Mass spectrometric analysis of rat liver cytosolic glutathione S-transferases: modifications are limited to N-terminal processing

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Cytosolic glutathione S-transferases (GSTs) from rat livers were purified using an S-hexylglutathione affinity column. The GST subunits were resolved by reverse-phase HPLC and their molecular masses were determined by electrospray mass spectrometry. The major hepatic GSTs detected were subunits 1, 1', 2, 3 and 4, with molecular mass of 25 520, 25 473, 25 188, 25 782 and 25 571 Da respectively. Subunits 6, 7 and 10 are minor components, with molecular mass of 25 551, 23 308 and 25 211 Da respectively. Alternatively, the hepatic GSTs were purified using a glutathione affinity column. Subunits 1, 1', 2, 8 and 10 were

eluted from this column with GSSG, the oxidized form of glutathione. Subunit 8 has a molecular mass of 25 553 Da. The remaining proteins on the glutathione affinity column were removed with glutathione and S-hexylglutathione. Subunits 2, 3, 4 and 6 could be detected in the eluate. We could not detect any significant difference in molecular mass between GSTs isolated from male and female rat livers. Cytosolic GSTs were isolated from livers of buthionine sulfoximine-treated female rats for MS analysis. The molecular masses obtained were identical to those determined for the controls.

INTRODUCTION

Glutathione S-transferases (GSTs; EC 2.5.1.18) are dimeric enzymes that catalyse both GSH-dependent conjugation and reduction [1,2]. The reactions are considered as one of the initial steps in the biotransformation of xenobiotics [3].

Rat GSTs have been the subject of numerous studies. The primary structures of more than 13 different GST subunits from rat tissues have been reported. Combinations of these subunits give at least 16 homo- or hetero-dimeric enzymes. These isoenzymes can be distinguished by their physical, chemical, immunological, enzymic and structural properties. The cytosolic enzymes are grouped into four distinct gene families, designated class-Alpha (subunits 1, 2, 8 and 10), class-Mu (subunits 3, 4, 6, 9 and 11), class-Pi (subunit 7) and class-Theta (subunits 5, 12 and 13) GSTs [4–6]. A separate membrane-bound transferase, designated microsomal GST, has been described by Morgenstern et al. [7].

Among the cytosolic GSTs, the complete amino acid sequences of 11 subunits are known. The primary structures of GST 4 [8] and GST 8 [9] have been elucidated by the techniques of protein chemistry. The other full-length sequences have been deduced from the corresponding cDNA structures. A search of the GenBank database reveals that multiple cDNA sequences have been reported for subunits 1 [10,11], 2 [12,13] and 3 [14,15]. In addition, the amino acid sequences of GSTs 4 [16] and 8 [17] deduced from the genomic and cDNA clone respectively are not identical to those obtained by protein sequencing. The expression of all these variants in tissues has not been established. The complete sequences, and hence the molecular masses, of GSTs 9 [18] and 11 [19] are not known.

The *in vivo* N-terminal processing and modification of GSTs have been well documented [8,9]. Under *in vitro* conditions, class-Alpha GSTs can act as substrates for protein kinase C, and the phosphorylated enzymes show decreased affinity for bilirubin [20]. The class-Mu GSTs can be modified with methyltransferase [21]. Methylation of GST 11-11 inhibits the conjugating activity of this enzyme towards 1-chloro-2,4-dinitrobenzene. The results

suggest that GST activity may be post-translationally regulated by protein modification(s). However, the occurrence of these modifications *in vivo* has not been established.

Over the past decade, electrospray mass spectrometry (ES/MS) has been developed into an accurate technique in molecular mass determination of biological macromolecules. Mass accuracy of about 1 part in 10 000 and resolutions of about 1 in 1000 are routinely obtained in an electrospray-quadrupole mass spectrometer [22]. Using this technique, the molecular masses of most GSTs (~ 25 kDa) can be determined with an accuracy of approximately ± 3 –5 Da under optimal conditions.

