Rat kidney glutathione S-transferase 1 subunits have C-terminal truncations

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Cytosolic glutathione S-transferases (GSTs) from rat kidneys were purified by a combination of glutathione and *S*-hexylglutathione affinity columns. The isolated GSTs were subjected to reverse-phase HPLC and electrospray MS analysis. The major GST isoenzymes expressed in kidney are subunits 1, 2, 7 and 8. GST 1', 3, and 4 are expressed in minor amounts. GST 10 is barely detectable in the male kidney cytosol. The molecular masses of these rat kidney GST subunits were determined by MS. The values obtained for subunits 1', 2, 3, 4, 7, 8 and 10 are identical with those obtained for rat liver GSTs. Rat kidney GST 1 consists of three polypeptides, with molecular masses of 25517, 25372 and 24982 Da. Results from peptide mapping, MS and amino-acid-sequencing analyses indicate that the major components were generated by deleting the C-terminal phenylalanine

(24982 Da) and the C-terminal IFKF tetrapeptide (25372 Da) from the GST 1 subunit, respectively. The 1-chloro-2,4-dinitrobenzene-conjugating and peroxidase activities of kidney GST 1 are substantially lower than for its counterpart from rat liver. In addition, rat kidney GST 1 has an arginine and a valine residue at positions 151 and 207 respectively. The results are in contradiction with the SWISS-PROT and GenBank rat liver GST 1 cDNA-sequencing data, which give a lysine and a methionine at the corresponding positions. Further analyses indicate that rat liver GST 1 also has C-terminal phenylalanine deletion, and an arginine and a valine residue at positions 151 and 207 respectively. However, the C-terminal-tetrapeptide-deleted form was not observed for rat liver GST 1.

INTRODUCTION

Glutathione S-transferases (GSTs; EC 2.5.1.18) are dimeric proteins that catalyse the conjugation of various electrophilic compounds with glutathione. The reactions are considered as one of the initial steps in transforming and eliminating the xenobiotics [1,2]. These isoenzymes are also involved in the reduction of organic hydroperoxides [3], synthesis of leukotriene C₄ [4], and binding and possibly transport of bilirubin [5] and thyroid hormones [6].

Mammalian GSTs have been extensively studied. On the basis of their enzymic, immunological and structural properties, GSTs from rat tissues were designated class-Alpha (subunits 1, 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 [7]. Of these isoenzymes, cDNA sequences have been reported for 12 of them [8]. With the exception of GST 6 [9], all these cDNA sequences were obtained from hepatic tissues.

Although GST activity has been demonstrated in various tissues, characterization of GSTs has usually been carried out with hepatic tissues. Kidney is the major organ to eliminate the hydrophilic products of xenobiotics and to re-uptake biologically important substances. However, only a few studies have focused on kidney GSTs [10–12]. The major GST subunits expressed in rat kidneys are 1, 2, 7 and 8 [12]. The identity of these isoenzymes was established by their chromatographic attributes [12], electrophoretic mobility [11], or enzymic and immunological properties [10]. The enzymic properties of kidney GSTs have not been fully characterized [10]. Interestingly, the inducibility of these isoenzymes with respect to xenobiotics and growth hormone apparently differs from those in rat liver [12,13]. Furthermore, it has also been shown that putative identical GST enzyme isolates from different mouse tissues or from corresponding tissues of male and female mice can have significant differences in their catalytic properties [14,15]. Those authors suggested that tissue-

specific expression of closely related but distinct GST subunits could be one of the causes. Therefore, the possibility that rat liver and kidney GSTs are closely related, but not identical, genes could not be disregarded.

Recently we have isolated rat liver cytosolic GSTs by HPLC on a reverse-phase column and characterized the proteins by electrospray MS (ES/MS) [8]. We have correlated the expressed GSTs with the various cDNA clones isolated in different laboratories. We defined N-acetylation as the major form of modification observed in rat liver GSTs. Using similar approaches, we report the characterization of rat kidney GSTs by ES/MS in this paper. We address the question of whether rat liver and kidney GSTs are identical or closely related gene products.

MATERIALS AND METHODS

Materials

Glutathione, *S*-hexylglutathione, epoxy-activated Sepharose 6B, 1-chloro-2,4-dinitrobenzene (CDNB) and cumene hydroperoxide were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The glutathione [16] and *S*-hexylglutathione [17] affinity columns were coupled as previously described. Superose 12 prepac columns were obtained from Pharmacia (Uppsala, Sweden). 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). *Staphylococcus aureus* V8 protease was from Boehringer-Mannheim (Mannheim, Germany). 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.

Abbreviations used: GST, glutathione S-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; ES/MS, electrospray MS; LC/MS, liquid chromatography/MS; SIR, selected ion recording.

