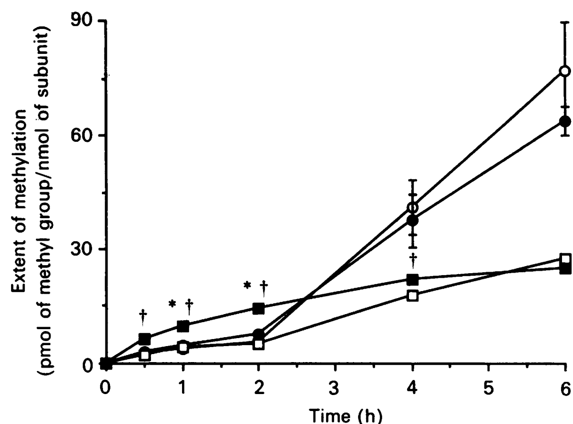


Fig. 4. Effect of increasing time of incubation on methylation *in vitro* of GST subunits 3 and 4

GSTs were methylated as described in the Materials and methods section. The final concentration of S-adenosyl-L-methionine in all incubations was 100 μM. A gradient from 50% solvent B (70% acetonitrile) to 62.5% solvent B over 50 min separated the GST subunits (see the Materials and methods section). The extents of subunit 3 methylation with (●) and without (○) added calmodulin and subunit 4 methylation with (■) and without (□) added calmodulin are expressed as pmol of methyl group incorporated/nmol subunit. Individual values are represented as the means ± S.E.M. ($n = 3$) and points without error bars indicate that the S.E.M. is less than the radius of the symbol. * Significant stimulation of subunit 3 methylation by addition of calmodulin ($P < 0.05$); † significant stimulation of subunit 4 methylation by addition of calmodulin ($P < 0.05$).

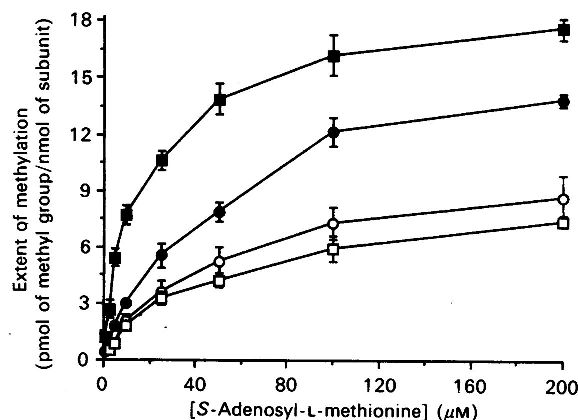


Fig. 5. Effect of increasing S-adenosyl-L-methionine concentrations on methylation *in vitro* of GST subunits 3 and 4

GSTs were methylated as described in the Materials and methods section. All samples were incubated for 2 h. A gradient from 50% solvent B (70% acetonitrile) to 62.5% solvent B over 50 min separated the GST subunits (see the Materials and methods section). The extents of subunit 3 methylation with (●) and without (○) added calmodulin and subunit 4 methylation with (■) and without (□) added calmodulin are expressed as pmol of methyl group incorporated/nmol of subunit. Individual values are represented as the means ± S.E.M. ($n = 3$) and points without error bars indicate that the S.E.M. is less than the radius of the symbol.

enhanced the methylation of both subunit 3 (2 h, 1.4-fold) and subunit 4 (2 h, 2.8-fold). Subunit 4 was the preferred substrate, principally owing to calmodulin stimulation (Fig. 4). However, at incubation times of 4 h and more dramatically at 6 h the methylation of subunit 3 was predominant; and, although calmodulin induced a slight significant increase in subunit 4 methylation at 4 h (1.2-fold), by 6 h calmodulin had no effect on the methylation of either subunit (Fig. 4). Maximum stoichiometry for both subunits was achieved at 6 h. Subunit 3 was methylated to 7.6% and subunit 4 to 2.8% on a mole-to-mole basis. The addition of fresh partially purified GST methyltransferase during the incubation did not enhance the extent of GST subunit methylation (results not shown).