In this study we purified GSTs from male, female and GSH-depleted rat livers by affinity chromatography. The GSTs were analysed by HPLC on a reverse-phase column and the identity of each isoenzyme was confirmed by protein sequencing. The molecular masses of the isoenzymes were determined by ES/MS to address the question of post-translational modification of GSTs.

MATERIALS AND METHODS

Materials

Buthionine sulfoximine (BSO), S-hexylglutathione, glutathione and epoxy-activated Sepharose 6B were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and coupled as previously described [23,24]. Vydac C₁₈ reverse-phase columns were obtained from H-P Separations Group (Hesperia, CA, U.S.A.). *Achromobacter* proteinase I was from Wako (Osaka, Japan). Trifluoroacetic acid, chemicals and the reverse-phase column used in sequencing were obtained from Applied Biosystems (Foster City, CA, U.S.A.). Sprague-Dawley rats were reared in the breeding colony of the Institute of Biomedical Sciences, Academia Sinica, Taiwan, ROC. All other chemicals used were reagent grade or better.

Glutathione depletion by BSO treatment

BSO was used to lower the hepatic glutathione levels in rats

Abbreviations used: GST, glutathione S-transferase; BSO, buthionine sulfoximine; ES/MS, electrospray MS; LC/MS, liquid chromatography/MS; TIC, total ion current.

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[25]. Adult female Sprague–Dawley rats were injected intraperitoneally with 4 mmol of BSO/kg body wt. at time zero and again after 2 h. The animals were killed 2 h after the second injection. The glutathione levels in the hepatic preparations were assayed by the method of Tietze [26].

Isolation of GSTs

Adult Sprague–Dawley rats (160–200 g) were used throughout the experiments. All purification steps were performed at 4 °C. Tissues were homogenized in 4 vol. of buffer A (10 mM Tris, pH 8.0, 6 mM β -mercaptoethanol, 2 mM EDTA and 0.5 mM PMSF) with 20 % sucrose. Debris was removed by centrifugation at 200 000 *g* for 1 h. The supernatant was then dialysed against buffer A overnight.

GSTs were purified using an *S*-hexylglutathione–Sepharose column as described by Mannervik and Guthenberg [23], with minor modifications. The cytosolic fractions were loaded on to an *S*-hexylglutathione–Sepharose column that had been pre-equilibrated in buffer A. The column was washed with buffer A containing 0.2 M NaCl (buffer B) before eluting with buffer B containing 5 mM each of GSH and *S*-hexylglutathione (buffer C).

Alternatively, the isoenzymes were isolated by coupled affinity chromatography. Cytosolic fractions were loaded on to a GSH–Sepharose affinity column that was connected in tandem with an *S*-hexylglutathione–Sepharose affinity column. The columns were pre-equilibrated in buffer A. After sample loading, the columns were disconnected and washed separately with buffer B. The *S*-hexylglutathione–Sepharose column was eluted with buffer C as outlined above. The GSH–Sepharose column was further washed with buffer B lacking β -mercaptoethanol (buffer D). The column was then eluted with buffer D containing 5 mM GSSG [27]. After washing with Buffer B, the remaining GSTs were eluted from the GSH–Sepharose column with Buffer C.

GSTs eluted from the affinity column were used directly in HPLC and ES/MS analysis. Alternatively, the proteins were passed through a Superose 12 gel filtration column that was eluted with 150 mM potassium phosphate, pH 7.5. Before storage, GSH was added to the protein solution to a final concentration of 5 mM.

HPLC analysis of GSTs

The GST isoenzymes were resolved on a Vydac (C_{18} ; 4.6 mm $\times$ 250 mm) reverse-phase column at a flow rate of 0.6 ml/min. The column was equilibrated with 40 % solvent 1 (0.08 % trifluoroacetic acid in water) and 60 % solvent 2 (0.05 % trifluoroacetic acid in 80 % acetonitrile). A linear gradient of 40–75 % solvent 2 was developed over 70 min. The elution profile was monitored by UV absorption at 214 nm. The amount of each GST subunit eluted from the column was determined from the peak area at 214 nm, using the method of Johnson et al. [21].

