Purification and characterization of a labile rat glutathione transferase of the Mu class

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A labile GSH transferase homodimer termed 11–11 was purified from rat testis by GSH–agarose affinity chromatography followed by anion-exchange f.p.l.c. The enzyme is unstable in the absence of thiol(s) and has relatively low affinity for both 1-chloro-2,4-dinitrobenzene (K_m 4.4 mM) and GSH ($K_{m(app.)}$ 4.4 mM). Its mobility on SDS/polyacrylamide-gel electrophoresis is slightly less than that of subunits 3 and 4 and its pI is 5.2. Subunit 11 has a blocked N-terminal amino acid residue, but after CNBr cleavage fragments accounting for 113 amino acid residues were sequenced and showed 65% homology with corresponding sequences in subunit 4, indicating that it is a member of the Mu family. GSH transferase 11 is a major isoenzyme in testis, epididymis, prostate and brain and present at lower concentrations in other tissues.

INTRODUCTION

GSH transferases are enzymes that utilize both endogenous and exogenous substrates. Among the former are lipid hydroperoxides (Tan *et al.*, 1984; Meyer *et al.*, 1985a), 4-hydroxyalkenals (Danielson *et al.*, 1987), prostaglandins (Meyer & Ketterer, 1987b; Burgess *et al.*, 1987; Ujihara *et al.*, 1988), leukotriene A₄ (Mannervik *et al.*, 1985a; Tsuchida *et al.*, 1987) and DNA hydroperoxide (Tan *et al.*, 1988). Exogenous substrates are electrophiles arising during the metabolism of xenobiotics, including drugs such as paracetamol (Coles *et al.*, 1988) and carcinogens such as polycyclic aromatic hydrocarbon epoxides (Robertson *et al.*, 1986; Robertson & Jernström, 1986).

These enzymes are dimers of which the subunits form a gene superfamily comprised of at least three multigene families, referred to as Alpha, Mu and Pi (Mannervik *et al.*, 1985b), which in the rat comprise subunits 1, 2, 8 and 10, subunits 3, 4, 6 and 9 and subunit 7 respectively (Ketterer *et al.*, 1988).

We have previously identified a novel GSH transferase subunit from the seminiferous tubules of rat testis by using reverse-phase h.p.l.c. (Ostlund Farrants *et al.*, 1987). The present paper describes the isolation and characterization of the native homodimer, which, using the nomenclature of Jakoby *et al.* (1984), we have named GSH transferase 11–11 and shown to be a member of the Mu multigene family.

EXPERIMENTAL

Physical methods

Some of the physical properties of GSH transferase subunit 11 were obtained as follows. Apparent M_r was determined by SDS/polyacrylamide-gel electrophoresis in 12.5% polyacrylamide/0.25% bisacrylamide according to the procedure of Laemmli (1970), with total rat liver GSH transferases as a marker. Isoelectric-point determination by horizontal isoelectric focusing in polyacrylamide gel was carried out at 15 °C with

Ampholines of pH range 3.5–9.5 and coloured protein pI markers (Pharmacia–LKB, Bromma, Sweden) according to the manufacturer's instructions. Analysis of GSH transferase subunits by reverse-phase h.p.l.c. was made by the method of Ostlund-Farrants *et al.* (1987) modified for improved yield and resolution by use of a 30 nm-pore-size Dynamax C₁₈ column (25 cm × 4.6 mm) (Rainin Instrument Co., Woburn, MA, U.S.A.).

Immunological methods

The immunological characterization of GSH transferase was carried out by using immuno dot-blot in accordance with Towbin *et al.* (1979) with antisera raised to rat GSH transferases 1–1, 2–2, 3–3, 4–4, 7–7, 8–8 and human GSH transferase π obtained from Medlabs, Dublin, Ireland. The antigen detection limits by dot-blotting at 1–10000 dilution of antisera were 10, 10, 25, 25, 10, 5 and 10 ng respectively. The antisera show weak cross-reaction with transferases of the same class (Alpha class 1–1, 2–2 and 8–8; Mu class 3–3 and 4–4; Pi class 7–7 and π) but do not cross-react with transferases of a different class.

Enzymic assays

The specific activity of GSH transferase 11–11 towards the substrates 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene, *p*-nitrobenzyl chloride, 1,2-epoxy-3-(*p*-nitrophenoxy)propane and ethacrynic acid was determined by the method of Habig *et al.* (1974) and towards 4-hydroxynon-2-enal by the method of Alin *et al.* (1985). Inhibition studies were carried out with tetrabromosulphophthalein, Cibacron Blue, linoleic acid and ethanol. The pH-dependence of GSH transferase activity with 1-chloro-2,4-dinitrobenzene was measured with 0.1 M-potassium phosphate buffer in the range pH 4.68–8.16 and 0.1 M-Tris/HCl buffer at pH 8.5.

