Glutathione S-transferases of the yeast *Yarrowia lipolytica* have unusually large molecular mass

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Two similar glutathione S-transferases (GSTs), which do not bind to glutathione- or S-hexylglutathione-agarose affinity resins, have been purified from the yeast *Yarrowia lipolytica*. An approx. 400-fold purification was obtained by a combination of DEAE-Sephadex, phenyl-Sepharose, hydroxyapatite and Mono-Q anion-exchange chromatography. The native molecular mass of both proteins was estimated as approx. 110 kDa by both Superose-12 gel-filtration chromatography and non-denaturing electrophoresis. SDS/PAGE indicated a subunit mass of 50 kDa. Reverse-phase HPLC of purified proteins gave a single, well-resolved, peak, suggesting that the proteins are homodimers.

Identical behaviour on HPLC, native electrophoresis and SDS/PAGE, N-terminal sequencing, sensitivity to a panel of inhibitors and identical specific activities with 1-chloro-2,4-dinitrobenzene as substrate suggest that the two isoenzymes are very similar. The enzymes do not immunoblot with antisera to any of the main GST classes, and N-terminal sequencing suggests no clear relationship with previously characterized enzymes, such as that of the fungus, *Phanerochaete chrysosporium* [Dowd, Buckley and Sheehan (1997) Biochem. J. 324, 243–248]. It is possible that the two isoenzymes arise as a result of post-translational modification of a single GST isoenzyme.

INTRODUCTION

The glutathione S-transferases (GSTs; EC 2.5.1.18) are an important family of drug-detoxification enzymes which catalyse a number of reactions, including conjugation of glutathione (GSH) to electron-deficient centres [1–3]. In addition, the enzymes are capable of extensive binding of non-substrate ligands, some of which are endogenous molecules [4,5]. GSTs are principally cytosolic enzymes which are dimers of subunits in the molecular mass range 16–27 kDa. Mammalian cytosolic GSTs have been classified into five main classes, Alpha, Mu, Pi, Theta and Sigma, based on a variety of immunological, sequence, kinetic and structural criteria [6–8]. It is thought that these classes arose from a primordial GST, similar to Theta class, which later underwent gene duplication and divergence [9]. Such a model is supported by kinetic comparisons of enzymes from the various classes [10].

Representative three-dimensional structures are now available from all of the cytosolic classes and these indicate that, despite limited sequence similarity between the classes, the proteins have similar general folds [11–15]. Important class-specific structural features have been identified from such studies, however, especially at the inter-subunit interface [3,14]. These findings provide structural confirmation of independent divergence of the Sigma and Theta classes from Alpha, Mu and Pi classes [14], and have focused attention on inter-subunit interactions in GSTs. Although individual subunits are thought to be kinetically independent [1], evidence for kinetic and structural interactions between Pi-class subunits has been published [16,17]. Moreover, single subunits of GSTs are normally not found since it appears that they rapidly fold into stable dimers [18]. Interestingly, a common structural attribute of cytosolic GSTs appears to be their relatively small subunit mass (16–27 kDa) and dimeric quaternary structure.

A fruitful approach to the study of the evolution of this complex enzyme family has been to investigate enzymes from a range of species rather than just those from better-characterized

mammalian sources. Good examples of this are the three-dimensional structures obtained from the blowfly *Lucilia cuprina* [13], the squid *Loligo vulgaris* [14] and the plant *Arabidopsis thaliana* [15]. A 52 kDa subunit, GST-like, dihydrotrypanothione-GSH thioltransferase has been purified from *Trypanosoma cruzi*, but this protein lacked GST-conjugation activity [19]. To better understand the evolutionary pressures on GSTs we have previously studied affinity-purified enzymes from a range of fungi. We have found that these proteins are expressed at low levels relative to mammalian cells and that they often immunoblot with class-specific antibodies to mammalian enzymes [20,21]. Recently, we have affinity-purified a GST from the white-rot fungus *Phanerochaete chrysosporium*, which is remarkably similar to plant Theta-class enzymes and which immunoblots with a human Theta class GST [21]. This similarity may arise either from common evolutionary origins or from the fact that *P. chrysosporium*, an extensive lignin degrader, and many plants share a common environment. In the present paper, we report the purification of GSTs from a yeast, *Yarrowia lipolytica*, which do not bind to affinity resins. These enzymes have subunit and native molecular masses (50 and 110 kDa, respectively) that are approximately twice the normal values for GSTs. Because of their unusually large size, they may be of particular interest in understanding subunit packing in GSTs.