Mass spectrometry

ES/MS analyses were performed on a VG Quattro-BIO-Q mass spectrometer (Fisons Instruments, VG Biotech, Altrincham, U.K.). The instrumental set-up of the liquid chromatography/mass spectrometry (LC/MS) system has been described elsewhere [28]. The instrument was set in the positive ion mode. The multiply charged ion peaks from myoglobin (molecular mass 16 951 Da) [29] were used as an external reference for mass scale calibration. Scanning was in the multichannel analyser mode from *m/z* 500 to *m/z* 1500 at 12 s/scan. Data were summed

according to the total ion current (TIC) profile and processed by the MaxEnt program [30]. UV absorbance of the samples at 214 nm was also monitored simultaneously for direct comparison with the TIC profiles.

Protein sequencing

Automated cycles of Edman degradation were performed using an Applied Biosystems gas/liquid-phase model 470/900A sequencer equipped with an on-line model 120A phenylthiohydantoin amino acid analyser [31].

RESULTS

Isolation of rat GSTs using a single *S*-hexylglutathione affinity column

Cytosolic GSTs were isolated using an *S*-hexylglutathione–Sepharose column. Routinely, we recovered approx. 0.6 mg of GST isoenzyme per g of tissue from either male or female rat liver. The resulting affinity fractions were analysed on a reverse-phase column. The chromatograms for male and female rat hepatic GSTs are presented in Figures 1(a) and 1(b) respectively.

The chromatographic peaks were assigned by peptide

Figure 1 HPLC profile of rat liver cytosolic GSTs

Male (a) and female (b) rat liver GSTs were isolated using an *S*-hexylglutathione–Sepharose column and analysed on a C_{18} reverse-phase column. The GST subunit numbers are indicated.

Table 1 HPLC quantification of rat liver cytosolic GSTs in affinity fractions

Results are expressed as percentages of total proteins isolated by affinity chromatography.

Subunit	GST levels (%)					
	<i>S</i> -Hexylglutathione column			Coupled affinity chromatography		
	Normal male	Normal female	BSO-treated female	Normal male	Normal female	BSO-treated female
1	12.7	10.0	4.1	5.2	5.6	4.7
1'	9.8	13.4	12.2	3.9	12.7	13.6
2	17.0	30.6	35.5	11.2	38.2	45.3
3	27.8	20.4	19.5	33.8	13.9	14.8
4	29.2	20.9	25.3	40.7	21.7	14.7
6	1.6	1.5	1.0	1.2	0.5	0.4
7	0.5	0.8	0.5	0.6	0.4	0.5
8	0.5	0.6	0.2	1.2	4.4	3.7
10	1.0	1.8	1.8	1.4	2.2	2.4

Figure 2 HPLC profile of female rat liver cytosolic GSTs

Female rat liver cytosolic GSTs were purified by coupled affinity chromatography and the fractions were analysed on a C_{18} reverse-phase column. (a) GSTs eluted from the GSH-Sepharose column with GSSG, (b) GSTs eluted from the GSH-Sepharose column with GSH and *S*-hexylglutathione, (c) GSTs eluted from the *S*-hexylglutathione column that was connected in series with the GSH-Sepharose column during sample loading. The GST subunits and glyoxalase I (G) are indicated.