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Isolation of GSTs

Adult male Sprague-Dawley rats (200–250 g) were used throughout the experiments. All purifications were performed at 4 °C. The GSTs were purified on an *S*-hexylglutathione–Sephacrose column or by coupled affinity chromatography as described in [8]. The GST subunits were further separated by gel-filtration chromatography. Samples were concentrated with Centriprep-10 concentrators (Amicon, MA, U.S.A.) before loading on a Superose 12 gel-filtration column that was equilibrated and eluted at 0.5 ml/min with 10 mM Tris/HCl (pH 8.0)/6 mM β -mercaptoethanol/2 mM ethylenedinitrilotetra-acetic acid. The elution profile was monitored by UV absorption at 280 nm. The subunits were collected and used immediately for enzyme assay.

Enzyme assay

GST activity with CDNB [18] and cumene hydroperoxide [19] as second substrates was determined at 25 °C by the respectively referenced methods. Protein concentration was determined by the method of Lowry et al. [20], with BSA as protein standard.

Liquid chromatography (LC)/MS analysis of GSTs

The GST isoenzymes were resolved on a Vydac (C_{18} ; 1 mm $\times$ 150 mm) reverse-phase column. Samples were introduced from a Prodigix-4P (Microtech Scientific, Sunnyvale, CA, U.S.A.) solvent delivery system. The UV absorbance of the same at 214 nm was monitored with a model UVIS 204 detector (Linear Instruments, Reno, NV, U.S.A.) equipped with a micro flow-cell. The column was equilibrated with 45 % Solvent 1 (0.08 % trifluoroacetic acid in water) and 55 % Solvent 2 (0.05 % trifluoroacetic acid in 80 % acetonitrile). A linear gradient of 45 % to 75 % solvent 2 was developed over 60 min at a flow rate of 28 μ l/min.

ES/MS analyses were performed on a VG Quattro-BIO-Q mass spectrometer (Fisons Instruments, VG Biotech, Altrincham, Cheshire, U.K.). The HPLC system, the detector and the electrospray probe of the mass spectrometer were connected with 6.35 μ m (0.0025 in) internal-diam. Polyether ether ketone (PEEK) capillary tubing. A splitter (1:2) was employed after the detector flow-cell for sample collection, and approx. 8–9 μ l of sample/min was introduced into the mass spectrometer. The instrument was set in the positive-ion mode. For molecular-mass determination of GSTs, the multiply charged ion peaks from myoglobin (16951 Da) [21] were used as an external reference for mass-scale calibration. Scanning was in the multichannel analyser mode from m/z 500–1500 in 10 s/scan. Data were summed according to the total ion current (TIC) profile and processed by the MaxEnt program [22].

Peptide mapping with LC/MS

GST subunits purified by the reverse-phase column were freeze-dried for removal of organic solvents and trifluoroacetic acid. The GST 1 subunits were digested with *Achromobacter* protease I in 0.3 M $(NH_4)_2CO_3$ (pH 8.8)/2 M urea at 45 °C for 18 h. Digestion with *Staph. aureus* V8 protease was carried out in 0.3 M $(NH_4)HCO_3$ (pH 7.8)/2 M urea, at 30 °C for 18 h. The protease/enzyme ratio was maintained at 1:20 for both reactions.

The resulting peptides were separated on a microbore C_{18} reverse-phase column with a linear gradient of 0–70 % Solvent 2 developed over 175 min. The mass spectrometer was calibrated in the positive-ion mode with a poly(ethylene glycol) mixture (300–1000 Da) as external standard. Data were collected in

centroid mode with a scanning rate of m/z 250–1500 in 6 s/scan for mapping analyses [23]. Specific peptide fragments were located by the selected ion recording (SIR) mode [24]. Each selected ion has a dwell time of 0.08 s and a span of ± 1 Da. The inter-channel delay was set at 0.02 s. The ions selected for peptide detection are: 910, 455.6 and 304 for ARKIFKF; 783 and 382 for ARKIFK; 374.5 and 188 for ARK.

Amino acid sequencing

Automated cycles of Edman degradation were performed with an Applied Biosystems gas/liquid-phase model 470/900A sequencer equipped with an on-line model 120A phenylthiohydantoin amino acid analyser [25].

RESULTS

Isolation of rat kidney GSTs by using an *S*-hexylglutathione affinity column

Cytosolic GSTs from male rat kidneys were isolated with an *S*-hexylglutathione–Sephacrose column. Individual GSTs were further resolved by HPLC (Figure 1A). GST subunits 1, 2 and 7 are the major GSTs isolated from male rat kidneys. GST 1', 3, 4 and 10 are identified with less abundance. GST subunits on the chromatogram were identified by their retention time and confirmed by amino acid sequence analysis in most cases (subunits 2, 3, 4, 7 and 10).

