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***Saccharomyces cerevisiae* CELLS HAVE THREE OMEGA CLASS
GLUTATHIONE TRANSFERASES ACTING AS 1-CYS THIOL
TRANSFERASES**

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Short title: Yeast Omega Class Glutathione Transferases

Abbreviations used: GST, glutathione transferase; GSH, reduced glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; HED: β -hydroxyethyl disulfide; DHA: dehydroascorbate; DMA^V: dimethylarsinic acid; *t*-BOOH: *tert*-butyl hydroperoxide; IPTG: isopropyl thio- β -D-galactopiranoside; ORF: open reading frame;

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SYNOPSIS

The *Saccharomyces cerevisiae* genome codes for three proteins that display homology with human Omega class glutathione transferases (GST) 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 β -hydroxyethyl disulfide (HED), as dehydroascorbate reductases and as dimethylarsinic acid reductases, while they are not active against the standard GST substrate 1-chloro-2,4-dinitrobenzene (CDNB). 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. Substitution of the active site of the yeast monothiol glutaredoxin Grx5 by the proposed Gto2 active site containing Cys46 makes Grx5 to recover 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: glutathione transferase, glutaredoxin, glutathione peroxidase, thiol oxidoreductase, deglutathionylation, redox regulation

INTRODUCTION

Glutathione transferases (GSTs) are enzymes that conjugate reduced glutathione (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 ABC transporters. Initially, most studies were carried out with mammalian GSTs, which have been classified into cytosolic, mitochondrial and membrane-associated forms [3]. Based on primary sequence and substrate specificity, cytosolic GSTs (both of 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 hGSTK1 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 1-chloro-2,4-dinitrobenzene (CDNB) is commonly used in standard GST activity determinations. However, 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 Tyr/Ser residue characteristic of other classes. Omega class GSTs therefore have different characteristics from most other GSTs, and functionally they resemble glutaredoxins. Some GSTs, such as Alpha class ones, also display glutathione peroxidase activity against organic hydroperoxides, including phospholipids hydroperoxides [10-12].

Omega class GSTs are widely distributed among living organisms, although only the human (hGSTO1-1 and hGSTO2-2), pig and *Schistosoma mansoni* species have been enzymatically studied extensively [6-9,13]. The three-dimensional structure of hGSTO1-1 has been determined [6]. It has the basic characteristics of a GST, having an amino terminal domain with a thioredoxin-fold structure that includes the reactive cysteine, and a carboxy domain with several α -helix regions that are important for substrate specificity. Omega class GSTs could act as redox modulators of the activity of various proteins. Thus, hGSTO1-1 modulates the activity of calcium channels in

different cell types [14]. Other observations support the role of hGSTO1-1 in the post-translational processing of interleukin-1 β [15]. A genetic study associates the human Omega class GSTs with the age-of-onset of Alzheimer's disease [16], which may point to the relationship between these GSTs and oxidative stress protection.

Compared to 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]. Sequence analysis does not allow including them into 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 glutaredoxins 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 proteins in a *grx1 grx2* mutant exacerbates the sensitivity of the latter 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/Gtt2 [21].

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

The observations relating to: (i) the glutaredoxin activity of Omega class GSTs, (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 open reading frames that would code for proteins homologous to known Omega class GSTs. In this study we demonstrate that purified recombinant products of the three ORFs are indeed Omega class GSTs, confirming the presence of this GST class in lower eukaryotes. We show that a single cysteine residue is essential for the thiol transferase

activity and that it can not be substituted by residues that are required for the 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 Omega GST molecules is also described. Finally, we also demonstrate that yeast Grx5 can gain activity against HED when the Gto2 active site motif replaces the CGFS motif, therefore pointing to the importance of the residues surrounding the active cysteine for the thiol transferase specificity of monothiol glutaredoxins and Omega GSTs.

EXPERIMENTAL

Materials

GSH, glutathione reductase, NADPH, hydrogen peroxide, *t*-BOOH, ethacrynic acid, DHA, sodium cacodylate, IPTG, glycyl betaine, phenylmethane-sulfonyl fluoride, N-p-tosyl-L-phenyl-alanine chloromethyl ketone and pepstatin A were from Sigma. HED, CDNB and cumene hydroperoxide were from Aldrich. Amylose resin was from New England Biolabs and Ni-NTA 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 by the ExSite method [28]. Oligonucleotides for PCR amplification and introduction of the mutations were designed in such a way that a restriction not altering the translation product was created near to or at the desired point mutation and used as a marker for it. Mutations were confirmed by DNA sequencing. The *S. cerevisiae* *GTO* genes plus about 600 and 200 base pairs, respectively located upstream and 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*, between EcoRI and HindIII sites) and pMM611 (*GTO3*, 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 medium plus 100 µg/ml ampicillin. Glucose at 0.2% was also added in cultures of transformants carrying pMAL-c2X derivatives. Yeast cells were grown at 30°C in synthetic SC medium [30] without uracil for plasmid selection.