MATERIALS AND METHODS

Materials

GSH-agarose, DEAE-Sephadex, phenyl-Sepharose and hydroxyapatite were purchased from Sigma (Poole, Dorset, U.K.). Antisera against Alpha-, Mu- and Pi-class GSTs were from Biotrin (Dublin, Ireland). Antiserum against rat GST 5-5 was a generous gift from Professor J. D. Hayes (Biomedical Research Centre, Ninewells Hospital and Medical School, Dundee, U.K.). Cultures of *Y. lipolytica* (NCYC 825) were obtained from the

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione S-transferase.

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National Collection of Yeast Cultures (AFRC Institute of Food Research, Norwich, U.K.). HPLC solvents were from Rathburn Chemicals Co. (Walkerburn, Scotland, U.K.). All other reagents were of analytical grade and obtained from Sigma or BDH (Poole, Dorset, U.K.).

GST Purification

Y. lipolytica was rehydrated in 0.5 ml of sterile YEPD broth [1 % (w/v) yeast extract/2 % (w/v) Bacto-peptone/2 % (w/v) glucose], which was then transferred to a culture bottle containing 10 ml of the same medium and incubated at 25 °C with constant gentle agitation until it appeared to be cloudy. *Y. lipolytica* was then plated on sterile 1.5 % (w/v) YEPD-agar plates, incubated at 25 °C for 3 days and stored at 4 °C. Cells were collected for protein purification from plates with a sterile loop and inoculated into 10 ml broth precultures that were incubated overnight at 25 °C with constant agitation. Precultures were then inoculated into 4 × 1.25 litres of broth and incubated under the same conditions for a further 3 days. Yeast cells were harvested by centrifugation at 2500 *g* for 40 min. Cell pellets (approx. 10 g) were resuspended in 20 ml of ice-cold sonication buffer [100 mM potassium phosphate buffer (pH 7.0) containing 2 mM EDTA and 0.25 mM PMSF]. Cell lysis was performed by sonication on ice (six times for 20 s each at an amplitude of 20 μ with 40 s intervals). The homogenate was centrifuged at 100 000 *g* for 1 h at 4 °C. The supernatant was filtered through glass wool and applied directly to DEAE-Sephadex (16 × 4 cm) equilibrated in 20 mM potassium phosphate (pH 7.0) containing 2 mM EDTA and 0.25 mM PMSF (buffer A). The column was developed with a gradient of 0–700 mM NaCl in buffer A and fractions were analysed for GST activity with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. Active fractions were pooled and applied to a column of phenyl-Sepharose (25 × 1.5 cm) equilibrated in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM EDTA and 0.25 mM PMSF (buffer B) containing 500 mM NaCl. The column was developed with a 300 ml gradient of 0–85 % (v/v) ethylene glycol in buffer B. Active fractions were pooled and dialysed overnight against 5 litres (three changes) of buffer A. Dialysed material was then applied to a column of hydroxyapatite (4 × 3 cm) equilibrated in buffer A. This column was developed with a 160 ml gradient of 0.02–1 M phosphate (pH 7.0) containing 2 mM EDTA and 0.25 mM PMSF. Active fractions were again pooled and dialysed overnight against 5 litres (three changes) of 50 mM potassium phosphate (pH 6.0) containing 2 mM EDTA and 0.25 mM PMSF (buffer C). This was applied to a 1 ml Mono-Q anion-exchange column (Pharmacia-LKB) that was developed with a 0–1 M NaCl gradient in buffer C. Peaks of protein containing GST activity with CDNB were collected and stored at –20 °C.

Enzyme assay

GST conjugation assays were performed with CDNB, 1,2-dichloro-4-nitrobenzene (DCNB), ethacrynic acid and 1,2-epoxy-3-(*p*-nitrophenoxy)propane, as described previously [22]. GSH peroxidase measured with H₂O₂ and cumene hydroperoxide was determined by the method of Lawrence and Burk [23]. Oxidized glutathione (GSSG) reductase was measured as described by Carlberg and Mannervik [24]; glyoxylase 1 activity was determined by the method of Mannervik et al. [25]. Catalase activity was determined by the method of Aebi [26]. Protein concentrations were determined by the method of Bradford [27] using BSA as a standard. All spectroscopic measurements were performed in a Uvikon 940 spectrophotometer.