The *S*-hexylglutathione–Sephacrose column purified GSTs were analysed by LC/MS, and the results are summarized in Table 1. Molecular masses of GSTs obtained from rat livers [8] and predicted from cDNA sequences [26–33] are also listed for comparison. The molecular masses of GST 1', 2, 3, 4, 7 and 10 were determined to be 25470, 25186, 25785, 25572, 23308 and 25215 Da respectively. All these GST isoenzymes have molecular masses identical with those of the corresponding enzymes obtained from rat livers [8]. The GST 1 fraction after HPLC purification contains three polypeptides, with molecular masses of 25517, 25372 and 24982 Da (Figure 2A). Except for the 25517 Da protein, most of the proteins in the kidney GST 1 fraction have a molecular mass 150–540 Da less than that of its counterpart from rat liver. This raises the intriguing question of whether the GST 1 expressed in kidney is a new isoenzyme or a post-translationally modified protein from the same gene.

Besides glyoxalase I (20730 Da), we have identified an additional protein on the chromatogram (Figure 1A). The peak marked H has a molecular mass of 15202 Da. Edman degradation of this protein yielded a sequence of VLSADDKTNIKN/WGK, which is identical with the N-terminal sequence of haemoglobin α -1 chain [34].

It has been reported that approx. 25 % of the GST activity in rat kidney cytosol does not bind to the *S*-hexylglutathione affinity matrix [10], whereas only a small fraction (1.5–3.5 %) failed to bind to the glutathione affinity matrix [11]. Therefore, the rat kidney GSTs were alternatively isolated by coupled affinity chromatography for MS analysis.

Isolation of rat kidney GSTs by coupled affinity chromatography

Male rat kidney cytosolic GSTs were purified by a combination of glutathione and *S*-hexylglutathione affinity columns arranged in tandem [8]. This method combines the benefit of class-selective elution and high GST-binding efficiency. Generally, we can recover about 0.3 mg of GST isoenzymes/g of kidney tissue.

The glutathione affinity column retained more than 90 % of the total isoenzymes. Elution of the column with GSSG recovered

Figure 1 HPLC profile of rat kidney cytosolic GSTs

(A) Male rat kidney cytosolic GSTs were isolated with an *S*-hexylglutathione-Sepharose column and analysed on a C_{18} reverse-phase column. The same cytosolic fractions were purified by coupled affinity chromatography and the fractions were analysed on a C_{18} reverse-phase column. (B) GSTs eluted from the GSH-Sepharose column with GSSG. (C) GSTs eluted from the GSH-Sepharose column with GSH and *S*-hexyl glutathione, after the GSSG elution. (D) GSTs eluted from the *S*-hexylglutathione column that was connected in series with the GSH-Sepharose column during sample loading. The GST subunits, haemoglobin α -1 chain (H) and glyoxalase I (G) are indicated.

Figure 2 Transformed mass spectrum of rat GST 1

Male rat kidney and liver cytosolic GSTs were purified by affinity chromatography and the fractions were analysed by LC/MS. Transformed mass spectra are shown for rat kidney GST 1 subunits eluted from: (A) the *S*-hexylglutathione-Sepharose column with GSH and *S*-hexylglutathione; (B) the GSH-Sepharose column with GSSG; and (C) the *S*-hexylglutathione affinity column that was connected in series with a GSH-Sepharose column during sample loading. Panels (D)–(F) are the transformed mass spectra of the corresponding samples from rat liver.

approx. 44 % of the total GSTs (Figure 1B). Class-Alpha GSTs (subunits 1, 2 and 8) were the major proteins recovered. Other isoenzymes identified were subunits 1', 4 and 7. The GST 8 subunit has a molecular mass of 25551 Da. The GST 1 fraction consisted of mainly proteins with molecular mass of 24982 Da. Proteins with molecular masses of 25517 and 25372 Da were also identified in this fraction. The transformed mass spectra of the GST 1 fraction are presented in Figure 2(B).

The remaining GSTs were eluted from the GSH-Sepharose column with a mixture of GSH and *S*-hexylglutathione. The

Table 1 Molecular masses of rat kidney and liver cytosolic GSTs determined by ES/MS

GST	Kidney	Liver*	Predicted	Modification	Reference
1	25517 $\pm$ 4	25520 $\pm$ 2	25518 [†] _‡	C-terminal (F) deletion C-terminal (IFKF) deletion	pGTR261 [26]
	25372 $\pm$ 4		25371 [†] _‡		
	24982 $\pm$ 3		24982 [†] _‡		
1'	25470 $\pm$ 3	25473 $\pm$ 2	25469 [†]		
2	25186 $\pm$ 2	25188 $\pm$ 1	25188		
3	25785 $\pm$ 2	25782 $\pm$ 2	25782		pGTB38 [27]
4	25572 $\pm$ 4	25571 $\pm$ 2	25571		pGTB42 [28]
7	23308 $\pm$ 3	23308 $\pm$ 2	23307		pGTR200 [29]
8	25551 $\pm$ 3	25553 $\pm$ 3	25550 [†]		λ GTR4-1 [30]
10	25215 $\pm$ 4	25211 $\pm$ 2	25216		pGP5 [31]
					λ GTRA-8 [32]
					pET-rGST Yc ₂ -K [33]

* Data from Yeh et al. [8].