Table 2 Molecular masses of rat liver cytosolic GSTs determined by ES/MS

GST	Molecular mass (Da)			Modification	Reference
	Normal	BSO-treated*	Predicted		
1	25 520 ± 2	25 516 ± 2	25 522†	<i>N</i> -acetylation	pGTR261 [11]
1'	25 473 ± 2	25 469 ± 4	25 469†	<i>N</i> -acetylation	pGTB381 [10]
2	25 188 ± 1	25 185 ± 2	25 188†		pGTB42 [12]
3	25 782 ± 2	25 781 ± 4	25 782†		pGTR200 [14]
4	25 571 ± 2	25 568 ± 3	25 571†		λGTR4-1 [16]
6	25 551 ± 3	25 548 ± 4	25 549†		Yb ₃ [38]
7	23 308 ± 2	23 309 ± 1	23 307†		pGP5 [39]
8	25 553 ± 3	25 549 ± 1	25 550	<i>N</i> -acetylation	λGTRA8 [17]
10	25 211 ± 2	25 213 ± 4	25 216†		pET-rGST Yc ₂ -k [43]

* Data obtained from female rat liver preparations.

† The N-terminal initiator methionine is excluded.

Figure 3 HPLC profile of BSO-treated female rat liver cytosolic GSTs

Female rats were treated with BSO and the hepatic GSTs were isolated and analysed on a reverse-phase column. (a)–(c) GSTs were purified by coupled affinity chromatography and analysed as in Figure 2. (d) Chromatogram of GSTs purified on a single *S*-hexylglutathione–Sepharose column.

sequencing. The N-terminal sequences of subunits 2, 3, 4, 6 and 7 can be obtained directly by Edman degradation. Subunit 1 was isolated from the reverse-phase column and digested with *Achromobacter* proteinase I. The resulting peptides were separated by HPLC and collected for sequence analysis. Peptides with the sequence K⁴⁵DGNLMFDQVPMV⁵⁷ and L⁶⁴AQTRAILNYIA-TKYDLYGK⁸³ were identified for subunit 1. Subunits 2 and 10 were poorly separated on the reverse-phase column, making meaningful sequence analysis difficult. Consequently, subunit 10 (Yc₂) was assigned according to Hayes et al. [32].

The amount of each GST subunit was determined from the peak area at 214 nm, using the molar absorption coefficients calculated by Johnson et al. [21]. The major hepatic GSTs observed were subunits 1, 1', 2, 3 and 4. In male rat liver, the class-Mu GSTs (subunits 3 and 4) represent 57% of the isoenzymes in the affinity fraction. The same class accounted for only 41% of female cytosolic rat liver GSTs; the remainder of female rat liver GSTs (56%) belong mostly to the Alpha family. GST 2 in female rat liver was present at a level double that of GST 1 and 1' combined. GST 10 was barely measurable in male rat liver, but quite discernible in female rat hepatic preparations (Figure 1 and Table 1). Subunits 6, 7 and 8 were detectable, but each represented only 0.5–2% of the total GSTs in the affinity fraction (Table 1).

The affinity-purified GSTs were analysed by LC/MS (Table 2). The molecular masses of GSTs 1, 1', 2, 3, 4, 6, 7 and 10 were determined to be 25520, 25473, 25188, 25782, 25571, 25551, 23308 and 25211 Da respectively. The molecular masses determined for GSTs isolated from male and female rat livers were similar, with deviations of the results less than 3 Da.

Isolation of rat GSTs by coupled affinity chromatography

Rat liver cytosolic GSTs were purified using a combination of glutathione and *S*-hexylglutathione affinity columns. Routinely, we can recover approx. 0.5 mg of GSTs/g of tissue from both columns. In a typical experiment, 88–93% of the isoenzymes are retained on the glutathione affinity column. GSSG elution recovers approx. 47 and 60% of the total isoenzymes from the male and female rat livers respectively.

GSTs eluted with GSSG from the GSH–Sepharose column

were analysed by HPLC, and the chromatogram for a female rat liver preparation is shown in Figure 2. The class-Alpha isoenzymes (GSTs 1, 1', 2 and 8) were the major proteins recovered. The identity of GST 8 was confirmed by sequencing peptides generated by proteinase digestion. Peptides with the sequences N¹³⁰RYFPVFEK¹³⁸ and K¹⁹⁶FLQPGSQRK²⁰⁵ were identified. The second peptide (residues 196–205) has a sequence that is common among all class-Alpha GSTs. Fortunately, the first peptide (residues 130–138) can distinguish GST 8 from subunits 1, 1' and 2. Mass spectrometric analysis of GST 8 gave a molecular mass of 25553 Da (Table 2).