Initial velocities with GSH and CDNB [22] were determined in the concentration range 0.5–5 mM and appearance of the product, dinitrophenol-GSH, was measured at 340 nm (ϵ = 9.6 mM^{–1}·cm^{–1}). These data were fitted to the Michaelis–Menten equation by non-linear least-squares regression analysis using ENZFITTER [28]. At least three independent determinations were made. *I*₅₀ values (i.e. the concentration of inhibitor giving 50 % inhibition of conjugation activity) for a panel of GST inhibitors were determined in two independent experiments. Triplicate measurements of conjugation activity at 1 mM GSH and CDNB were made at 8–10 different concentrations of inhibitors [1].

Electrophoresis

SDS/PAGE was performed as described by Laemmli [29]. The SDS concentration was 0.1 % (w/v) and the stacker and resolving gels were formed from 4.5 % and 15 % (w/v) acrylamide, respectively, containing 0.12 % and 0.4 % (w/v) *N,N'*-methylenebisacrylamide cross-linker, respectively.

Native-size determination

Purified samples (50 μ g) eluting from Mono-Q chromatography were applied to a Superose-12 (Pharmacia-LKB) gel-filtration column equilibrated in 50 mM sodium phosphate buffer (pH 7.0), containing 150 mM NaCl, at a flow rate of 0.3 ml/min. Elution volumes were determined by monitoring *A*₂₈₀ and compared with those of standard proteins of known size.

Non-denaturing electrophoresis was also used to determine native size [30]. Briefly, electrophoretic mobilities of test and standard proteins of known size were measured in gels formed from the following acrylamide concentrations: 5 %, 7.5 %, 10 %, 12.5 % and 15 % (w/v). Acrylamide (4.5 %, w/v) was used in all stackers, with 0.12 % (w/v) *N,N'*-methylenebisacrylamide. The method of Laemmli [29] was used, with the modification that SDS was omitted. A retardation factor for each protein was determined as the negative of plot slopes of the logarithm of mobility against the acrylamide percentage. These factors were then plotted against the standard proteins' molecular sizes to construct a standard curve from which the native sizes of *Y. lipolytica* GSTs were estimated.

Reverse-phase HPLC

Reverse-phase HPLC analysis of *Y. lipolytica* GSTs from peaks B and C was performed on a Vydac C₁₈ column (5 μ m particle size; 300 Å pore size; 0.46 × 250 mm) using a Varian model 9012 solvent-delivery system with a Waters model 481 LC spectrophotometer. The solvents were 0.1 % (v/v) trifluoroacetic acid in water (solvent A) and 0.1 % (v/v) trifluoroacetic acid in 100 % (v/v) acetonitrile (solvent B). The column was developed using the following stepped linear gradient at a flow rate of 1 ml/min: 0–40 % (v/v) solvent B in 10 min; 40–60 % (v/v) solvent B in 15 min; 60–100 % (v/v) solvent B in 10 min. The column temperature was 20 °C and *A*₂₁₄ was monitored continuously.

Isoelectric focusing

The isoelectric point (pI) was determined by comparing the mobility of the test proteins in a stable pH gradient with standard proteins of known pI [31]. Samples (40 μ g) were applied to pre-cast polyacrylamide gels with a pH range of 3.5–9.5 (ampholine PAGplate, Pharmacia-LKB) on a horizontal electrophoresis system (Bio-Rad). Gels were run at 1500 V, 50 mA and 30 W for 2 h. After focusing, gels were placed in a fixing solution of 11.5 % (w/v) trichloroacetic acid/3.5 % (w/v) sulphosalicylic acid for

0.5 h. They were then washed in destain [25 % (v/v) ethanol/8 % (v/v) acetic acid] for 5 min before staining in 0.12 % (w/v) Coomassie Brilliant Blue R-250 dissolved in destain for 10 min at 60 °C. Gels were then placed in destain until the background was clear.