[†] N-Acetylated protein.

_‡ Molecular mass was predicted from pGTR261 with arginine and valine at position 151 and 207, respectively.

major protein recovered was GST 7 (Figure 1C). Small amounts of GST 1, 2, 3 and 4 were also identified in this fraction. The composition of the GST 1 fraction was similar to that of the sample prepared by eluting the GSH affinity matrices with GSSG.

Approx. 10% of the GSTs was retained on an *S*-hexylglutathione–Sephacrose column that was connected in tandem with the glutathione–Sephacrose column. HPLC analyses indicated that the major isoenzyme detected in this fraction is GST 1 (Figure 1D). Other GST isoenzymes (subunits 1', 2, 3, 4 and 7) and glyoxalase I were also identified. The GST 1 in this fraction, as analysed by LC/MS (Figure 2C), is a mixture of three polypeptides with molecular masses of 25518, 25370 and 24982 Da. These values match the molecular masses of the intact GST 1, GST 1 with a C-terminal phenylalanine deletion and GST 1 with a C-terminal 4-residue deletion (IFKF), respectively.

Peptide mapping and identification of the C-terminal fragments of rat kidney GST 1

GST 1 subunits isolated from the *S*-hexylglutathione column of the coupled affinity chromatography were digested with endoprotease. The resulting peptides after *Staph. aureus* V8 or *Achromobacter* proteinase I digestion were separated on a microbore C₁₈ reverse-phase column, and the elution profile was monitored at 214 nm (Figure 3). One-third of the sample was analysed by on-line LC/MS, and the rest was collected for amino acid sequencing analysis. Peaks on the chromatograms selected for sequencing analysis were marked, and the results are summarized in Figure 4.

From the *Achromobacter* proteinase I digests (Figure 3A), we sequenced 18 peptides covering 104 residues (Figure 4). Peptide K6 has a molecular ion of $m/z = 641.7$, and a sequence of L²⁰⁵PVDAK²¹⁰. The sequence of this peptide is different from that deduced from clone pGTR261, as reported by Lai et al [26]. For pGTR261, residue 207 was depicted as methionine instead of valine. The assignment of residue 207 is confirmed by sequencing peptide K12. K12 has the sequence F¹⁹⁶LQPGSQRKLPVDAK²¹⁰ and molecular mass of 1685 Da.

Peptide K16 and K17 have the same primary sequence (D⁴⁶GNLMFDQVPMVEIDGMK⁶³). Peptide K16 ($m/z = 2056$) is 16 Da larger than K17. Presumably, one of the methionines in K16 was oxidized. The rest of the peaks on the chromatogram that have not been marked did not yield any meaningful sequencing data upon Edman degradation, and the molecular ions could not be matched with the N-terminal acetylated fragments.

The HPLC chromatogram of the *Staph. aureus* V8 protease digests of rat kidney GST 1 is presented in Figure 3(B). We have selected 17 peptides for amino acid sequencing, and the results cover 207 residues.

Peptide V5 has a blocked N-terminus and a molecular mass of 1905 Da. The results suggest that V5 was generated from the acetylated N-terminal 16 residues of GST 1.

Peptide V10 has the sequence K⁴²LKKDGNLMFDQVPMVE⁵⁸ and a measured molecular mass of 2009 Da. The resulting mass is 16 Da higher than prediction. Together with the MS and sequencing data of peptides K16 and K17, we tentatively conclude that either Met-50 or Met-56 was oxidized.

Peptide V14 has 25 residues (positions 137–161) and a molecular mass of 2904 Da. Sequencing analysis indicates that residue 151 is arginine, as reported for the rat liver cDNA clone pGTR261 [26]. The sequence obtained from SWISS-PROT and GenBank has a lysine at that position. With these two substitutions (Lys-151 → Arg and Met-207 → Val), the molecular

Figure 3 HPLC profile of protease digests of rat kidney GST 1

Male rat kidney GST 1 subunits eluted from the *S*-hexylglutathione column of the coupled affinity chromatography were digested with (A) *Achromobacter* protease I or (B) *Staph. aureus* V8 protease. Resulting peptides were subjected to LC/MS analysis. Fractions collected for amino acid sequencing analysis are indicated. Bottom continuous line, A_{214} ; broken line, [Solvent 2]. (C) Mass spectrum of the sample in (B). The mass spectrometer was set in SIR mode.

mass of rat kidney GST 1 is 25518 Da, slightly less than that deduced from pGTR261 (25522 Da).

Peptide V17 has a molecular mass of 5174 Da, covering residues 169–214 of GST 1. The MS data suggest that this peptide has valine at position 207. We could not confirm the methionine-to-valine substitution by Edman degradation, since Val-207 is 39 residues from the N-terminus of this peptide.