Kinetic parameters for S-adenosyl-L-methionine

Since calmodulin-stimulated methylation of subunits 3 and 4 was greatest at 2 h, the K_m and $V_{max.}$ for S-adenosyl-L-methionine were determined by varying the S-adenosyl-L-methionine concentration at an incubation time of 2 h. There was a dose-dependent increase in methylation of both subunits 3 and 4 in the presence and in the absence of calmodulin with increasing S-adenosyl-L-methionine concentrations (Fig. 5). The K_m and $V_{max.}$ were determined from three independent experiments. The means ± S.E.M. of these three determinations for each subunit are presented in Fig. 5. There was no significant difference between the K_m for subunit 3 with or without added calmodulin (Table 3). However, the $V_{max.}$ for subunit 3 was significantly increased 1.6-fold by calmodulin. In contrast, both the K_m and $V_{max.}$ for subunit 4 were changed by addition of calmodulin. The K_m was decreased 3.6-fold and $V_{max.}$ increased 2-fold (Table 3). Comparison of subunits 3 and 4 indicated that there was no significant difference between the maximum rate of methylation either with or without added calmodulin (Table 3). Similarly, the K_m values for subunits 3 and 4 in the absence of calmodulin were not different. The only significant difference between the methylation of subunits 3 and 4 was the K_m in the presence of calmodulin (Table 3).

Table 3. Steady-state S-adenosyl-L-methionine Michaelis–Menten kinetic values for the methylation of GST subunits 3 and 4

The GST pool eluted from the S-hexylglutathione–Sepharose affinity column was methylated for 2 h at various S-adenosyl-L-methionine concentrations. The subunits were separated by h.p.l.c. Values of K_m and $V_{max.}$ represent the means ± S.E.M. ($n = 3$) and are expressed as μM-S-adenosyl-L-methionine and pmol of methyl group incorporated/nmol of subunit respectively.

	Calmodulin absent		Calmodulin present	
GST subunit	K_m (μM)	$V_{max.}$ (pmol of methyl group/nmol)	K_m (μM)	$V_{max.}$ (pmol of methyl group/nmol)
3	54.2 ± 11.3	11.0 ± 2.51	57.5 ± 7.65	18.0 ± 0.34*
4	54.8 ± 12.2	9.28 ± 0.74	15.2 ± 1.59*†	18.5 ± 0.78*

* Significantly different from the corresponding value in the absence of calmodulin ($P < 0.05$).
† Significantly different from the corresponding value for subunit 3 ($P < 0.05$).

DISCUSSION

The use of reverse-phase h.p.l.c. and f.p.l.c. chromatofocusing in the separation of GST subunits and dimers respectively has been previously documented (Ostlund Farrants *et al.*, 1987; Kispert *et al.*, 1989; Ålin *et al.*, 1985). Modification of these methods resulted in improved resolution of GST isoenzymes by f.p.l.c. chromatofocusing (Fig. 2) and GST subunits by reverse-phase h.p.l.c. (Fig. 1). Characterization of Mono P-purified GST dimer peaks by reverse-phase h.p.l.c. yielded some interesting results, especially with regard to Alpha class isoforms. Subunit 8 was present in the first three protein peaks (1–1, 1–2a and 1–2b) eluted from the Mono P column (Fig. 3). The data for GST peak 1–1 implied that a pair of different dimers, 1–1 and 8–8, were eluted simultaneously from the Mono P column or that a heterodimer of subunits 1 and 8 exists. In addition, comparison of the h.p.l.c. profiles for 1–2a and 1–2b suggested that more of a 8–8 dimer was co-eluted with 1–2b than with 1–2a, or that the isoenzyme peak 1–2b contained more of a 2–8 heterodimer than did 1–2a. Since the pH of elution of 8–8 has been shown to be approx. 6.0 (Ålin *et al.*, 1989), the existence of basic heterodimers 1–8 and 2–8 appears to be the most probable explanation for the presence of subunit 8 in the basic GST peaks 1–1, 1–2a and 1–2b.

Methylation data indicated that the neutral (Mu class) GSTs 3–3, 3–4 and 4–4 were the preferred substrates for methylation *in vitro*. The stimulatory effect of calmodulin on methylation was limited to the Mu class isoenzymes and associated more with subunit 4 than with subunit 3. Isolation of GST dimers by chromatofocusing before methylation, rather than after methylation, virtually eliminated the calmodulin-stimulated methylation of Mu class subunits and also increased the calmodulin-independent methylation of Alpha class subunits (Table 2). These observations were attributed to an interaction between the GSTs and Pharmalytes used for chromatofocusing.