The remaining GSTs bound to the GSH–Sepharose column were eluted from the column with a mixture of GSH and *S*-hexylglutathione. The major proteins recovered were GSTs 3 and 4 (Figure 2b). GSTs 2 and 6 were detectable in minor quantities. This fraction represents 42 and 32% of the total GSTs in male and female rat liver preparations respectively.

Approx. 7–11% of the GSTs were retained on an *S*-hexylglutathione–Sepharose column that was connected in series with the glutathione–Sepharose column. The HPLC pattern of this fraction prepared from female rat liver is presented in Figure 2(c). The major isoenzymes detected were subunits 1, 1', 2, 3 and 4; subunits 6 and 8 could also be found. Internal sequencing of the protein that eluted in front of GST 3 yielded the sequence FSLYFLAYE. This peptide has high sequence identity to human glyoxalase I [33]. The molecular mass of this protein as determined by LC/MS is 20735 ± 4 Da.

The relative recoveries of rat cytosolic GSTs, as purified by coupled affinity chromatography and determined by HPLC analysis, are summarized in Table 1. Using this approach, GSTs 2 and 8 can be recovered at substantially higher yields than with the single column method. Using LC/MS analysis the molecular masses of the GSTs purified by coupled affinity chromatography are identical to those isolated using a single *S*-hexylglutathione–Sepharose column.

GSTs from the livers of BSO-treated female rats

In female rats treated with BSO, the glutathione levels in the hepatic preparations were approx. 30% of those in the controls. The GSTs were purified using an *S*-hexylglutathione–Sepharose

column. The recovery of GSTs from the hepatic preparation was 0.58–0.62 mg/g of tissue, implying that BSO treatment did not change the amount of GSTs. The affinity fraction was analysed by HPLC and the chromatogram is presented in Figure 3(d). The relative amounts of isoenzymes identified on the chromatogram are summarized in Table 1. Using this purification method, we observed a decrease in GST 1 and glyoxalase I expression. The amount of GST 2 expressed was slightly increased.

The GSTs in the hepatic preparations from BSO-treated females were also purified using the coupled affinity chromatography method. The HPLC chromatograms of the various affinity fractions are presented in Figure 3, and the quantifications of the isoenzymes are summarized in Table 1. GST 8 was eluted from the GSH–Sepharose column with GSSG, together with subunits 1, 1' and 2. This fraction represents 68% of the total GSTs recovered from both columns. The remaining GSTs (26% of the total) on the GSH–Sepharose column were mainly subunits 3 and 4. Subunits 2, 6 and 7 can be clearly identified. Approx. 6% of the GSTs were recovered from the *S*-hexylglutathione column. This fraction was enriched in GST 10.

These four affinity fractions were analysed by LC/MS, and the molecular masses obtained for the GSTs are summarized in Table 2. The results obtained are similar to those of the controls.

DISCUSSION

Cytosolic GSTs from male, female and GSH-deficient female rat livers were isolated by affinity chromatography, and the resulting proteins were analysed on a reverse-phase column. The relative amounts of the GST isoenzymes were determined according to their absorption at 214 nm and are listed in Table 1. The recovery of the isoenzymes from the *S*-hexylglutathione column was slightly different from that with the coupled affinity method. The reason for this discrepancy needs further investigation. Our results are similar, but not identical, to those obtained by Johnson et al. [21]. Using a single *S*-hexylglutathione column, we have recovered twice as much subunit 1' as did Johnson et al. [21]. Our data were obtained from samples that had not gone through a freeze–thawing step, and analyses were usually performed within 72 h of killing the animals. After storage at –20 °C, we observed a decrease in class-Alpha GSTs on HPLC analysis (results not shown).