Immunoblotting analysis

Proteins were separated by SDS/PAGE and analysed by Western blotting with antisera to mammalian Alpha-, Mu-, Pi- and Theta-class GSTs as described previously [21,32].

N-terminal amino acid sequencing

Proteins were transferred electrophoretically to a polyvinylidenedifluoride membrane and sequenced directly by the Edman degradation in a gas-phase sequencer (Applied Biosystems, Model 477A) at the Department of Molecular and Cell Biology, Marischal College, University of Aberdeen, Scotland, U.K. Phenylthiohydantoin amino acids were separated by C_{18} reverse-phase HPLC and detection was performed online with a phenylthiohydantoin analyser.

RESULTS

GST purification

Y. lipolytica expresses a range of antioxidant and GSH-related enzyme activities. In crude extracts, these include catalase ($145 \pm 12.7 \mu\text{mol/min/mg}$), Glyoxylase 1 ($0.095 \pm 0.009 \mu\text{mol/min/mg}$), GSSG reductase ($0.017 \pm 0.008 \mu\text{mol/min/mg}$) and GSH peroxidase ($0.011 \pm 0.002 \mu\text{mol/min/mg}$ with cumene hydroperoxide as substrate; $0.82 \pm 0.24 \text{ nmol/min/mg}$ with H_2O_2 as substrate). Modest GST conjugation activity with CDNB as substrate was also found ($0.125 \pm 0.008 \mu\text{mol/min/mg}$) and, in order to study this activity in more detail, a crude extract from *Y. lipolytica* was applied to GSH- and S-hexyl-GSH-agarose affinity resins. However, no activity was found to bind to either of these columns. A purification scheme was therefore developed consisting of chromatography on DEAE-Sephadex followed by phenyl-Sepharose and hydroxyapatite chromatography. These steps resulted in an approx. 160-fold purification of the enzyme (Table 1). To achieve final purification, the material resulting from hydroxyapatite chromatography containing activity with CDNB was applied to a Mono-Q anion-exchange column and fractionated into three main protein peaks (Figure 1). Two of these (peaks B and C) accounted for over 98 % of the total activity with CDNB and peak A was, therefore, not studied further. Peak B contained two thirds of CDNB activity while peak C contained approximately one third. Purified GSTs were

Figure 1 Fractionation of *Y. lipolytica* GSTs by Mono-Q anion-exchange chromatography

Material from hydroxyapatite chromatography was fractionated by Mono-Q anion-exchange chromatography. A three-step 0–1 M NaCl gradient was applied to develop the column as shown (dashed line). Protein elution was monitored by A_{280} (solid line). Peaks were assayed for GST activity with CDNB and by SDS/PAGE and designated A–C as shown.

found to be homogeneous and identical with each other, giving a single visible band by the criterion of SDS/PAGE (Figure 2). They had similar specific activities to CDNB and had been purified approx. 400-fold by the purification scheme outlined in Table 1. Henceforth, these will be designated GST B and GST C, respectively.

Quaternary structure of *Y. lipolytica* GSTs

Purified GSTs had unexpectedly large subunit molecular masses (Figure 2 and Table 2). They were both approx. 50 kDa and the same value was observed when electrophoresis was performed in the presence of reducing agents such as 2-mercaptoethanol, thus excluding the possibility of disulphide bridges between subunits of the usual size range (16–27 kDa). Native molecular mass was determined both by Superose-12 gel filtration and by non-

Table 1 Purification of *Y. lipolytica* GSTs

Experimental details are shown in the text. GST activity with CDNB as substrate was measured as described by Habig et al. [22].

Purification step	Volume (ml)	[Protein] (mg/ml)	Activity ($\mu\text{mol/min/ml}$)	Total activity ($\mu\text{mol/min}$)	Specific activity ($\mu\text{mol/min/mg}$)	Yield (%)	Purification factor
Crude extract	22	37.0	2.65	58.3	0.07	100	1
DEAE-Sephadex	140	0.069	0.250	35.0	3.62	60.1	50.3
Phenyl-Sepharose	125	0.015	0.171	21.4	11.4	36.7	158
Hydroxyapatite	37	0.040	0.470	17.4	11.8	29.9	163
FPLC Mono-Q							
Peak A	2.3	0.003	0.08	0.19	27.3	0.32	390
Peak B	5.0	0.056	1.25	6.24	22.3	10.7	319
Peak C	5.5	0.017	0.56	3.08	32.9	5.28	470