Kinetic analyses were carried out with 6 mM-GSH and 1-chloro-2,4-dinitrobenzene as the varied-concentration substrate and with 1 mM-1-chloro-2,4-dinitrobenzene and GSH as the varied-concentration substrate. Since

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ethanol (above 0.6 mM) was observed to cause substantial inhibition of activity, the stock solution of 1-chloro-2,4-dinitrobenzene in ethanol used (114 mg/5 ml) was twice the usual concentration. Assays were carried out at 37 °C. Enzyme protein concentration was determined by the method of Bradford (1976), with ovalbumin as standard.

Protein sequencing

Reactive thiol groups were blocked by reaction of the enzyme protein (50 µg) with 4-vinylpyridine (Cavins & Friedman, 1970). Peptides suitable for *N*-terminal sequence analysis were prepared by reaction of the modified protein with excess CNBr (0.5 mg) in 300 µl of 70% (v/v) formic acid. Peptides were purified by reverse-phase h.p.l.c. and sequenced by using the procedure of Taylor *et al.* (1988).

Purification of GSH transferase 11-11

Tissue samples were obtained from brain, liver, spleen, kidney, testis, epididymis, prostate, lung, cardiac and skeletal muscle, frozen in liquid N₂ and stored at -70 °C before use. The most abundant source of GSH transferase 11-11 proved to be the testis, which was therefore used for its purification as follows. A 3 g portion of tissue was thawed, homogenized in 11 ml of 10 mM-sodium phosphate buffer, pH 7.0, containing 0.15 M-KCl, 2 mM-dithiothreitol, 25 µM-phenylmethanesulphonyl fluoride and 1 mM-EDTA (homogenization buffer) and centrifuged at 70 000 *g*_{av} for 90 min.

The GSH transferase fraction in the supernatant was isolated by GSH-agarose affinity chromatography after the method of Vander Jagt *et al.* (1985). The eluate was concentrated 5-fold by dialysis against poly(ethylene glycol) 6000 and transferred into 20 mM-Tris/HCl buffer, pH 7.5, containing 5% (w/v) mannitol, 2 mM-dithiothreitol and 0.5 mM-GSH by using the PD-10 column (Pharmacia-LKB, Milton Keynes, Bucks., U.K.). The eluate was then applied to an anion-exchange f.p.l.c. Mono Q HR5/5 column equilibrated with the same buffer, and the GSH transferases were fractionated by using a linear gradient of 0-0.6 M-NaCl in the same buffer. GSH transferase 11-11 was eluted at approx. 150 mM-NaCl, the yield being in the region of 0.3 mg.

Tissue distribution

Tissue samples were homogenized in 2 vol. of homogenization buffer, and a total GSH transferase fraction was obtained as described above. Samples appropriate in volume to the GSH transferase concentrations of the tissue concerned were analysed by reverse-phase h.p.l.c. as described above, and the subunit protein content was quantified from the integrated absorbance at 214 nm as described by Meyer *et al.* (1989).

RESULTS

Purification of GSH transferase 11-11

Unlike other GSH transferases, it is essential to carry out the purification of GSH transferase 11-11 in the presence of a thiol in order to avoid complete loss of activity and protein yield. The purification procedure is rapid and gives a GSH transferase 11-11 fraction well resolved from the other GSH transferases present in testis (see Fig. 1). The enzyme gave a single peak on reverse-phase h.p.l.c. with a retention time of 38 min and

Fig. 1. Separation of GSH transferases from testis by f.p.l.c. anion-exchange chromatography

A sample (2.1 mg) of rat testis GSH transferases in 20 mM-Tris/HCl buffer, pH 8.0, containing 5% (w/v) mannitol, 2 mM-dithiothreitol and 0.5 mM-GSH was applied to a Mono Q HR 5/5 column equilibrated with this buffer. The column was eluted with a linear gradient of 0-0.6 M-NaCl in 60 ml (-----). Fractions were monitored for absorbance at 280 nm (—), and peaks were collected and analysed by reverse-phase h.p.l.c. Glutathione transferase 11-11 was eluted by approx. 150 mM-NaCl.

distinct from that of any other characterized GSH transferase (Fig. 2a), a single band on SDS/polyacrylamide-gel electrophoresis, with a mobility slightly less than that of subunits 3 and 4 (*M*_r 26800) (Fig. 3a), and two components on slab-gel isoelectric focusing, having isoelectric points of 5.15 and 5.25 (Fig. 3b).