Increased incubation times resulted in the loss of calmodulin-stimulated methylation and a switch in preferred subunit methylation (Fig. 4). In addition, *S*-adenosyl-L-methionine kinetics, determined at 2 h incubation, revealed that the $V_{max.}$ of methylation was approximately doubled for both subunits 3 and 4 by calmodulin (Table 3). The K_m for subunit 4 was also significantly decreased by calmodulin, and the only significant difference between the methylation of subunits 3 and 4 was a lower K_m for subunit 4, compared with subunit 3, in the presence of calmodulin (Table 3). Finally, methylation was substoichiometric, and on a mole-to-mole basis ranged from 2.8 % to 7.6 % for subunits 4 and 3 respectively (Fig. 4).

The low stoichiometry of GST methylation is probably not due to inherent instability of the methyl group on GST, since treatment with 0.2 M-NaOH for 4 h at 85 °C did not result in loss of radiolabel (results now shown). In addition, it does not appear that a subpopulation of highly methylated GSTs do not bind to the affinity column matrix. This conclusion was based on reverse-phase h.p.l.c. of the unbound fractions (wash-through and salt wash) from the *S*-hexylglutathione–Sepharose affinity column. These data indicated that neither protein nor radioactivity, significantly different from background, was eluted at the retention times corresponding to Mu class subunits (results not shown). Two more probable causes of substoichiometric methylation are that optimum incubation conditions have not yet been achieved and/or that the partially purified enzyme preparation did not possess sufficient GST methyltransferase activity. Increasing the time of incubation substantially increased the stoichiometry of methylation for subunit 3 from 0.5 % at 1 h to 7.8 % at 6 h (Fig. 4). Further manipulation of incubation conditions at 6 h (i.e. *S*-adenosyl-L-methionine and partially purified GST methyltransferase concentrations, buffers and ionic strength) may increase stoichiometry.

Another possible explanation for the low extents of methylation is that the rat liver GSTs may be methylated *in vivo* before isolation. This in itself would restrict the pool of GSTs available for methylation and would account for the observed substoichiometry. Calmodulin is an example of such a phenomenon (Siegel & Wright, 1985). Bovine testis calmodulin is highly methylated *in vivo* and is substoichiometrically methylated *in vitro*. Thus addition of this form of calmodulin to incubation mixtures inhibits methylation of other forms of calmodulin that are unmethylated by product inhibition of the enzyme (Siegel & Wright, 1985). The techniques required to separate methylated from unmethylated or to quantify the fraction of GSTs methylated *in vivo* do not presently exist.

Finally, GST methylation may actually be substoichiometric, but this does not eliminate the possibility of a regulatory function. Substoichiometric methylation would be consistent with the literature on substrates of carboxymethyltransferases (Clark, 1985). Although substoichiometric, carboxymethylation is proposed to participate in protein repair and targeting of a subpopulation of proteins to lysosomes for subsequent degradation (Clark, 1988).

The finding that GSH inhibited methylation *in vitro* of GSTs at concentrations lower than that found in normal rat liver cytosol brings into question the relevance of this process *in vivo* (Neal *et al.*, 1988). It would appear, on the basis of these data, that GSTs would not be methylated under normal conditions. However, under conditions of oxidative stress induced by a large number of toxic compounds and therapeutic agents, as well as metal ions and radiation, tissue concentrations of GSH are lowered (Meister, 1988; Meister & Anderson, 1983). GSH depletion may also be achieved by use of inhibitors that interfere with GSH synthesis. Buthionine sulphoximine blocks γ-glutamylcysteine synthetase activity and significantly lowers GSH concentrations *in vivo* (Meister & Anderson, 1983), and depletion of rat liver GSH by injection of buthionine sulphoximine resulted in increased methylation of the GSTs *in vitro* (Neal *et al.*, 1988). These data suggest that conditions that lower the normal concentrations of GSH may allow for methylation of the GST protein *in vivo*. Further characterization of the methylation process under conditions relevant *in vivo* is required before its physiological relevance can be established.

Lack of stoichiometric levels of methylation and, as of yet, the inability to separate unmethylated and methylated GSTs precludes an attempt to evaluate the functional significance of GST methylation. However, the specificity of methylation and calmodulin stimulation of methylation for Mu class GSTs suggest that methylation may be involved in the regulation of these enzymes. Whether that regulation be at the level of endogenous substrate binding, protein turnover, membrane targeting, enzyme activity or other physiological phenomena remains to be determined.

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