We could not detect residues 17–30 of GST 1 by LC/MS analysis of the protease digests. However, the *Staph. aureus* V8 protease digests have been subjected directly to laser-desorption MS on a Bruker Reflex instrument, with α -cyano-4-hydroxycinnamic acid as matrix. We detected a peptide of molecular mass 1302 Da (results not shown). The result matched that of a peptide covering residues 17–28 of GST 1. Therefore, we identified 219 out of 221 residues on GST 1.

Assuming that GST 1 has both one- and four-residue deletions, we should have the following C-terminal peptides after *Staph. aureus* V8 protease digestion: A²¹⁵RK²¹⁷ (373.5 Da), A²¹⁵RKIFK²²⁰ (762.1 Da) and A²¹⁵RKIFKF²²¹ (909.3 Da). The C-terminal fragments are rather hydrophilic and could be eluted from the reverse-phase column with a low percentage of organic solvent. Under this condition, the ionization efficiency of these peptides is low in the electrospray ion source. Therefore, the

Figure 4 Peptide map of rat kidney and liver GST 1

Peptides from *Achromobacter* protease I and *Staph. aureus* V8 protease digestion were designated according to the numbering in Figures 3 and 5.

mass spectrometer was set in SIR mode to enhance the sensitivity of the instrument. The mass spectrum of the SIR experiment is presented in Figure 3(C). The A²¹⁵RK²¹⁷ (V1), A²¹⁵RKIFK²²⁰ (V2) and A²¹⁵RKIFKF²²¹ (V7) peptides were eluted from the column at 21, 23 and 32.9 % of Solvent 2, respectively (Figure 3C). These three peptides were in doubly charged form, with observed molecular ions of 187.7, 382 and 455.6 Da respectively. The ARK peptide, which could not be recognized by regular peptide mapping, was identified by this method. The sequences of these peptides were all confirmed by Edman degradation (results not shown).

LC/MS analysis of protease digests of rat liver GST 1

In order to determine whether the gene for rat kidney GST 1 is a novel gene that expressed specifically in kidney tissues, rat liver GST 1 subunits were isolated and subjected to LC/MS and peptide-mapping analyses. The transformed mass spectrum of rat liver GST 1 isolated with a single *S*-hexylglutathione column (panel D), a GSH-Sepharose affinity column (panel E) and an *S*-hexylglutathione-Sepharose affinity column connected in tandem with the GSH-Sepharose affinity column (panel F) are presented in Figure 2. The results show clearly that these preparations were contaminated with a minor amount of GST 1 subunits with C-terminal phenylalanine deletion. We could not detect rat liver GST 1 subunits with the C-terminal tetrapeptide deletion. Interestingly, we observed in some preparations a form of GST1 with a molecular mass 76 Da higher than prediction (25595 Da; Figure 2F).

Peptides that resulted from *Achromobacter* protease I digestion of rat liver GST 1 subunits were separated on a reverse-phase

column, and the chromatogram is presented in Figure 5(A). The elution profile is similar to, if not identical with, that of the *Achromobacter* protease I digests of the rat kidney sample. The peptides in these fractions were identified by their molecular mass and confirmed by Edman degradation. The results are summarized in Figure 4. We identified peptides (LK6 and LK12) with valine at position 207. We could not detect any peptide with methionine at that position.

In a separate experiment, the *Achromobacter* protease I digests were subjected to LC/MS, and the instrument was set at SIR mode for the detection of the following peptides: L²⁰⁵PVDAK²¹⁰ (LK6), LPMDAK, F¹⁹⁶LQPGSQRKLPVDAK²¹⁰ (LK12), FLQPGSQRKLPMDAK, SHGQDYLVGNK and S¹⁴¹HGQDYLVGNRRLTRVDIHLLELLLYVEEFDASLLTSFPLLK¹⁸¹ (LK19). We could only identify peptides LK6, LK12 and LK19. Peptides LK6 and LK12 have been sequenced in their entirety, whereas peptide LK19 has been sequenced for 15 cycles.

To confirm our observation further, rat liver GST 1 subunits were subjected to *Staph. aureus* V8 protease digestion and LC/MS analyses. The chromatogram is presented in Figure 5(B) and the results are summarized in Figure 4. We identified peptides containing arginine at position 151 (LV14) and valine at position 207 (LV17). We have not detected peptides with lysine or methionine at these two positions. In addition, we observed a peptide with the sequence A²¹⁵RKIFK²²⁰ (LV2). The result suggested that some of the rat liver GST 1 subunits have C-terminal phenylalanine deletion.

Peptide LV15 has the sequence M¹⁰⁴IMQLVICPPDQKE¹¹⁷ by Edman degradation and a molecular mass of 1721 Da, which is 76 Da higher than prediction. The result suggested the modification of this peptide by β -mercaptoethanol that was present in

Figure 5 HPLC profile of protease digests of rat liver GST 1

Male rat liver GST 1 subunits eluted from the *S*-hexylglutathione column of the coupled affinity chromatography were digested with (A) *Achromobacter* protease I or (B) *Staph. aureus* V8 protease. Resulting peptides were subjected to LC/MS analysis. Fractions collected for amino acid sequencing analysis are indicated. Bottom continuous line, A_{214} ; broken line, [Solvent 2].

