Saccharomyces cerevisiae cells have three Omega class glutathione S-transferases acting as 1-Cys thiol transferases

Ana GARCERÁ, Lina BARRETO, Lidia PIEDRAFITA, Jordi TAMARIT and Enrique HERRERO¹

Departament de Ciències Mèdiques Bàsiques, Facultat de Medicina, Universitat de Lleida, Montserrat Roig 2, 25008-Lleida, Spain

The *Saccharomyces cerevisiae* genome encodes three proteins that display similarities with human GSTOs (Omega class glutathione S-transferases) hGSTO1-1 and hGSTO2-2. The three yeast proteins have been named Gto1, Gto2 and Gto3, and their purified recombinant forms are active as thiol transferases (glutaredoxins) against HED (β -hydroxyethyl disulphide), as dehydroascorbate reductases and as dimethylarsinic acid reductases, while they are not active against the standard GST substrate CDNB (1-chloro-2,4-dinitrobenzene). Their glutaredoxin activity is also detectable in yeast cell extracts. The enzyme activity characteristics of the Gto proteins contrast with those of another yeast GST, Gtt1. The latter is active against CDNB and also displays glutathione peroxidase activity against organic hydroperoxides such as cumene hydroperoxide, but is not active as a thiol transferase.

Analysis of point mutants derived from wild-type Gto2 indicates that, among the three cysteine residues of the molecule, only the residue at position 46 is required for the glutaredoxin activity. This indicates that the thiol transferase acts through a monothiol mechanism. Replacing the active site of the yeast monothiol glutaredoxin Grx5 with the proposed Gto2 active site containing Cys⁴⁶ allows Grx5 to retain some activity against HED. Therefore the residues adjacent to the respective active cysteine residues in Gto2 and Grx5 are important determinants for the thiol transferase activity against small disulphide-containing molecules.

Key words: deglutathionylation, glutaredoxin (Grx), glutathione peroxidase, glutathione S-transferase (GST), redox regulation, thiol oxidoreductase.

INTRODUCTION

GSTs (glutathione S-transferases) are enzymes that conjugate GSH to a variety of exogenous and endogenous electrophilic compounds, including environmental xenobiotics, anticancer drugs and products of oxidative stress [1–4]. GSH conjugates are usually more soluble than the original compounds, and are removed from the cell by membrane-associated ATP-binding-cassette transporter. Initially, most studies into GST function were carried out using mammalian GSTs, which have been classified into cytosolic, mitochondrial and membrane-associated forms [3]. On the basis of primary sequence and substrate specificity, cytosolic GSTs (of both mammalian and non-mammalian origins) have been divided into a number of classes: Alpha, Beta, Delta, Epsilon, Kappa, Lambda, Mu, Phi, Pi, Sigma, Tau, Theta, Omega and Zeta. The human Kappa class GST protein is located both in the peroxisomes and mitochondria [5], widening the range of organelle locations for mammalian GSTs. Most GST classes share a number of substrates, among which CDNB (1-chloro-2,4-dinitrobenzene) is commonly used in standard GST activity determinations. However, GSTOs (Omega class GSTs) display low or null activity with standard GST substrates, while they have significant thiol transferase (glutaredoxin) activity [6–9]. In most GST classes, an N-terminal tyrosine or serine residue is essential for the nucleophilic attack on substrates. In contrast, Omega and also Beta class GSTs form a mixed disulphide involving GSH and an N-terminal domain cysteine residue from the GST molecule [2,6–9]. These GSTs lack the active site tyrosine/serine residue characteristic of the other classes. GSTOs therefore have different characteristics from those of most other GSTs, and functionally they resemble Grx (glutaredoxin) proteins. Some GSTs,

such as the Alpha class, also display glutathione peroxidase activity against organic hydroperoxides, including phospholipid hydroperoxides [10–12].

GSTOs are widely distributed among living organisms, although only the human (hGSTO1-1 and hGSTO2-2), pig and *Schistosoma mansoni* species have been studied extensively with regard to their enzymatic functions [6–9,13]. The three-dimensional structure of hGSTO1-1 has been determined [6], which revealed that it has the basic characteristics of a GST molecule, having an N-terminal domain with a thioredoxin-fold structure that includes the reactive cysteine and a carboxy domain with several α -helix regions, which are important for substrate specificity. GSTOs could potentially act as redox modulators of the activity of various proteins. It has been observed that hGSTO1-1 modulates the activity of calcium channels in different cell types [14], and also plays a role in the post-translational processing of interleukin-1 β [15]. A genetic study associates the hGSTOs with the age of onset of Alzheimer's disease [16], which may point to the relationship between these GSTs and oxidative stress protection.

Compared with GSTs from other organisms, those from fungal species are both more functionally and structurally diverse [17]. The yeast *Saccharomyces cerevisiae* has two GSTs (Gtt1 and Gtt2) that act on standard GST substrates [18]. Analysis of their sequences shows that they cannot be assigned to any of the above-mentioned GST classes. Gtt1 is associated with the endoplasmic reticulum and is involved in thermotolerance [18]. More recently, it has been shown that Gtt1 and Gtt2 are functionally related to Grx1 and Grx2, and provide defence against oxidants and other stresses [19]. Grx1 and Grx2 are classical dithiol glutaredoxins required to protect yeast cells against external oxidants [20]. Purified Grx1 and Grx2 are active as GSTs, and the absence of the Gtt

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; DHA, dehydroascorbate; DMA^V, dimethylarsinic acid; Grx, glutaredoxin; GST, glutathione transferase; GSTO, Omega class GST; Gtt1, *Saccharomyces cerevisiae* GST1; HED, β -hydroxyethyl disulphide; hGSTO, human GSTO; Gto, glutathione transferase Omega-like; Ni-NTA, Ni²⁺-nitrilotriacetate; IPTG, isopropyl β -D-thiogalactoside; SC, synthetic complete; t-BOOH, t-butyl hydroperoxide; ORF, open reading frame; TPCK, N-p-tosyl-L-phenylalanine chloromethyl ketone.