Figure 2 SDS/PAGE analysis of *Y. lipolytica* GSTs

SDS/PAGE of proteins purified by Mono-Q chromatography. Lanes 1 and 9, standard proteins with molecular masses of 66 (BSA), 45 (ovalbumin), 36 (glyceraldehyde 3-phosphate dehydrogenase), 29 (carbonic anhydrase), 24 (trypsinogen), 20.1 (soya bean trypsin inhibitor) and 14.2 kDa (α -lactalbumin); lane 2, crude extract; lane 3, post-DEAE-Sephadex chromatography; lane 4, post-phenyl-Sepharose chromatography; lane 5, post-hydroxyapatite chromatography; lanes 6–8, peaks A–C.

Table 2 Subunit structure of *Y. lipolytica* GSTs

Apparent subunit masses were estimated by SDS/PAGE, whereas native molecular masses were measured by both Superose-12 FPLC gel filtration and non-denaturing electrophoresis. Experimental details are given in the text.

Enzyme	Subunit molecular mass by SDS/PAGE (kDa)	Native molecular mass	
		Superose-12 (kDa)	Electrophoresis (kDa)
GST B	50	113	108
GST C	50	115	108

Figure 3 Reverse-phase HPLC of *Y. lipolytica* GST C

Purified protein fractions (50 μ g) were applied to a Waters μ Bondpak C₁₈ column. The column was developed by an acetonitrile gradient in aqueous 0.1% (w/v) trifluoroacetic acid (dashed line). A_{214} was continuously monitored (solid line). Both GSTs B and C were eluted at 53.5% (v/v) acetonitrile.

Table 3 I_{50} values for *Y. lipolytica* GSTs

The I_{50} values shown were measured using CDNB as substrate. They are the means $\pm$ S.E.M. of two independent experiments, each of which was performed in triplicate. Statistical analysis was carried out using an unpaired Student's *t*-test. No significant difference was found between values for GSTs B and C.

Inhibitor	I_{50} (μ M)	
	GST B	GST C
Bromosulphophthalein	9.0 ± 1.1	6.0 ± 0.4
Cibacron Blue	1.3 ± 0.2	2.8 ± 0.3
Ethacrynic acid	78.7 ± 20.5	71.8 ± 18.5
Hematin	1.6 ± 0.9	1.4 ± 0.7
Indomethacin	373 ± 17.0	310 ± 73.7
S-hexyl GSH	23.0 ± 1.6	16.1 ± 1.1

denaturing electrophoresis at a range of acrylamide concentrations. Data obtained from these experiments are summarized in Table 2 and suggest that both purified GSTs have a dimeric quaternary structure. However, subunit and native molecular masses are approximately twice the value normally observed with both mammalian GSTs [1] and previously characterized fungal GSTs [20,21].

It is known that some GSTs can exist as heterodimers, the subunits of which may be electrophoretically indistinguishable. Reverse-phase HPLC analysis may be used to separate such distinct subunits [33]. Figure 3 shows C₁₈ reverse-phase HPLC of purified GST C from *Y. lipolytica*. This analysis showed a single, well-resolved peak for this protein which had identical retention to that of GST B (not shown). This indicates that GSTs B and C are homodimers with structural similarity to each other.

Enzymatic properties of *Y. lipolytica* GSTs

Substrate specificity is a good criterion for both characterizing newly discovered GSTs and also for allocating GSTs to particular classes [1]. Activity of the two purified proteins with a range of GST substrates was, therefore, determined. There is a remarkable similarity in substrate specificity between the two enzymes. Specific activities with CDNB were 37 ± 3.3 and 29 ± 5.1 μ mol/min/mg for GSTs B and C, respectively. Neither enzyme displayed activity with DCNB, ethacrynic acid, *trans*-4-phenyl-3-buten-2-one, or with peroxides (i.e. H₂O₂ and cumene hydroperoxide). Although activity was occasionally detected with 1,2-epoxy-3-(*p*-nitrophenoxy)propane (results not shown), this was found to be highly variable.