Figure 6 Gel-filtration chromatography of affinity-purified rat cytosolic GSTs

(A) Gel-filtration profile of a sample of male rat kidney GSTs obtained by eluting the GSH-Sepharose column with GSSG. (B) Gel-filtration profile of a sample of male rat kidney GSTs obtained from the *S*-hexylglutathione column of the coupled affinity chromatograph. (C) Gel-filtration profile of a sample of rat liver GSTs obtained by eluting the GSH-Sepharose column with GSSG. Fractions were collected for SDS/PAGE or enzymic-assay analyses as indicated by horizontal bars.

the buffers for tissue homogenization and affinity chromatography.

Isolation of GST 1 and GST2 subunits by gel filtration

The GST subunits after affinity chromatography can be further separated on a gel-filtration column. Presumably, the subunits

interact ionically with the gel matrix under low salt condition. The subunits were eluted from the column in multiple broad peaks between 0.8 and 1.6 bed vol. (Figure 6). With our regular GST purification protocol (150 mM potassium phosphate, pH 7.5), the GSTs were eluted from the column in a single peak at 0.4 bed vol.

Rat kidney GSTs eluted with GSSG from the GSH-Sepharose

Figure 7 PAGE of GSTs eluted from the gel-filtration column

Samples were analysed on a SDS/12.5%-polyacrylamide gel, and proteins were revealed by Coomassie Blue staining: lane 1, total rat liver cytosolic GSTs; lanes 2 and 3, rat liver GST 2-2 and 1-2, respectively, as indicated in Figure 6(C); lane 4, rat kidney GST 1-1 from Figure 6(B); lanes 5 and 6, rat kidney GST 2-2 and 1-2, respectively, as indicated in Figure 6(A).

Table 2 Enzymic activities of rat kidney and liver GST 1 and 2 subunits

Substrate	Kidney			Liver	
	1-1*	1-2†	2-2	1-1‡	2-2
CDNB	17.0	13.9	12.4	38.6 33.5§	12.0 13.7§
Cumene hydroperoxide	1.4	1.8	2.0	2.2 1.4	2.1 3.0

* Fraction consists of normal and truncated forms of GST 1.

† Most of the GST 1 subunits have a C-terminal 4-amino-acid deletion.

‡ Sample is a mixture of GST 1 and 1' homo- and hetero-dimers.

§ Data from Hayes et al. [33].

|| Data from Coles and Ketterer [35].

column were separated on a Superose 12 column, and the elution profile is presented in Figure 6(A). Fractions containing GST 2-2 and 1-2 subunits were collected as indicated in Figure 6(A) for enzyme assay. The purity of these samples was analysed by SDS/PAGE and is presented in Figure 7 (lanes 5 and 6 respectively). The possibility that the GST 1-2 preparation is a mixture of GST 1-1 and 2-2 homodimers could not be excluded. Analyses by LC/MS indicate that the GST 1 subunits thus isolated consisted mainly of proteins with a C-terminal 4-residue deletion (results not shown). An equivalent sample was prepared with rat liver tissues, and the GST 2-2 and 1-2 subunits were isolated (Figure 6C and Figure 7, lanes 2 and 3). The kidney GST 1-1 isolated from the *S*-hexylglutathione column of the coupled affinity chromatography were treated similarly. The gel-filtration elution profile and the SDS/PAGE pattern are presented in Figure 6(B) and Figure 7 (lane 4), respectively. The GST 1 in this preparation is a mixture of intact and C-terminally truncated proteins (Figure 2C).

Enzyme assay of GST 1 and 2 subunits from rat kidney

The enzymic activities of these GST subunits were assayed with CDNB and cumene hydroperoxide as second substrates. The results are summarized in Table 2. The specific CDNB-conjugating activity of GST 2-2 from rat kidney and liver is 12.4 and 12.0 $\mu\text{mol}/\text{min}$ per mg respectively. These values are close to that reported by Hayes et al. [33] for rat liver GST 2-2 (13.7 $\mu\text{mol}/\text{min}$ per mg). The peroxidase activity of the kidney and liver GST 2-2 is 2.0 and 2.1 $\mu\text{mol}/\text{min}$ per mg respectively. The experimental

value for the liver sample is slightly less than that reported by Coles and Ketterer (3.0 $\mu\text{mol}/\text{min}$ per mg) [35].