¹ To whom correspondence should be addressed (email enric.herrero@cmb.udl.es).

proteins in a *GRX1 GRX2* deletion mutant exacerbates the sensitivity of the yeast to oxidants [19]. These data therefore support that in *S. cerevisiae* Gtt1 and Gtt2 could participate in the defence against several stress conditions, including oxidant action, in close relationship with dithiol glutaredoxins. A role in defence against hydroperoxides has also been described for the *Schizosaccharomyces pombe* homologues of Gtt1 and Gtt2 [21].

In addition to the two dithiol glutaredoxins, *S. cerevisiae* cells contain three monothiol glutaredoxins (Grx3, Grx4 and Grx5) that have homologues in organisms from bacteria to humans and are characterized by the presence of a CGFS motif at the active site [22,23]. The cysteine residue is required for the biological activity of yeast Grx5 in the synthesis of Fe–S clusters in mitochondria [23]. In contrast with dithiol glutaredoxins, monothiol glutaredoxins are not active in the standard thiol transferase assay when the low-molecular mass disulphide molecule HED (β -hydroxyethyl disulphide) was used as the substrate [24–26].

The observations relating to: (i) the glutaredoxin activity of GSTOs; (ii) the activity of Alpha class and other GSTs as glutathione peroxidases; and (iii) the fact that Grx1 and Grx2 are active as glutathione peroxidases [27] help to depict the complex interplay between GSTs, glutaredoxins and glutathione peroxidases in oxidative stress responses. Analysis of the *S. cerevisiae* genome shows that it contains three ORFs (open reading frames) that could code for proteins similar to known GSTOs. In the present study, we demonstrate that purified recombinant products of the three ORFs are indeed Gto (glutathione transferase Omega-like) proteins, which confirms the presence of this class of GST protein in lower eukaryotes. We show that a single cysteine residue is essential for the thiol transferase activity and that it cannot be replaced by residues that are required for thiol transferase activity in other GST classes. This therefore defines a monothiol mechanism of action for the Gto proteins as thiol transferases. The role of other residues conserved in the yeast GSTO proteins are also described. Finally, we also demonstrate that yeast Grx5 can retain activity against HED when the Gto2 active site motif replaces the CGFS motif, therefore pointing to the importance of the residues surrounding the active site cysteine for the thiol transferase specificity of monothiol glutaredoxins and GSTOs.

EXPERIMENTAL

Materials

GSH, NADPH, H_2O_2 , t-BOOH (t-butyl hydroperoxide), ethacrynic acid, DHA (dehydroascorbate), sodium cacodylate, IPTG (isopropyl β -D-thiogalactoside), glycyl betaine, PMSF, TPCK (N-p-tosyl-L-phenylalanine chloromethyl ketone) and pepstatin A were purchased from Sigma. HED, CDNB and cumene hydroperoxide were from Aldrich. Amylose resin was from New England Biolabs and Ni-NTA (Ni²⁺-nitrilotriacetate)-agarose was from Qiagen. Factor X was purchased from Amersham Biosciences.

Strains and plasmids

The plasmids employed for expression of recombinant proteins in *Escherichia coli* BL21 cells (Novagen) are described in Table 1. Two expression vectors were used: pET-21a (Novagen) and pMAL-c2X (New England Biolabs). Derivatives of the wild-type genes mutated in the *GTO2* or *GRX5* sequences (Table 1) were constructed using the ExSite method [28]. Oligonucleotides for PCR amplification and introduction of the mutations were designed to insert a restriction enzyme site (that did not alter the translation product) near to or at the desired point mutation and therefore could be used as a marker for it. Mutations were con-

Table 1 Plasmids employed in this work

Plasmid	Original vector	Comments
pMM494	pET-21a	<i>GTO2</i> cloned between NdeI and XhoI sites of vector
pMM496	pET-21a	<i>GTO3</i> cloned between NdeI and XhoI sites of vector
pMM498	pET-21a	<i>GTT1</i> cloned between NdeI and XhoI sites of vector
pMM543	pET-21a	pMM494 derivative, with C46G substitution
pMM545	pET-21a	pMM494 derivative, with C67S substitution
pMM547	pET-21a	pMM494 derivative, with C46Y substitution
pMM563	pET-21a	pMM494 derivative, with C307G substitution
pMM566	pMAL-c2X	<i>GTO1</i> cloned between EcoRI and HindIII sites of vector
pMM640	pET-21a	pMM563 derivative, with C67S and C307G substitutions
pMM644	pET-21a	pMM640 derivative, with C46G, C67S and C307G substitutions
pMM646	pMAL-c2X	<i>GTO2</i> cloned between EcoRI and PstI sites of vector
pMM647	pMAL-c2X	pMM646 derivative, the CPWA sequence beginning at position 46 of Gto2 protein replaced by CGFS
pMM652	pMAL-c2X	<i>GTO2</i> sequence coding for amino acids 1 to 200 cloned between EcoRI and PstI sites of vector
pMM677	pMAL-c2X	pMM646 derivative, with L246A substitution
pMM679	pMAL-c2X	pMM646 derivative, with G280L substitution
pMM681	pMAL-c2X	pMM646 derivative, with D287G substitution
pMM684	pMAL-c2X	pMM646 derivative, with E173A substitution
pMM686	pMAL-c2X	pMM646 derivative, with S174A substitution
pMM688	pMAL-c2X	pMM646 derivative, with R51A substitution
pMM690	pMAL-c2X	pMM646 derivative, with E173D substitution
pMM694	pMAL-c2X	<i>GRX5</i> with the coding sequence for CPWA instead of the wild sequence for C60FPS, cloned between BamHI and PstI sites of vector
pMM696	pMAL-c2X	pMM690 derivative, with R51A and E173D substitutions
pMM731	pET-21a	pMM494 derivative, with C46A substitution
pMM742	pET-21a	pMM494 derivative, with C46S substitution