Purification of recombinant proteins

Bacterial cultures transformed with the desired plasmid (derived from pET-21a or pMAL-c2X) were grown exponentially until they reached an A_{600} value of 0.5. IPTG was then added to a final concentration of 1 mM. After 4 h of growth in these conditions, cells were recovered by centrifugation and pellets were processed according to the respective purchaser recommendations 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 protein inside bacteria. Proteins were purified by affinity chromatography. Ni-NTA agarose columns were used for proteins expressed from plasmids based on pET-21a, and amylose resin columns for proteins expressed from plasmids based on pMAL-c2X.

Lysis of yeast cultures

Yeast cells were grown exponentially to a concentration of about 2×10^7 cells per ml. They were recovered by centrifugation, washed with lysis buffer (150 mM phosphate buffer pH 6.5) and resuspended in lysis buffer containing a mixture of protease inhibitors (phenylmethanesulfonyl fluoride, N-p-tosyl-L-phenylalanine chloromethyl ketone and pepstatin A, each at a final concentration of 1 mM). An equal volume of glass beads was added and cells were broken by shaking (for two periods of 45 seconds each separated by 1 min in ice). The liquid extract was recovered and centrifuged at 12000g x 30 min. The supernatant was kept for further analyses.

Enzyme activity analyses

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 disulfide formed between HED and GSH, as described in Ref. 32. DHA reductase and DMA^V reductase activities were measured as in Ref. 9. Peroxidase activity with different peroxides as substrates was assayed as in Ref. 27. Protein concentration was determined by the Bradford method.

Intrinsic fluorescence measurements

Intrinsic fluorescence measurements of wild type and mutant Gto2 proteins were performed at 25 °C using a spectrofluorometer system RF-5000 (Shimadzu) equipped with a temperature-controlled cell holder. Measurements were carried out with protein solutions at 2.5 µM concentration in Tris-HCl buffer pH 8.0. The excitation wavelength was set to 280 nm, and the range of emission wavelength was set to 270–400 nm.

***In silico* analyses**

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

RESULTS

Three ORFs of the *S. cerevisiae* genome code for proteins of the GST Omega class

Analysis of the *S. cerevisiae* genome indicated that it contains three ORFs whose putative products display homology with the human Omega class GST hGSTO1-1 and other proteins of the same GST class. We therefore named the three ORFs *GTO1* (*YGR154c*), *GTO2* (*YKR076w*) and *GTO3* (*YMR251w*), for Glutathione Transferase Omega-like. *GTO2* has also been named *ECM4* by other authors; it was proposed to be involved in cell wall biogenesis, based on the hypersensitivity of the corresponding null mutant to chemical agents that sense cell wall defects [35]. Homology between the three Gto proteins extends throughout the entire amino acid sequence (Figure 1A). Gto1 is

50% identical to Gto2 and 62% to Gto3, while Gto2 and Gto3 are 50% identical. Semblance 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 (in position 31 in Gto1 and 46 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 substituted by a tryptophan residue in Gto2 and Gto3. Compared to hGSTO1-1, *S. cerevisiae* Gto proteins are larger since they contain additional amino acid stretches without homology in hGSTO1-1. As an example, the Gto1 region from amino acid 179 to 227 (and its equivalent regions in Gto2 and Gto3, Figure 1A) has not counterpart in hGSTO1-1. BLAST searches reveal the presence of proteins homologous 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 Omega class proteins and 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 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 Omega class GSTs

We tried to purify the Gto1, Gto2 and Gto3 proteins from *E. coli* cells that overexpressed them based on 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 sorbitol plus glycyl betaine to the LB medium may result in the solubilization of some recombinant proteins that in other conditions are insoluble inside *E. coli* cells [36]. We therefore tested the expression of Gto1 and Gto3 under these growth medium 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, for Gto1. This allowed us to obtain a soluble protein with the vector-coded maltose binding protein N-terminally fused to Gto1, 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-based columns (for pET-21a-based constructs) or amylose resin columns (for pMAL-c2X-based constructs). In the latter case, the Gto1 moiety was released from the maltose binding protein by digestion with Factor X. Purity of the final Gto preparations was checked by SDS-acrylamide electrophoresis, followed by Coomassie staining and scanning of the stained gels. In the above cases and also in those described in later 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 reductases and DMA^V reductases (Table 2). The specific activity of the recombinant Gto proteins against the different substrates tested was at about the same level as that reported for hGSTO1-1 [6,13]. In contrast, hGSTO2-2 displays considerably higher activity as DHA reductase [13].