We observed quantitative differences in hepatic GST expression between male and female rats. The class-Mu GSTs (subunits 3 and 4) were predominant in male livers, comprising at least 57% of the total GSTs, depending on the purification method. In female livers, GST 2 was the major component (30%). On adding GSTs 1 and 1', the class-Alpha GSTs constituted 54–56% of the GSTs in female rat livers. These results are consistent with the finding of Igarashi et al. [34] that liver class-Mu proteins are more abundant in male compared with female rats, while the GST 2 level is higher in females.

The molecular masses of the GSTs were determined by LC/MS and the results are summarized in Table 2. We obtained values of 25520 and 25473 Da for GSTs 1 and 1' respectively. Hayes et al. [35] have obtained partial amino acid sequences for GST 1 and 1'. They suggested that GSTs 1 and 1' are encoded by the cDNA inserts in pGTR261 [11] and pGTR38 [10] respectively. The molecular masses of the proteins deduced from the sequences of pGTR261 (25479.6 Da) and pGTR38 (25427.8 Da) are 40–45 Da less than those obtained by MS. GST 1 has been suggested to be an N-terminally blocked protein [36]. Results from MS analyses imply that GSTs 1 and 1' are *N*-acetylated after removing the initiator methionine. Furthermore, the experimentally obtained molecular mass of GST 1 confirms that residue 151 on

pGTR261 should be a lysine instead of the reported arginine [11]. The deduced molecular mass of the *N*-acetylated protein derived from the sequence of pGTR261 containing arginine-151 would be 25550 Da. This value was not detected in our mass spectrum for the GST 1 peak.

GST 2 has a molecular mass of 25188 Da. This value is identical to that deduced from the sequence of pGTB42 [12], with the initiator methionine removed. A variant of GST 2, encoded by pGTR262, has been reported by Tu et al. [13]. Clone pGTR262 differs from pGTB42 in having isoleucine and lysine instead of leucines at residues 101 and 183 respectively. The protein deduced from clone pGTR262 would have a molecular mass of 25203 Da. We could not detect this protein in our mass spectrum.

A search of the literature shows that there are at least three forms of GST 3. Lai et al. [14] published the sequence of pGTR200, which encodes a protein of 25782 Da. Ding et al. [15] isolated a GST 3 variant (pGTA/C44) that has an isoleucine-168-to-asparagine substitution; the resulting protein should have a molecular mass of 25781 Da. A second variant, with lysine-198-to-asparagine and serine-199-to-cysteine replacements, was also reported by Ding et al. [37]. This protein has a deduced molecular mass of 25784.5 Da. We obtained a molecular mass of 25782 Da from MS; the experimental result is not precise enough to differentiate which variants are expressed, or whether they all are.

GST 4 has been sequenced at the protein level [8]. The initiator methionine was removed to give a calculated molecular mass of 25457 Da. The GST 4 gene (λ GTR4-1) was isolated by Lai et al. [16]. The protein encoded by λ GTR4-1 differs from the GST 4 protein sequence of Ålin et al. [8] by having tryptophan instead of serine at residue 146. Consequently, the protein should have a molecular mass of 25571 Da. Our mass spectrometric data (Table 2) confirm the expression of the λ GTR4-1 gene.

GST 6 has been isolated from rat testis [18] and its cDNA has been cloned from a rat brain library [38]. The protein is not *N*-terminally blocked and has a molecular mass of 25549 Da (excluding the initiator methionine). Only a small quantity of subunit 6 can be seen in the liver samples (Figure 1). We obtained a molecular mass of 25551 Da for this protein. The same value was obtained for GST 6 isolated from rat brain and testis (results not shown).

The molecular mass of subunit 7 was determined to be 23308 Da by LC/MS. The value obtained is identical to that reported by Suguoka et al. [39] for a GST P cDNA clone. To confirm our result, GST 7 was also isolated from rat kidneys and brains for analyses. These isoenzymes have an identical retention time on the reverse-phase column and the same molecular mass as that obtained from the liver samples (results not shown).