firmed by DNA sequencing. The *S. cerevisiae* *GTO* genes plus approx. 600 bp upstream and 200 bp downstream of the coding sequence were cloned in the multicopy plasmid YEplac195 [29]. The resulting plasmids were pMM579 (*GTO1*, cloned between EcoRI and BamHI sites of the vector polylinker), pMM584 (*GTO2*, cloned between EcoRI and HindIII sites) and pMM611 (*GTO3*, cloned between BamHI and PstI sites).

S. cerevisiae W303-1A (*MATa ura3-1 ade2-1 leu2-3,112 trp1-1 his3-11,15*) was used as standard wild-type yeast strain [22]. MML752 is an isogenic derivative with the *GRX1::kanMX4* and *GRX2::LEU2* gene disruptions. These were successively introduced in the wild-type strain by standard genetic methods.

Growth conditions

E. coli BL21 cells carrying the recombinant plasmids were grown at temperatures ranging from 25 to 37 °C in LB (Luria–Bertani) medium plus 100 μ g/ml ampicillin. For transformants carrying pMAL-c2X derivatives, 0.2% glucose was added to the culture medium. For plasmid selection, yeast cells were grown at 30 °C in synthetic complete medium [30] without uracil.

Purification of recombinant proteins

Bacterial cultures transformed with the desired plasmid (derived from pET-21a or pMAL-c2X) were grown exponentially until they reached a D_{600} of 0.5. IPTG was then added to a final concentration of 1 mM and the cells were grown for a further 4 h. The cells were recovered by centrifugation at 8000 g for 20 min, and the pellets were processed according to the manufacturer's instructions for pET-21a- and pMAL-c2X-based recombinant proteins. In some cases, 1 M sorbitol plus 2.5 mM glycyl betaine was added to the LB/ampicillin medium to favour the recovery of soluble recombinant proteins. Proteins were purified by affinity chromatography following the manufacturer's instructions. Ni-NTA-agarose

Figure 1 Comparison of GSTO protein sequences

(A) ClustalW multiple sequence alignment of the protein products of *S. cerevisiae* ORFs *GTO1* (YGR154c), *GTO2* (YKR076w) and *GTO3* (YMR251w). Residues identical in the three sequences are shown in black boxes. (B) Sequence comparison of the region around the putative active site of several GSTOs, after ClustalW alignment. SwissProt accession numbers of the respective proteins are: Gto1, P48239; Gto2, P36156; Gto3, Q04806; *Chaenorhabditis elegans* Omega, P34345; *Schistosoma mansoni* Omega, Q86LC0; mouse Omega O1-1, Q09131; pig Omega O1-1, Q9N1F5; human Omega O1-1, P78417; human Omega O2-2, Q9H4Y5. Residues identical in the compared sequences are in black boxes and residues with conserved changes are in grey boxes. The conserved region corresponding to the putative active site is underlined. Dots mark residues described as important for the activity of the hGSTO1-1 enzyme [6,9].

columns were used for proteins expressed from plasmids based on pET-21a, and amylose resin columns were used for proteins expressed from plasmids based on pMAL-c2X.

Lysis of yeast cultures

Yeast cells were grown exponentially to a concentration of approx. 2×10^7 cells/ml. They were recovered by centrifugation at 6000 *g* for 10 min, washed with lysis buffer (150 mM phosphate buffer, pH 6.5) and resuspended in lysis buffer containing a mixture of protease inhibitors (PMSF, TPCK and pepstatin A, each at a final concentration of 1 mM). An equal volume of glass beads was added and the cells were broken by shaking using a FastPrep FP120 (Thermo Savant), for two periods of 45 s each separated by 1 min on ice. The liquid extract was recovered and centrifuged at 12000 *g* for 30 min. The resultant supernatant was kept for further analyses.

Enzyme activity analysis

GST activities were spectrophotometrically determined for different substrates [31]. Thiol transferase (glutaredoxin) activity with HED as substrate was determined through the reduction of the mixed disulphide formed between HED and GSH, as described previously [32]. DHA and DMA^V (dimethylarsinic acid) reductase activities were measured as described previously [9]. Peroxidase activity with different peroxides as substrates was assayed as described previously [27]. Protein concentration was determined using the Bradford method.

Intrinsic fluorescence measurements

Intrinsic fluorescence measurements of wild-type and mutant Gto2 proteins were performed at 25 °C using a spectrofluorimeter

system RF-5000 (Shimadzu) equipped with a temperature-controlled cell holder. Measurements were carried out using 2.5 μ M protein in Tris/HCl, pH 8.0. The excitation wavelength was set at 280 nm, and the range of emission wavelength was set to between 270 and 400 nm.

In silico analysis

Homology searches were performed using the BLASTP program with default parameters (NCBI; <http://www.ncbi.nlm.nih.gov/BLAST>). Multiple sequence alignments were performed with ClustalW [33], using the European Bioinformatics Institute tools (<http://www.ebi.ac.uk>). Protein secondary structure was predicted using a Multivariate Linear Regression Combination analysis that combined the SOPMA, GOR4 and SIMPA programs [34] (Network Protein Sequence Analysis, PBIL, <http://npsa-pbil.ibcp.fr>).