Kinetic parameters were determined for the conjugation activity with CDNB of each purified GST. K_m^{GSH} was found to be 0.85 ± 0.12 and 1.31 ± 0.19 mM, while K_m^{CDNB} was 1.06 ± 0.27 and 1.04 ± 0.25 mM for GSTs B and C, respectively. The turnover numbers (k_{cat}) for GSTs B and C were 4412 ± 68 and 5694 ± 180 min⁻¹, respectively. Statistical analysis (unpaired Student's *t*-test) showed no significant difference between the K_m values of the two GSTs, whereas the difference in k_{cat} numbers was found to be significant ($P < 0.05$). Despite this latter finding, there is clearly a remarkable kinetic similarity between the two enzymes.

Another useful kinetic criterion often used for comparison of GSTs is sensitivity to inhibitors. This has also been used to allocate GSTs to particular classes and to demonstrate the catalytic independence of GST subunits [1]. The I_{50} values of purified *Y. lipolytica* GSTs with a panel of GST inhibitors are shown in Table 3. There is considerable similarity between the two enzymes by this criterion as well.

Table 4 Comparison of the N-terminal amino acid sequences of *Y. lipolytica* GSTs B and C with other fungal proteins and with Alpha-, Mu-, Pi-, Theta- and Sigma-class GSTs

Alignment of sequences is performed so as to emphasize the conservation of Ser-10 of Theta class. The *Y. lipolytica* GST sequences B and C are aligned with those of *Proteus mirabilis* [34], *Serratia marcescens* [35], *Escherichia coli* [36], *Pseudomonas* sp. LB400 and dichloromethane dehalogenases from *Methylophilus* sp. DM II and *Methylobacterium* sp. DM4 [37], *Trypanosoma cruzi* [19], *Issatchenkia orientalis* GST and EF1 γ [38], *Phanerochaete chrysosporium* GST I and maize GST II [21], *Y. lipolytica* URA5 [39], *Saccharomyces cerevisiae* URE2 [40], *Arabidopsis thaliana* GST [15] and GSH peroxidase [41], tobacco (*Nicotiana tabacum*) *parB* [42], maize GSTs I and III [43], *Hyoscyamus muticus* [44], *Silene cucubalus* [45], *Dianthus* [46], *Drosophila melanogaster* and *Lucilia cuprina* [13], *Musca domestica* GST [47], *Pleuronectes platessa* GST A [48], rat subunits 5 and 12 and human GST TI [7], human GST T2 [49], rat subunits 1, 3 and 7 [1] and squid [50].