Classification

The relationship of GSH transferase 11-11 to other GSH transferases was determined by the use of immunological dot blots and the determination of primary sequence.

GSH transferase 11-11 cross-reacted with antisera raised against GSH transferases 3-3 and 4-4 (Mu class) but not with antisera raised against GSH transferases 1-1, 2-2 and 8-8 (Alpha class), GSH transferase 7-7 (Pi class) or human GSH transferase π (Fig. 4).

It was not possible to obtain the *N*-terminal sequence by automated Edman degradation, presumably owing to a blocked *N*-terminus. After protection of reactive thiol groups with 4-vinylpyridine and subsequent cleavage of the protein with CNBr, a number of peptides were generated and separated by reverse-phase h.p.l.c. Seven peptides were sequenced at approx. 100 pmol level and their positions in the protein were deduced by comparison with sequences in subunit 4 (Alin *et al.*, 1986; Lai *et al.*,

Fig. 2. Analysis of GSH transferase subunit 11 by reverse-phase h.p.l.c.

(a) Analysis of purified GSH transferase 11. (b) Separation of the total GSH transferase from rat testis. Subunits are numbered in accordance with Jakoby *et al.* (1984) and Ketterer *et al.* (1988).

1988) (Fig. 5). The sequence of subunit 11 so obtained (113 residues) is 65% identical with the corresponding sequence in subunit 4. No residue was obtained at positions corresponding to residues 86, 114, 173 and 201 in subunit 4. On the basis of homology with subunits 3 and 4, the first three at least are likely to be cysteine residues that were not accessible for reaction with 4-vinylpyridine under the conditions used and were therefore destroyed during sequencing. However, one cysteine residue was recovered as a 4-ethylpyridine adduct at position 184 in subunit 11, a position occupied by an alanine residue in subunits 3 and 4.

Enzymic properties

Results of assays of GSH transferase 11-11 with a number of substrates are shown in Table 1. Significant GSH transferase activity is obtained with 1-chloro-2,4-dinitrobenzene, 4-hydroxynon-2-enal, *p*-nitrobenzyl chloride and 1,2-dichloro-4-nitrobenzene.

Inhibition of activity towards 1-chloro-2,4-dinitrobenzene was obtained with Cibacron Blue (I_{50} 0.07 μ M), tetrabromosulphophthalein (I_{50} 12 μ M), linoleic acid (I_{50} 120 μ M) and ethanol (I_{50} 0.8 M).

Kinetic analysis showed that the K_m for 1-chloro-2,4-

Fig. 3. (a) SDS/polyacrylamide-gel electrophoresis and (b) flat-bed electrofocusing of GSH transferase 11-11

(a) SDS/polyacrylamide-gel electrophoresis: lane 1, the total GSH transferase fraction from rat liver; lane 2, purified GSH transferase 11-11. (b) Flat-bed isoelectric focusing: GSH transferase 11-11 (lane 1) is compared with standards of proteins of known pI (lane 2).

Fig. 4. Immunological relationships of GSH transferase subunit 11

GSH transferase subunit 11 purified by ion-exchange chromatography (see Fig. 1) was tested for immuno-cross-reactivity by dot-blotting with antisera raised to GSH transferases 1-1, 2-2, 3-3, 4-4, 7-7, 8-8 and π [(a)-(g) respectively]. The left-hand circles contain GSH transferase 11-11, the right-hand circles are controls lacking antigen.

dinitrobenzene is in the region of 4.4 mM, far higher than its aqueous solubility; the V_{max} is 240 μ mol/min per mg. The apparent K_m for GSH obtained with 1 mM-1-chloro-2,4-dinitrobenzene is 4.4 mM.

Since the K_m for GSH is so high, the specific activities obtained under standard conditions (0.25-1 mM-GSH)

Fig. 5. Primary structure of GSH transferase subunit 11

Sequences of CNBr-derived peptides of subunit 11 are aligned for comparison with the homologous sequences of subunit 4 (Ålin *et al.*, 1986; Lai *et al.*, 1988). Deduced methionine residues are in square brackets; residues in low yield are in parentheses, and a hyphen shows where no residue was obtained.

Table 1. Specific activities of GSH transferase 11-11 towards various substrates

Activities towards various model substrates were measured under standard conditions (Habig *et al.*, 1974).