RESULTS

Three ORFs of the *S. cerevisiae* genome code for GSTO proteins

Analysis of the *S. cerevisiae* genome indicated that it contains three ORFs whose putative products display similarity with hGSTO1-1 and other GSTO proteins. We therefore named the three ORFs *GTO1* (YGR154c), *GTO2* (YKR076w) and *GTO3* (YMR251w). *GTO2* has also been named *ECM4* by other authors, where it was proposed to be involved in cell wall biogenesis, on the basis of the hypersensitivity of the corresponding null mutant to chemical agents that sense cell wall defects [35]. Similarity between the three Gto proteins extends throughout their entire amino acid sequence (Figure 1A). Gto1 is 50 % identical with

Figure 2 Dendrogram after ClustalW multiple alignment of the indicated GST proteins

SwissProt accession numbers of the compared proteins are (see also legend of Figure 1): *Brucella melitensis*, Q8Y1I9; *Rhizobium meliloti*, Q92N54; *Xanthomonas campestris*, Q8P4Y7; *E. coli*, P42620; *Pseudomonas aeruginosa*, Q9IOM8; *Streptococcus agalactiae*, Q8E608; *Gloeobacter violaceus*, Q7NG00; *Halobacterium* sp., Q9HN26; *Arabidopsis thaliana* Omega, Q8H121; *A. thaliana* Phi1, P42760; *Brassica juncea* Phi2, Q7X737; *Oryza sativa* Phi1, Q65857; *C. elegans* Zeta, Q18938; *Homo sapiens* Zeta1, Q43708; *Gallus gallus* Alpha, Q08392; *H. sapiens* Alpha, P09210; *Xenopus laevis* Sigma, Q8JHA7; *Rattus norvegicus* Sigma, Q35543; *H. sapiens* Sigma, Q6FHT9; *R. norvegicus* Pi, P04906; *H. sapiens* Pi, Q5TZY3; *G. gallus* Mu2, P20136; *H. sapiens* Mu1, Q5TZY5; *Drosophila melanogaster* Theta, P20432; *Mus musculus* Theta1, Q64471; *H. sapiens* Theta1, P30711; *S. cerevisiae* Gtt1, P40582; *S. cerevisiae* Gtt2, Q12390; *Schizosaccharomyces pombe* SPCC1281.07c, Q94524; *Candida albicans* ECM4, Q5A953; *Neurospora crassa* NCU04368.1, Q7RWR2.

Gto2 and 62 % with Gto3, while Gto2 and Gto3 are 50 % identical. Similarity with the metazoan homologues is less marked, although it also extends throughout the sequence. The cysteine residue essential for GSH-dependent activity in hGSTO1-1 [6] is conserved in the three Gto proteins and in eukaryotic homologues (Cys³¹ in Gto1 and Cys⁴⁶ in Gto2 and Gto3; Figure 1B). Other important residues for the activity of the hGSTO1-1 species such as Pro³³, Leu⁵⁶ and Lys⁵⁹ [6] are conserved in the three yeast Gto proteins. hGSTO1-1 Phe³⁴ also has its corresponding residue in Gto1, while it is replaced by a tryptophan residue in Gto2 and Gto3. Compared with hGSTO1-1, *S. cerevisiae* Gto proteins are larger as they contain additional amino acid stretches that do not correspond to any part of the hGSTO1-1 sequence. As an example, the Gto1 region spanning amino acids 179–227 (and its equivalent regions in Gto2 and Gto3; Figure 1A) has no counterpart in hGSTO1-1. BLAST searches reveal the presence of proteins similar to the three *S. cerevisiae* products in other fungal species and in many bacteria, especially proteobacteria and cyanobacteria. Multiple alignment analysis indicated that fungal (including the *S. cerevisiae* Gto proteins) and bacterial sequences are grouped close to the metazoan GSTO proteins and are significantly separated from other classes of GSTs (Figure 2). In this tree, the *S. cerevisiae* Gto proteins and their fungal relatives group together as a subcluster with their bacterial homologues. Remarkably, yeast Gtt1 does not become grouped with other known GST classes, while Gtt2 forms a cluster with sequences of the GST Zeta class (Figure 2). This confirms the previously observed divergence between the Gtt proteins (and in general most fungal GSTs) and mammalian GST classes [17].

Purified Gto proteins have enzyme activities characteristic of GSTOs

We attempted to purify the Gto1, Gto2 and Gto3 proteins from *E. coli* cells when they were overexpressed using the pET-21a expression vector. It was possible to obtain Gto2 in a soluble form from bacterial cultures at 37 °C without further additions. However, neither recombinant Gto1 nor Gto3 became soluble under these conditions. It has been reported that addition of 1 M sorbitol plus glycyl betaine to the growth medium may result in the solubilization of some recombinant proteins that under other conditions are insoluble in *E. coli* cells [36]. We therefore tested the expression of Gto1 and Gto3 under these growth conditions at a temperature range from 25 to 37 °C. Gto3 was obtained in a soluble form with the above additions even at 37 °C, while Gto1 remained insoluble under all conditions. We therefore used a different expression vector, pMAL-c2X, to overexpress Gto1. This allowed us to obtain a soluble protein in which the maltose-binding protein was N-terminally fused to Gto1. This was achieved in LB medium without additions at 37 °C. Soluble recombinant Gto proteins obtained under the different conditions reported above were further purified by affinity chromatography using Ni-NTA resin columns (for pET-21a-based constructs) or amylose resin columns (for pMAL-c2X-based constructs). In the latter case, the Gto1 moiety was cleaved from the maltose-binding protein by digestion with Factor X. Purity of the final Gto preparations were checked by SDS/PAGE (10 % gels), followed by Coomassie Blue staining and scanning of the stained gels (using a GS-800 Bio-Rad densitometer). In the above cases and also in those described in later

Table 2 Activity of recombinant GSTs of *S. cerevisiae* with various substrates

Results are means $\pm$ S.D. of three determinations with separate protein preparations. ND, not detectable.