Enzyme source		N-terminal sequence
<i>Y. lipolytica</i>		I A K I Y D L Q T P R Y T S I K A L I K H F K L D V E V V
Bacteria		
<i>Proteus mirabilis</i>		K L Y Y T P G S C S L S P H I V L R Q T G L D F S I E
<i>Serratia marcescens</i>		K L F Y K A G A C S L S P H I V L R E L G L D F T A X
<i>Escherichia coli</i> GST	MK	L F Y K P G A C S L A S H I T L R E S G K D F T L V S V
<i>Pseudomonas</i> sp. LB400	MK	L Y Y S P G A C S L S P H I A L R E A G L N F E L V Q V D
<i>Methylophilus</i> sp. DM II	STK	L R Y L H H P A S Q P C R A V H Q F M L E N N I E F Q E E
<i>Methylobacterium</i> sp. DM4	SPNPTNIHTGKT	L R L L Y H P A S Q P C R S A H Q F M Y E I D V P F E E E
Protozoa		
<i>Trypanosoma cruzi</i>	MKA	L K L F K D R L C P F C Q R V L I T A K E K R V T L E E V
Fungi		
<i>Issatchenkia orientalis</i> GST		A T V Y I K P H T P R G D W L A S L G Q Y V G L E I K T V
<i>Issatchenkia orientalis</i> EF1 γ		G T L Y A N F R I R T W V P R G L V K A L K L D V K V V
<i>Phanerochaete chrysosporium</i>		V L K L Y G N P M S T C T K R V A T V L H K G V P F E L
<i>Yarrowia lipolytica</i> URA5	QAY	K K D F L D L A T K Y E A L K F G S F T L K S G R T S P Y
<i>Saccharomyces cerevisiae</i> URE2		L E G Y T L F S H R S A P N G F K V A I V L S E L G V H
Plant		
<i>Arabidopsis thaliana</i> GST 2	AG	I K V F G H P A S I A T R R V L I A L H E K N L D F E L V
<i>Arabidopsis thaliana</i> peroxidase		V K L Y G M A Y S T C T K R V Y T T A K E I G V D V K I V
Tobacco <i>parB</i>	A	I K V H G S P M S T A T M R V A A C L I E K E L D F E F V
Maize GST I	AP	M K L Y G A V M S W N L T R C A T A L E E A G S D Y E I V
Maize GST II	ATPA	V K V Y G W A I S P F V S R A L L A L E E A G V D Y E L V
Maize GST III	AP	L K L Y G M P L S P N V V R V A T V L N E K G L D F E I V
<i>Hyoscyamus muticus</i>	G	M K L H G P A M S P A V M R V I A T L K E K D L D F E L V
<i>Silene cucubalus</i>	TT	I K V H G N P R S T A T Q R V L V A L Y E K H L E F E F V
<i>Dianthus</i>	SSSETQK	M Q L Y S F S L S S C A W R V R I A L H L K G L D F E Y K
Insects		
<i>Drosophila melanogaster</i> GST		V D F Y Y L P G S S P C R S V I M T A K A V G V E L N K K
<i>Lucilia cuprina</i>		D F Y Y L P G S T P Y H S V L M T A K A L G I E L N K K
<i>Musca Domestica</i> GST I		M D F Y Y L P G S A P C R S V L M T A K A L G I E L N K K
Fish		
<i>Pleuronectes platessa</i> GST A	MAKD	M T L L W G S G S P P C W R V M I V L E E K N L Q A Y N S
Mammalian Theta		
Rat subunit 5	V	L E L Y L D L L S Q P C R A I Y I F A K K N N I P F Q M H
Rat subunit 12	G	L E L Y L D L L S Q P C R A V Y I F A K K N G I P F Q L R
Human GST TI	G	L E L Y L D L L S Q P X R A V Y I F A K K N D I P F E L R
Human GST T2	V	L E L F L D L L S Q P S R A V Y I F A
Alpha, Mu, Pi and Sigma		
Rat subunit 1		P V L H Y F I A R G R M E C I R W L A A A G V E F E E K L
Rat subunit 3	P	M I L G Y W N V R G L T H P I R L L E Y T D S S Y E E K
Rat subunit 7	PP	Y T I V Y F P V R G R C E A T R M L L A D Q G Q S W K E E
Squid	PK	Y T L H Y F P L M G R A E L C R F V L A A H E E F T D R V

Structural comparison of *Y. lipolytica* GSTs

Comparison of GST structures show that some catalytically essential residues are located near the N-terminus, which forms part of the active site [11–15]. In the Alpha, Mu, Pi and Sigma classes, an N-terminal tyrosine is found which, in Theta class, is replaced by a serine. N-terminal sequencing therefore provides information useful for GST comparison and also helps in identifying similarity to particular classes. The first 30 N-terminal residues of purified *Y. lipolytica* GSTs were identified by Edman degradation and were found to be identical. This sequence is shown in Table 4 where it is compared with similar sequences from other fungal proteins and from a range of plant, bacterial,

insect, fish and mammalian GSTs. An N-terminal tyrosine is evident at position 5 whereas another hydroxyl residue, threonine, aligns with the serine of Theta-class GSTs. These data again show the high degree of similarity between the *Y. lipolytica* GSTs. However, it was not possible to allocate these proteins to a particular class based on sequence identity in this part of the protein.

Another technique that has proved useful for allocation of GSTs to particular classes is immunoblotting [51]. *Y. lipolytica* GSTs do not immunoblot with antibodies to Alpha, Mu, Pi or Theta classes. Unlike the *P. chrysosporium* GST, therefore, which both immunoblots and shows N-terminal-sequence simi-

larity to Theta class [21], *Y. lipolytica* GSTs show no obvious structural similarity to the mainstream cytosolic GST classes.

Are these multiple GSTs?