The protein and cDNA sequences of GST 8 have been reported by Ålin et al. [9] and Stenberg et al. [17] respectively. The protein begins with an *N*-acetylated methionine and has a molecular mass of 25622 Da [9]. With an *N*-acetyl group, the protein deduced from the cDNA sequence should have a molecular mass of 25552 Da. Our data (25553 Da) confirm the cDNA sequence.

Coles and Ketterer [3] reported that there are two forms of GST 8 in rat livers. In our hands, GST 8 was eluted from the reverse-phase column at 60 min (Figure 2a). Some UV-absorbing material was eluted from the column after GST 8; however, we could not obtain any useful mass spectrum from this peak.

The biosynthesis of GST 10 (Yc₂) is ethoxyquin-inducible. Hayes et al. [40] have reported the partial amino acid sequence for this protein. Meyer et al. [41] and Scott and Kirsch [42] have described a class-Alpha GST in fetal rat liver that contains a subunit with similar electrophoretic properties to GST 10. Recently the cDNA encoding this protein was isolated from a

fetal rat liver library [43]. The protein deduced from cDNA sequencing has a molecular mass of 25216 Da. We obtained a molecular mass of 25211 ± 2 Da for GST 10 isolated from adult rat livers. Therefore the GST 10 isoenzymes expressed in adult and fetal rat livers are probably the same protein.

The other protein that can be identified on the chromatogram is glyoxalase I (Figure 2c). Isolation of glyoxalase I using an *S*-hexylglutathione affinity column has been reported by Mannervik's group [44,45]. In the present study the identity of the protein was confirmed by sequencing of endoproteinase-generated peptide fragments. The sequence obtained was identical to residues 67–76 of human glyoxalase I [33]. The molecular mass of this protein is 20733 ± 4 Da.

The molecular mass data listed in Table 2 represent both normal male and female rat liver cytosolic GSTs. We could not detect any gender-related differences. GST π isolated from male mouse livers has a higher specific activity and greater thermostability compared with that from females [46]. The murine ovary and testis GSTs are structurally similar [47]; however, the reported k_{cat} values of some of the testicular isoenzymes were up to 10-fold higher than those of the corresponding ovarian isoenzymes. The substrate specificities were also significantly different for the corresponding isoenzymes from testis and ovary. The authors suggested that differential post-translational modifications and/or tissue-specific expression of closely related isoenzymes were the possible causes of these observations [47]. Recently, Bammler et al. reported the isolation of two actively transcribed murine Pi-class GST genes [48]. These two genes encoded proteins that differ by 72 Da. The observations made by Awasthi et al. [47] are possibly due to the differential expression of these two genes. For rat liver samples, our data exclude the existence of differential post-translational modifications of isoenzymes. Even though it is unlikely, we could not rule out the possibility of tissue-specific expression of closely related isoenzymes that differ by less than 4 Da.

GSTs purified from *Musca domestica* have been suggested to be post-translationally modified [49]. *In vitro* experiments have shown that rat class-Alpha and -Mu GSTs are substrates for protein kinase C [20] and methyltransferase [50] respectively. The methyltransferase is expressed in rat liver [21], and the reaction is stimulated by calmodulin and inhibited by glutathione [51]. Under glutathione-deficient conditions, we should expect an increase in methylation of hepatic GSTs. However, we could not detect any change in molecular mass among GSTs isolated from the normal and glutathione-deficient liver tissues (Table 2). Ji et al. [52] have addressed the same issue crystallographically. They could not detect the presence of methyl groups on lysine and arginine side chains of rat liver GST 3.

In summary, we have determined the molecular masses of cytosolic GSTs from rat livers by MS, and have shown that the isoenzymes are not post-translationally modified. We could not detect any differences in molecular mass among GSTs isolated from male and female rats.

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