Substrate	Enzyme			
	Gto1	Gto2	Gto3	Gtt1
Glutathione transferase activity ^a				
CDNB	ND	ND	ND	70 $\pm$ 8
1,2-Dichloronitrobenzene	ND	ND	ND	ND
Ethacrynic acid	252 $\pm$ 31	94 $\pm$ 6	104 $\pm$ 19	105 $\pm$ 11
4-Hydroxynonenal	ND	ND	ND	ND
Thiol transferase ^b	1.93 $\pm$ 0.118	3.27 $\pm$ 1.286	1.18 $\pm$ 0.240	ND
DHA reductase ^c	0.23 $\pm$ 0.031	0.11 $\pm$ 0.009	0.16 $\pm$ 0.004	ND
DMA ^v reductase ^d	0.17 $\pm$ 0.031	0.14 $\pm$ 0.009	0.15 $\pm$ 0.026	ND

^a Specific activity as nmol of glutathione conjugate formed per min per mg of protein.

^b Specific activity as μ mol of NADPH oxidized per min per mg of protein, with β -hydroxyethyl disulphide as a substrate.

^c Specific activity as μ mol of DHA reduced per min per mg of protein.

^d Specific activity as μ mol of NADPH oxidized per min per mg of protein.

sections, the purity of the recombinant Gto protein in the final preparation was at least 95 %.

Recombinant Gto proteins did not display activity against the GST substrates CDNB, 1,2-dichloronitrobenzene or 4-hydroxynonenal, while they were active on ethacrynic acid (Table 2). In contrast, they demonstrated thiol transferase activity against HED, which is a substrate of dithiol glutaredoxins [32]. Using recombinant Gtt1 (purified from a construction based on pET-21a, Table 1) as a control for standard GSTs, this protein showed measurable activity against CDNB, as previously reported [18]. However, it did not demonstrate any glutaredoxin activity (Table 2). The Gto proteins also displayed moderate activity as DHA and DMA^v reductases (Table 2). The specific activity of the recombinant Gto proteins against the different substrates tested was at approximately the same level as that reported for hGSTO1-1 [6,13]. In contrast, hGSTO2-2 displayed considerably higher activity as a DHA reductase [13].

Specific activity of the recombinant Gto proteins as a function of GSH concentration was determined (Figure 3). The data were best-fitted with a sigmoidal curve. The apparent Hill coefficients (h_{app}) were calculated to be 2.36, 2.43 and 4.29 for Gto1, Gto2 and Gto3 respectively, indicating strong positive co-operativity. These co-operative interactions are most frequently associated with multimeric proteins. The K_m (app) for GSH was similar for the three proteins (1.13–1.42 mM), while the V_{max} values exhibited larger differences (Figure 3).

Thiol transferase activity of the Gto proteins is expressed *in vivo*

To determine whether the thiol transferase activity observed with recombinant Gto proteins was also manifested *in vivo*, a strain (MML752) was used that lacked the two dithiol glutaredoxins Grx1 and Grx2, to lower the thiol transferase activity against the substrate HED in cell extracts. This strain exhibited approx. one-third of the thiol transferase activity present in extracts from wild-type cells. This was in accordance with previous observations for a different genetic background [22]. The origin of the residual thiol transferase activity in the absence of GRX1 and GRX2 was not determined. MML752 cells were transformed with multicopy plasmids carrying *GTO1*, *GTO2* or *GTO3*, in which these genes were expressed under their own promoters. Overexpression of

Figure 3 Thiol transferase activity of Gto proteins as a function of GSH concentration

Thiol transferase activity of recombinant proteins was assayed according to the method described previously [32], with GSH concentration varying in the range 0–5 mM. Best-fit plots were determined with non-linear regression functions included in the Mathematica 5.1 program. K_m (app) (labelled K_m ; mM), V_{max} (labelled V_m ; μ g/min per mg of protein) and Hill coefficient (labelled h_{app}) values are shown for each curve. Results correspond to a single representative experiment; differences between three experiments were less than 15 %.

Gto1 or Gto2 proteins *in vivo* caused a significant increase (60–70 %) in cellular thiol transferase activity above the level observed in cellular extracts from the void vector-transformed $\Delta grx1 \Delta grx2$ mutant (Figure 4). The increase in enzyme activity in the cell extracts from Gto3-overexpressing cells was more modest (approx. 20 %) but was still reproducible. One of the functions of GSTs is defence against oxidants [3,4]. We therefore hypothesized that treatment of the above *GTO* transformants with diamide (an oxidant of thiol groups) could promote a further increase in thiol transferase activity measured in cell extracts. This was, in fact, the case for Gto2-overexpressing cells, but not for those overexpressing Gto1 or Gto3 (Figure 4). It was concluded that thiol transferase activity of Gto proteins is present in yeast cells under normal growth conditions and that, in the case of Gto2, this activity is induced under oxidative stress conditions. This latter observation is in accordance with the reported activation of expression of the *GTO2* gene under oxidative stress conditions [37].

Figure 4 *In vivo* expression of glutaredoxin activity from *S. cerevisiae* Gto proteins

Cultures of *S. cerevisiae* cells transformed with the multicopy vector YEplac121 or the derived plasmids pMM529 (Gto1), pMM584 (Gto2) or pMM611 (Gto3) were grown exponentially in SC medium at 30°C to a cell concentration of about 2×10^7 cells/ml. In each case, half of the culture had been treated with 2.5 mM diamide for the previous 3 h (black bars), while the other half remained untreated (white bars). Total cell extracts were obtained and the glutaredoxin activity determined. Relative values of specific activity are shown over the bars. The unit value corresponds to untreated wild-type cells transformed with the vector (absolute specific activity: 3.65 ± 0.16 nmol/min per mg of protein). Results are means $\pm$ S.D. for three experiments.