It is clear that GSTs B and C are structurally and kinetically very similar to each other. It is possible that they may represent a single gene product that generates distinct proteins post-translationally. A number of potential causes of multiple peaks on ion-exchange chromatography were therefore investigated. Pre-incubation of each protein with 5 mM dithiothreitol prior to Mono-Q re-chromatography gave no alteration to the elution position of either isoenzyme (results not shown), suggesting that they are not mixed-disulphide variants of each other. Moreover, isoelectric focusing gave identical pI values of pH 8.3 for both proteins, suggesting that they are unlikely to result from extensive deamidation of glutamines/asparagines. The use of phosphate buffer with Mono-Q groups at pH 6 might potentially cause artefactual elution of multiple peaks of a single protein. This possibility was excluded by carrying out ion exchange with bis-Tris buffer, which gave identical elution profiles to those obtained using phosphate (results not shown). Based on our data, it is possible that these enzymes represent charge isomers of a single gene product or else are products of distinct genes.

DISCUSSION

There is very little, if any, GST activity detectable in yeasts such as *Saccharomyces cerevisiae* [20]. In the present work, modest conjugation activity using CDNB as substrate was found in extracts of the non-*Saccharomyces* yeast, *Y. lipolytica*, and two proteins were purified to homogeneity by non-affinity methods. These proteins had two remarkable features. First, both possess subunit and native molecular masses approximately twice the normal range of values usually found in GSTs. Secondly, with the exception of their behaviour on Mono-Q anion-exchange chromatography, they appear to be structurally and kinetically identical with each other.

There appears to be a remarkable conservation of subunit mass during GST evolution. Virtually all GSTs purified from bacterial (16 kDa; [52]), fungal (25 kDa; [21]), plant (24 kDa; [53]), invertebrate (22 kDa; [51]) and mammalian (18–25 kDa; [1]) sources possess subunit masses in the range 16–27 kDa. It is unclear why this should be so but three-dimensional structures determined for GSTs from a wide range of sources suggest that packing of subunits may be a crucial feature in defining particular GST classes. Since these classes are thought to have arisen from gene duplication and divergence, the ability of subunits to pack efficiently into dimers and the nature of structural interactions at the interface between subunits may have been important during evolution. It is intriguing that *Y. lipolytica* GSTs possess subunit-mass values twice that of the normal range found. A possible mechanism for this is artefactual crosslinking between subunits of molecular mass 25 kDa, either by disulphide bridges or by some bifunctional reagent present in the extract, perhaps by an analogous mechanism to the crosslinking of human placental Pi-class GST at cysteines by chlorambucil [54]. We investigated this by carrying out SDS/PAGE in the presence of reducing agents, but subunit masses of 50 kDa were still obtained with these samples. It is difficult to exclude the presence of some, as yet unknown, bifunctional reagent, but we feel that this is an unlikely possibility since there is no evidence for any residual 'unreacted' subunits of molecular mass 25 kDa in even heavily overloaded SDS/PAGE gels. If such a reagent were present, it would have to be highly reactive to crosslink virtually all available 25 kDa subunits. Secondly, the GSTs appear to be fully active cataly-

tically and this is not consistent with reaction with a bifunctional reagent, which would be expected to cause large conformational changes, and thus inactivation, to the protein. Interestingly, a GST-related dihydrotrypanothione-GSH thioltransferase from *Trypanosoma cruzi* has similar subunit and native masses to *Y. lipolytica* GSTs [19]. However, this protein lacks GSH conjugation activity, binds to *S*-hexylglutathione and shows little similarity in N-terminal sequence to *Y. lipolytica* GSTs, suggesting that it is quite distinct from these enzymes.

Although the two proteins separated as GSTs B and C (Figure 1) clearly have different affinities for Mono-Q, in every other respect they were almost indistinguishable. They possess similar substrate specificities, kinetic parameters with GST substrates and identical sensitivity to GST inhibitors. In addition, the proteins have identical pIs, N-terminal sequence, retention on reverse-phase HPLC and mobility in electrophoretic gels under a wide variety of conditions, including a range of acrylamide concentrations and the presence of reducing agents. In view of these strong kinetic and structural similarities it is tempting to speculate that these proteins may not be the products of distinct genes. Instead, it is possible that they arise from a single gene product and they may, in fact, be charge isomers of each other, perhaps arising from modification of specific residues during the purification procedure. Our results suggest that the proteins are not mixed-disulphide or pI variants, although we cannot exclude the possibility that subtle changes to charges on their surface might underlie their distinct behaviour on chromatography. It will be necessary to clone and sequence the genes for these proteins to give unambiguous answers to these questions.

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