The GST 1-1 from rat kidney (Figure 6B) has CDNB-conjugation and peroxidase activity of 17.0 and 1.4 $\mu\text{mol}/\text{min}$ per mg respectively. We are not able to purify the corresponding GST 1-1 fraction from rat liver by the gel-filtration method. A mixture of Ya components (GST 1 and 1') from rat liver was isolated with a cation exchanger (Mono S) from the affinity fraction (results not shown). This fraction can be either homo- or hetero-dimers, with CDNB-conjugating and peroxidase activity of 38.6 and 2.2 $\mu\text{mol}/\text{min}$ per mg respectively. The CDNB-conjugating and peroxidase activity of the kidney GST 1-2 subunits is 13.9 and 1.8 $\mu\text{mol}/\text{min}$ per mg respectively. The decreased activity of rat kidney 1-1 and 1-2 subunits probably attributes to the presence of C-terminal-deleted GST 1 subunits in the samples, as reported for the human class-Alpha GST [36].

DISCUSSION

Cytosolic GSTs from male rat kidneys were isolated with an *S*-hexylglutathione affinity column. The resulting proteins were analysed on a reverse-phase column (Figure 1A). Low recovery of kidney GST isoenzymes has been reported for affinity-chromatographic purification with *S*-hexylglutathione matrices [10]. The observation probably reflects the lower binding affinity of GST 7 and 8 towards this matrix. When the coupled affinity-chromatography method was used, the recovery of GST 7 increased by 33 % (results not shown). GST 8 binds preferentially to the glutathione-Sepharose matrices (Figures 1A and 1B).

GST 1 subunits expressed in rat kidney show a remarkable molecular difference from those found in livers by ES/MS studies (Table 1). The native form of GST 1 (25517 Da) can be identified. However, the major components of rat kidney GST 1 have molecular masses of 25372 and 24982 Da. Using peptide mapping, mass spectrometry and amino acid sequencing analyses, we identified these two major components of GST 1 as subunits having C-terminal one- and four-residue deletions. In addition, we provide evidence that rat kidney GST 1 has arginine and valine at position 151 and 207, respectively.

Two highly homologous Ya isoenzymes have been identified in rat livers by cDNA cloning. They are designated as GST 1 (Ya₁, pGTR261) [26] and GST 1' (Ya₂, pGTB38) [27] and they are products of separate genes with eight amino acid differences. Considerable divergence in the 3'-non-coding region of these two cDNAs has also been observed. The deduced protein sequence of pGTR261 encodes Arg-151 in the original paper [26], whereas the sequence obtained from SWISS-PROT and GenBank contains a lysine at residue 151. A third unique Ya cDNA (pGTB45) has also been identified from rat liver cDNA cloning [37]. The coding sequence of this gene is reportedly identical with that of pGTR261. However, upon close scrutiny, the published partial cDNA sequence of pGTB45 encodes a valine at position 207 [37]. We would like to point out that an N-acetylated GST 1 with arginine and valine at positions 151 and 207, respectively, would have a molecular mass of 25518 Da. Replacing the valine at position 207 with methionine, as encoded by the published sequence of pGTR261, would result in a protein of molecular mass of 25550 Da. Experimentally, we obtained a value of 25518–25522 Da for GT 1 from either kidney or liver tissues.

We have also characterized the rat liver GST 1 subunits using peptide mapping and LC/MS. We present irrefutable evidence that the normal rat liver GST 1 has arginine and valine at positions 151 and 207, respectively. More important, we could not detect GST 1 subunits with lysine and methionine at these two positions.

Recently, the gene structure and regulatory mechanism of glutathione S-transferases have been reviewed [38]. Induction of GST Ya (1 and 1') genes in liver by xenobiotics and drugs such as phenobarbital, 3-methylcholanthrene and β -naphthoflavone has been well established. However, the inductive effect of these compounds on GST 1 cannot be identified in kidney [12]. Furthermore, continuous growth-hormone infusion can lead to a 75% decrease in GST 1 mRNA in male rat liver, whereas this effect has not been observed in rat kidney [13]. These observations suggest the existence of different regulatory mechanisms and/or isoforms of GST 1 expressed in liver and kidney. The mRNA encoding pGTR261 was isolated from rat liver after pentobarbital induction [26]. Possibly, the protein encoded by pGTR261 cannot be detected without induction.

The rat kidney GST 1 subunits exist mainly in truncated forms. It should be noted that the S-hexylglutathione matrices interact weakly with the GST 1 with a C-terminal tetrapeptide deletion. Consequently, recovery of this enzyme from a single S-hexylglutathione affinity column is low. The structure of a human class-Alpha GST co-crystallized with S-benzylglutathione has been elucidated by the X-ray deflection technique [39]. The C-terminal 11 amino acid form an α -helix ($\alpha 9$) that traverses from Domain II to Domain I. The region immediately before and all of one edge of $\alpha 9$ form the benzyl-group-binding site. Similar conclusions have been reached by using photoaffinity labelling [40], or by constructing human class-Alpha deletion isoenzymes [36]. Therefore the C-terminal-truncated GST 1 probably has lower substrate-binding affinity, hence a decrease in observed activity.