Gtt1 has significant activity as glutathione peroxidase, in contrast with Gto proteins

Many Alpha class GSTs and also some members of other classes display glutathione peroxidase activity against organic hydroperoxides [10–12]. We analysed whether Gto proteins were also active as glutathione peroxidases. Purified recombinant Gtt1 was analysed in parallel. H_2O_2 and two organic hydroperoxides, t-BOOH and cumene hydroperoxide, were tested as substrates. Recombinant Gto1 showed no activity against these three substrates, while Gto2 and Gto3 exhibited only very modest activity against t-BOOH (Figure 5). Gto3 also showed low but reproducible activity against cumene hydroperoxide. In contrast, we detected significant peroxidase activity for purified Gtt1 against cumene hydroperoxide (Figure 5). Elimination of any single component in the *in vitro* reaction with Gtt1 decreased the measurements to almost undetectable levels (Figure 5, inset). This confirmed that the detected glutathione peroxidase activity could indeed be attributed to purified Gtt1. Specific peroxidase activity as a function of GSH concentration displayed a sigmoidal curve, with a K_m (app) value of 0.45 mM (results not shown).

The glutaredoxin activity of Gto2 operates through a single cysteine residue

Our attention was focused on Gto2 to analyse the residues and protein regions necessary for the glutaredoxin activity of the molecule. The three-dimensional structure of hGSTO1-1 has been determined experimentally [6]. However, this structure could not be used for obtaining a reliable spatial model of Gto2, since the similarity between the two proteins throughout the amino acid sequence is below 20%. A secondary structure model was determined for Gto2 (see the Experimental section). In accordance with the known structure of GSTOs and other GST classes

Figure 5 Glutathione peroxidase activity of *S. cerevisiae* GSTs

Recombinant Gtt1 (expressed from plasmid pMM498), Gto1 (pMM566), Gto2 (pMM494) and Gto3 (pMM496) proteins were assayed for glutathione peroxidase activity against cumene hydroperoxide (black bars), t-BOOH (grey bars) and H_2O_2 (white bars). Specific activity values are given as μ mol/min per mg of protein. The requirements for Gtt1 glutathione peroxidase activity against cumene hydroperoxide are shown in the inset, where the percentage of specific activity (relative to the complete assay mixture) is indicated when the mixture lacked the Gtt1 enzyme, GSH or glutathione reductase. Results are means $\pm$ S.D. for three independent experiments.

[1–4,6], the predicted secondary structure of Gto2 depicts an N-terminal domain in which β -strands alternate with α -helices and a C-terminal domain rich in α -helices (Figure 6A). The sequence has three cysteine residues (at positions 46, 67 and 307, as shown in Figure 1A) that could be required for the activity of Gto2 as a glutaredoxin. Cys⁴⁶ is located between a β -strand and an α -helix region in the model of Figure 6(A), and the adjacent proline residue could be important in causing a change in orientation of the following α -helix domain. Both residues are conserved in hGSTO1-1 (Figure 1B). The equivalent Cys³² in hGSTO1-1 is exposed at the surface of the molecule [6]. The other two cysteine residues of Gto2 do not have an equivalent in hGSTO1-1 or in Gto1 or Gto3. Point mutations were inserted into the sequence of Gto2 that altered the three cysteine residues. Only the absence of Cys⁴⁶ decreased the thiol transferase activity of Gto2 to the same undetectable levels as a molecule lacking all three cysteines (Figure 6B). A mutant form of Gto2 was made in which Cys⁴⁶ was the only one of the three cysteine residues left unchanged, and this protein had enzyme activity levels comparable with that of the wild-type protein, which indicated that the thiol transferase activity of Gto2 operates through a single cysteine residue at position 46, that is, using a monothiol mechanism.

In many GST classes, a serine or tyrosine residue at the active site is responsible for the nucleophilic attack on substrates such as CDNB [1–3]. We then tested the effect of replacing Cys⁴⁶ with a serine or tyrosine residue on the *in vitro* GST activity of Gto2. Besides being completely inactive as thiol transferases (Figure 6B), the mutated Gto2 versions (C46S and C46Y) did not recover the activity against CDNB, which remained at basal levels. Recently, it has been shown that replacing the active site cysteine with an alanine residue in hGSTO1-1 is able to restore activity with CDNB [9]. However, this was not the case for the Gto2 mutant C46A, which displayed no activity against CDNB (results not shown).

Figure 6 Effect of amino acid changes on the glutaredoxin activity of Gto2

(A) Secondary-structure prediction for Gto2 protein. Grey boxes correspond to proposed α -helices and black boxes to β -strands. The positions of the amino acid residues which were mutated in the present study are shown. (B) Percentage of specific enzyme activity (in the HED assay for glutaredoxin) of the recombinant Gto2 versions with the indicated amino acid changes, relative to the wild-type protein. Values are given on the right and are the means for three independent experiments. Proteins were expressed in *E. coli* cultures transformed with the respective plasmids indicated in Table 1. Results are means $\pm$ S.D. for three independent experiments.