Substrate	Specific activity ($\mu\text{mol}/\text{min per mg}$)
1-Chloro-2,4-dinitrobenzene	30
4-Hydroxynon-2-enal	2.1
p-Nitrobenzyl chloride	0.5
1,2-Dichloro-4-nitrobenzene	0.33
1,2-Epoxy-3-(p-nitrophenoxy)-propane	0.1
Ethacrynic acid	< 0.1

are considerably below the V_{max} . The enzyme is active with 1-chloro-2,4-dinitrobenzene between pH 6.0 and 8.5. Activity arises from nil at pH 6.0 to approx. 75 $\mu\text{mol}/\text{min per mg}$ at pH 7.9, and consequently the specific activity under standard conditions (pH 6.5) is approx. 50% that at the optimum pH.

Tissue distribution

The separation of the GSH transferase subunits in testis by reverse-phase h.p.l.c. is shown in Fig. 2, which also shows the behaviour of purified GSH transferase 11-11 in this system, thus enabling the peak representing subunit 11 in the testis to be identified. The quantitative analysis of the tissue distribution of subunit 11 (Table 2) shows that it occurs in greatest amounts in the testis

Table 2. Tissue distribution of rat GSH transferase subunit 11

The GSH transferase content of various tissues was determined by combined affinity chromatography/reverse-phase h.p.l.c. as described in the Experimental section.

Tissue	($\mu\text{g}/\text{g}$ of tissue)	(% of all subunits present)
Testis	93	16.5
Epididymis	40	10.6
Prostate	15	2.4
Liver	11	0.8
Brain	5.8	12.1
Kidney	5	1.0
Spleen	2.7	4.1
Cardiac muscle	1.8	2.5
Lung	0.9	1.2
Skeletal muscle	0.6	1.7

(93 $\mu\text{g}/\text{g}$), epididymis (40 $\mu\text{g}/\text{g}$) and prostate (15 $\mu\text{g}/\text{g}$) and is a relatively major component of GSH transferases in the brain (12.1%).

DISCUSSION

In order to name the GSH transferase characterized in this study we have referred to the nomenclature of Jakoby *et al.* (1984), which has since been extended to include subunit 7 (Meyer *et al.*, 1985b) and subunit 8 (Jensson *et al.*, 1986). The subunit originally termed 6 has since been shown to be composed of two distinct subunits (Ostlund Farrants *et al.*, 1987), characterized as Yn1 and Yn2 by Ishikawa *et al.* (1988), which are now termed subunits 6 and 9 respectively. Subunit 6 corresponds to Yb₃ (Abramovitz & Listowsky, 1987). Another GSH transferase subunit prominent in neonatal liver (Meyer *et al.*, 1985a; Scott & Kirsch, 1987) is now termed subunit 10, and hence the subject of the present study is termed subunit 11.

GSH transferase 11-11 has been overlooked in most previous studies, presumably because of its instability in the absence of a reducing agent, which results in total loss of activity on ion-exchange chromatography or isoelectric focusing. Recently a GSH transferase termed YoYo was purified from rat testis by the use of anion-exchange chromatography in the presence of 2-mercaptoethanol (Hayes, 1988). The chromatographic behaviour, subunit electrophoretic mobility and tissue source strongly suggest that YoYo and 11-11 GSH transferases are identical.

Immunological cross-reactivity, partial sequence identity and sensitivity to inhibition by Cibacron Blue put GSH transferase 11-11 into the Mu class (Mannervik *et al.*, 1985b). Like all Mu isoenzymes it has distinguishing characteristics including a poor K_m for GSH and 1-chloro-2,4-dinitrobenzene, poor inhibition by linoleate (Meyer & Ketterer, 1987a) and considerable lability except in the presence of low- M_r thiol. Also, sequence data so far available suggest that there is a relatively low level of homology of structure between subunit 11 and other subunits of the Mu class (3, 4, 6 and 9), and this may be the reason that heterodimers containing subunit 11 have not been observed.

Of particular interest is the cysteine residue at position

184, unique among Mu-class enzymes, which is accessible to 4-vinylpyridine. The reactivity of this cysteine residue may contribute to the instability of GSH transferase 11-11 in the absence of dithiothreitol. It may be noted that in the absence of reducing agent 11-11 not only loses activity but gives rise to several inactive forms during either ion-exchange chromatography or reverse-phase h.p.l.c.

The tissue distribution of subunit 11 is similar to that of subunit 6, being a major component in relatively few tissues among those that have been tested, namely testis, epididymis, prostate and brain (Ketterer *et al.*, 1988).

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