We isolated the class-Alpha GSTs for enzyme-activity assay. The GST 2-2 subunits prepared from rat kidney or liver tissues (Table 2) have similar activity, comparable with that reported for GST 2-2 from rat livers [33,35]. Hayes et al. [33] determined the peroxidase activity of GSTs at 37 °C, whereas we carried out our assay at 25 °C. Therefore, our peroxidase activity is comparable with that reported by Coles and Ketterer [35].

The GST 1-2 fraction prepared from rat kidney can be a mixture of 1-1, 2-2 and 1-2 homo- and hetero-dimers. Due to the high expression of GST 1' in rat liver, a similar preparation from rat liver can be a mixture of six different homo- or hetero-dimers. Therefore the GST 1-2 fraction from rat liver was not used for activity comparison.

The rat liver GST 1 preparation used in the enzymic assay is a mixture of GST 1 and 1' subunits in roughly equal proportion. The CDNB-conjugating activity of this fraction (Table 2) is similar to that reported by Hayes et al. [33]. The GST 1'-1' homodimer heterologously expressed in *Escherichia coli* has a CDNB-conjugating activity of 52 μ mol/min per mg [41]. Experimentally, we found that the CDNB-conjugating activity of GST 2-2 is 12 μ mol/min per mg. Considering that an individual component of a GST dimer is kinetically independent [42], GST 1' and 2 subunits would have a CDNB-conjugating activity of 26 and 6 μ mol/min per mg respectively. Assuming that our rat liver GST 1-1' fraction (38.6 μ mol/min per mg) was composed of heterodimers only, the calculated CDNB-conjugating activity for each GST 1 subunit would be 12.6 μ mol/min per mg. Therefore, the GST 1-1 and GST 1-2 dimers would theoretically have a CDNB-conjugating activity of 25 and 19 μ mol/min per mg respectively. The calculated values are perceptibly higher than the experimental results obtained for the rat kidney samples (Table 2).

It should be noted that the rat kidney GST 1 has a valine residue at position 207 as well as a C-terminal deletion. Widersten et al. [43] have pointed out that the methionine residue at that position participates in the aromatic substitution reaction, by

interacting with the delocalized negative charge of the substituted ring structure in the transition state. Therefore, the actual contribution of the C-terminal deletion to the enzymic activity of GST 1 remains to be investigated.

The GST 1 C-terminal deletions are not caused by non-specific protease degradation during the isolation procedure. Among the GSTs isolated from rat kidneys, C-terminal deletion is limited to the GST 1 subunits. The deletions occur at the carbonyl side of lysine residues. Careful examination of amino acid sequences of class-Alpha GSTs reveals that GST 1', 2 and 8 have lysine residues at position 217 and 220 respectively. None of these isoenzymes have C-terminal deletion.

The biological significance of this C-terminal deletion is not clear and remains to be investigated. However, Kilby et al. [44] reported the loss of the C-terminal serine residue on β B-2-crystallin as an age-related phenomenon.

Two forms of GST 7-7 have been identified in rat kidney, with similar catalytic activity toward ethacrynic acid [11]. Glycosylation has also been proposed for the rat GST 7 subunits [45]. With our present purification scheme and analyses, we can only identify the GST 7 with a molecular mass of 23308 ± 3 Da. We cannot detect the modified or alternative form of GST 7.

Coles and Ketterer [35] observed two forms of GST 8 in rat livers. For the rat kidney sample, GST 8 was eluted from the reverse-phase column in a single peak (Figure 1B). The transformed mass spectra of a sample stored without glutathione or reducing reagent gave two peaks, of molecular masses 25552 and 25632 Da (results not shown). Incubation of the sample with dithiothreitol before LC/MS analysis can eliminate most of the 25632 Da peak. Presumably, the 25632 Da represents the oxidized form of GST 8. Autoxidation of rat class-Mu [46,47] and *Schistosoma mansoni* GSTs [48] has been reported. However, this form of modification on class-Alpha GSTs has not been well documented.

Kidney is highly enriched in class-Alpha GSTs that have been shown as substrates *in vitro* for protein kinase C phosphorylation [49]. Results from LC/MS analyses indicate that these class-Alpha GSTs have not been phosphorylated (Table 2).

Kidney has the highest GST methyltransferase activity [50]. Class-Mu GSTs have been reported as the substrates for this enzyme, especially in the presence of calmodulin [50]. However, molecular-mass analyses of rat kidney GST 3 and 4 indicates that these subunits have not been methylated.

In conclusion, the GST 1 isolated from rat kidneys has an arginine and a valine residue at position 151 and 207, respectively. The same isoenzyme is found in normal rat liver tissues. Rat kidney GST 1 subunits exist mainly in C-terminal-truncated forms. Besides oxidation at methionine residue(s) and N-terminal acetylation, we have not detected any other forms of post-translational modification on rat kidney GSTs.

H.-I.Y. is the recipient of a NSC postdoctoral fellowship (NSC84-2811-B001-019). This research was supported in part by Grant NSC 84-2311-B001-067 from the National Science Council, Republic of China.

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