An analysis of fluorescence emission spectra was made to determine whether replacement of the active site Cys⁴⁶ residue had an effect on the global structure of Gto2 (Figure 7). The intrinsic fluorescence emission of a protein is affected by changes in the environment of aromatic residues and provides information on changes in its tertiary structure. No detectable changes were seen for the C46G and C46A substitutions, indicating that the lack of enzyme activity of the respective derivatives was strictly due to the absence of Cys⁴⁶. For the C46S and C46Y substitutions, a moderate decrease of emission intensity was detected, which may indicate some alteration of the tertiary structure of the protein or quenching of tryptophan fluorescence by the more polar environment caused by the hydroxylated amino acid.

We tested whether replacing Cys⁴⁶ plus the adjacent residues Pro⁴⁷, Trp⁴⁸ and Ala⁴⁹ in Gto2 (Figure 1A) with the CGFS sequence of the Grx5 active site had some effect on the thiol transferase activity of the protein. It must be stressed that, besides Cys⁴⁶, only Gto2 Pro⁴⁷ (but not Trp⁴⁸ and Ala⁴⁹) is conserved in other GSTOs (Figure 1B). The hybrid Gto2 molecule with the CGFS motif of Grx5 showed no detectable activity as a thiol transferase with HED (Figure 6B). This confirmed that, although

Figure 7 Intrinsic fluorescence emission spectra analysis of Gto2

Emission spectra of wild-type and recombinant Gto2 proteins with the indicated amino acid changes were recorded after excitation at 280 nm.

Cys⁴⁶ was required for Gto2 activity, the adjacent residues were also important.

It has been proposed that an electron-sharing network consisting of alternating ionic bridge interactions between negatively and positively charged amino acids is important for the catalytic activity of many, if not all, GST classes [38]. In hGSTO1-1, Arg³⁷, Glu⁸⁵ and Ser⁸⁶ would be part of in such a network, facilitating the formation of anionic glutathione as an essential step in the GST-mediated catalysis [38]. The equivalent residues in *S. cerevisiae* Gto2 are Arg⁵¹, Glu¹⁷³ and Ser¹⁷⁴. We individually replaced these residues with alanine residues and purified the new protein versions. None of the substitutions, or indeed an E173D change, affected the thiol transferase activity of Gto2 (Figure 6B). The double substitution R51A/E173D also had no effect on Gto2 activity. At least in the case of yeast Gto2, it therefore seems that those three residues are not required for the *in vitro* enzyme activity.

The C-terminal domain of Gto2 is also required for the activity of the protein

GSTs and both dithiol and monothiol glutaredoxins are members of the thioredoxin superfamily together with other proteins that interact with cysteine-containing substrates, such as thioredoxins, protein disulphide-isomerases and glutathione peroxidases [39,40]. A structural motif called the thioredoxin fold is shared by all members of this superfamily. This motif is formed by a four- or five-stranded β -sheet with a number of α -helices distributed on either side. This thioredoxin-fold structure constitutes the N-moiety of all GSTs, including those of the Omega class [1–4,6]. To test whether the first half of the Gto2 molecule, which includes all parts of the thioredoxin-fold structure (Figure 6A), was sufficient to provide the enzyme activity, a version of Gto2 was made that was truncated after amino acid 200. Although this truncated form of Gto2 still contained Cys⁴⁶, it was not active in the HED assay. This indicated that for Gto2 the thioredoxin-fold domain alone was not sufficient for the protein to be active as a thiol transferase. Three residues (Leu²⁴⁶, Gly²⁸⁰ and Asp²⁸⁷) in the C-terminal moiety of Gto2 are conserved at equivalent positions of Gto2 homologues from other species. We mutated these residues separately and studied the effects on activity of the respective recombinant proteins. The change D287G totally abolished enzyme activity, while the other two changes (L246A and G280L) produced no detectable effects (Figure 6B).

A hybrid Grx5 protein with the active site of Gto2 has some thiol transferase activity

As shown above, Gto2 operates through a monothiol mechanism of action for its thiol oxidoreductase activity on HED. However, Grx5 and other monothiol glutaredoxins are unable to reduce this substrate. To test whether the CPWA sequence of the proposed active site of Gto2 was able to provide Grx5 with activity in the HED assay, we made a mutant protein where the Grx5 CGFS active site was replaced with the CPWA sequence (plasmid pMM694; as shown in Table 1). This hybrid Grx5 protein displayed some activity in the complete assay (69 ± 14 nmol/min per mg of protein, mean $\pm$ S.D. for three experiments). The specific activity against HED of the modified Grx5 form was considerable lower than the activity of Gto2 in the same assay (Table 2). However, it was significantly above background levels and it was not detectable in the absence of GSH or the substrate. We conclude that the amino acids adjacent to the respective active site cysteine residues in Gto2 and Grx5 are important determinants to define the action on small disulphide-containing molecules such as HED.

DISCUSSION

Glutaredoxins utilize GSH as an electron donor in the reduction of disulphide bridges, while typical GSTs use GSH to conjugate it to electrophilic compounds [1–4,32]. The two types of enzymes are related both structurally and functionally. Thus the N-terminal moiety of GSTs and the complete structure of dithiol glutaredoxins share the thioredoxin-fold domain [39,40]. Dithiol glutaredoxins, but not monothiol ones, exhibit GSH-disulphide oxidoreductase activity when assayed with substrates such as HED [24–26,32]. This is also the case for eukaryotic GSTOs, including the three yeast Gto proteins. A single cysteine at the active site (Cys⁴⁶) is sufficient for the enzyme activity of Gto2, which therefore operates as thiol oxidoreductase through a monothiol mechanism of action. However, the differential activity on the small disulphide HED molecule indicates that monothiol glutaredoxins and GSTOs employ different mechanisms of enzyme activity. The present study shows that the amino acids adjacent to the active site cysteine residue in Gto2 and Grx5 are important for determining the activity of the two proteins against HED. A hybrid derivative of Grx5 with the CPWA sequence from the Gto2 active site retains some enzyme activity. This suggests that the glycine, phenylalanine and serine residues adjacent to Cys⁶⁰ in Grx5 establish some constraints for the deglutathionylation reaction. This does not discard the existence of additional structural constraints outside the active site of Grx5 [41], which would explain that the activity of the hybrid Grx5 derivative occurs at low levels.

Fungal GSTs display a wide variety of primary structures, which are different from structures of mammalian GST classes [3,17]. However, homologues of hGSTO1-1 and of other higher eukaryote GSTOs are also present in lower eukaryotes such as *S. cerevisiae*, as well as in other fungi and bacteria. The primary structure of the three yeast homologues (Gto1, Gto2 and Gto3) is more similar to bacterial GSTOs than to hGSTO1-1, although a number of residues that are important for the tertiary structure of the latter and for its interaction with GSH [6] are also present in the three yeast Gto proteins. This includes the N-terminal cysteine residue essential for thiol oxidoreductase activity (Cys³² and Cys⁴⁶ in hGSTO1-1 and Gto2 respectively). It is remarkable that, while most bacterial and fungal GSTs are structurally separated from mammalian GSTs [17], those with the basic structure of the Omega class are present along the whole evolutionary scale.

This, together with the fact that they operate as glutaredoxins, suggest their importance in the evolution from GSH-dependent redox enzymes to GST-conjugating enzymes on the basis of a thioredoxin-fold module. It has been suggested that the GST classes with GSH-conjugation ability could have evolved from thiol-disulphide oxidoreductases that use GSH as redox donor, and could be precursors of other GST classes [42]. GSTs with cysteine residues at their active sites could be representatives of these ancient GSTs.

The glutaredoxin activity of Gto1, Gto2 and Gto3 measured *in vitro* correlates with their *in vivo* expression. Of the total thiol transferase activity detected in cell extracts in our hands, approx. 70% was probably due to the two dithiol glutaredoxins Grx1 and Grx2. The remaining activity in the absence of Grx1 and Grx2 can be increased approx. 1.6- to 1.7-fold by separately overexpressing Gto1 or Gto2 (or more modestly in the case of Gto3) in otherwise normal growth conditions. This means that in yeast cells with a single copy of any of the *GTO* genes, the glutaredoxin activity of these GSTs would account for only a small percentage of the total glutaredoxin activity of the cell. The enzyme activity of the Gto proteins is probably required for specialized functions during growth on glucose medium, or they must otherwise be induced in specific situations. For instance, Gto2 activity increases significantly during stress caused by diamide. Whole-proteome analysis in yeast cells [43] and our own studies with green fluorescent protein-tagged forms of the three Gto proteins indicate that Gto1 has a peroxisomal location, while Gto2 and Gto3 are cytosolic. In spite of the peroxisomal location of Gto1, it is clear that its activity can even be expressed under growth conditions (such as on glucose-based medium) in which the number of peroxisomes per yeast cell is low. The low glutaredoxin activity of Gto proteins is compatible with their participation in specific functions inside the cell that require the maintenance of the adequate redox state of specific protein targets, instead of a general role in the defence against oxidative stress. These specific roles in determined cellular functions are also characteristic of monothiol glutaredoxins [44,45].

The C-terminal moiety of Gto2 is required for the activity of the enzyme in addition to the N-terminal region containing Cys⁴⁶. Asp²⁸⁷ is an essential residue in the C-terminal region. Aspartate residues are present at equivalent positions in Gto homologues, including hGSTO1-1. The equivalent in hGSTO1-1 (Asp¹⁷⁴) is located at the same α -helix as Arg¹⁸³. This has been proposed to participate (together with other residues at both the N- and C-terminal moieties) in the formation of the H-site responsible for the interaction with GST substrates [6]. hGSTO1-1 Arg¹⁸³ is also conserved in all the GSTO proteins analysed. However, it must be kept in mind that the *in vitro* assays for thiol transferase activity are not performed with natural GST substrates. This favours the hypothesis that Asp²⁸⁷ in Gto2, and its equivalents in Gto2 homologues, could participate in the maintenance of the general structure of the protein instead of being directly involved in the enzyme reaction. In hGSTO1-1, Asp¹⁷⁴ is part of the α 6-helix, which is located close to the α 1-helix. The α 1-helix is important for the structure of the active site and includes Arg³⁷, a residue that is conserved in the three Gto proteins [6] (Figure 1). This model for hGSTO1-1 suggests an interaction between Asp¹⁷⁴ and Arg³⁷. A similar situation in Gto2 would explain the requirement for Asp²⁸⁷ for the spatial conformation of the molecule.

S. cerevisiae Gtt1 and Gtt2 have structural similarities with Ure2, a regulator of nitrogen catabolic gene expression. Although lacking GST activity [18], the Ure2 protein has glutathione peroxidase activity [46]. Purified Gtt1 is active as a glutathione peroxidase against organic hydroperoxides, although not against H₂O₂. This combined with the peroxidase activity of Grx1 and

Grx2 [23] supports the *in vivo* role of the Gtt GSTs and the two dithiol glutaredoxins in the defence against hydroperoxides. Among the three *Schizosaccharomyces pombe* GSTs (Gst1, Gst2 and Gst3), Gst3 is the most similar to Gtt1 and exhibits *in vivo* activity as a glutathione peroxidase against organic hydroperoxides [18]. Therefore the peroxidase activity of Gtt1 and its possible role in protection against oxidative stress in co-operation with other antioxidant systems may be a conserved property in different fungi. Altogether, these observations support the existence of a complex relationship between GSTs, glutaredoxins and glutathione peroxidases in general protection against oxidative stress and in specific protein redox regulation.

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