Specific activity of the recombinant Gto proteins as a function of GSH concentration was determined (Figure 3). The data were best fit with a sigmoidal curve. The apparent Hill coefficients (n_{app}) were calculated to be 2.36 (Gto1), 2.43 (Gto2) and 4.29 (Gto3), indicating strong positive cooperativity. These cooperativity interactions are most frequently associated with multimeric proteins. The K_{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*

We determined whether the thiol transferase activity observed with recombinant Gto proteins was also manifested *in vivo*. To this end, a strain (MML752) was used that lacked the two dithiol glutaredoxins Grx1 and Grx2, in order to lower the thiol

transferase activity against HED in extracts. This strain exhibited about 30% 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 Gto1 or Gto2 proteins *in vivo* caused a significant increase (60-70%) in cellular thiol transferase activity above the observed in cellular extracts from the void vector-transformed *Dgrx1 Dgrx2* mutant (Figure 4). The increase in enzyme activity in the cell extracts from Gto3-overexpressing cells was more modest (about 20%) but 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 sulphhydryl 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].

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

Many Alpha class GSTs and also some members of other classes display glutathione peroxidase activity against organic hydroperoxides [10-12]. We analyzed whether Gto proteins were also active as glutathione peroxidases. Purified recombinant Gtt1 was analyzed in parallel. Hydrogen peroxide 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). The latter 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 reduced measured values 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_{app} value of 0.45 mM (data 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 homology between the two proteins throughout the amino acid sequence is below 20%. On the other hand, a secondary structure model was determined for Gto2 (see Experimental). In accordance with the known structure of Omega and other GST classes [1-4,6], the predicted secondary structure of Gto2 depicts a 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, Figure 1A) that could be required for the activity of Gto2 as a glutaredoxin. Cys46 is between a β -strand and a α -helix region in the model of Figure 6A, and the adjacent proline residue could be important in causing a change in orientation in the following α -helix domain. Both residues are conserved in hGSTO1-1 (Figure 1B). The equivalent Cys32 residue in hGSTO1-1 is exposed at the surface of the molecule [6]. The other two cysteine residues of Gto2 have not equivalent in hGSTO1-1 or in Gto2 or Gto3. Point mutants were constructed that individually lacked any of the three cysteines in Gto2. Only the absence of Cys46 reduced the thiol transferase activity of Gto2 to the same undetectable levels as a molecule lacking all three cysteines (Figure 6B). A version of Gto2 was made in which Cys46 was the only one of the three cysteine residues left. This molecule had enzyme activity levels comparable to the non-mutated molecule, which indicated that the thiol transferase activity of Gto2 operates through a single cysteine 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 substituting 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 substitution of the active site

cysteine by an alanine residue in hGSTO1-1 makes the latter to recover activity with CDNB [9]. However, this was not the case for the C46A Gto2 mutant, which displayed no activity against CDNB (data not shown).

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 tertiary structure. No detectable changes were detected 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 accompanying Pro, Trp and Ala residues in Gto2 (Figure 1A) by the CGFS sequence of the Grx5 glutaredoxin 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 Omega class GSTs (Figure 1B). The hybrid Gto2 molecule with the CGFS motif of Grx5 showed no detectable activity as thiol transferase with HED (Figure 6B). This confirmed that although 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, the 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 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 molecule

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 disulfide 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 surrounded by a number of α -helices distributed on either side of the β -sheet. This thioredoxin-fold structure constitutes the N-moiety of all GSTs, including those of the Omega class [1-4,6]. In order 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 still containing Cys46, this truncated Gto2 form was not active in the HED assay. This indicated that the thioredoxin-fold domain of Gto2 alone was not sufficient for the molecule to perform as 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 sulphhydryl oxidoreductase activity on HED. However, Grx5 and other monothiol glutaredoxins are unable to reduce this substrate. In order to know whether the CPWA sequence of the proposed active site of Gto2 was able to provide activity to Grx5 in the HED assay, we made a construction whether the Grx5 CGFS active site was replaced by the CPWA sequence (plasmid pMM694, Table 1). This hybrid Grx5 molecule displayed some activity in the complete assay (69 ± 14 nmols/min/mg protein, mean of 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 disulfide 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-disulfide oxidoreductase activity when assayed with substrates such as HED [24-26,32]. This is also the case for eukaryotic Omega class GSTs, 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 disulfide HED molecule indicates that monothiol glutaredoxins and Omega GSTs employ different enzyme mechanisms. This study has shown that amino acids adjacent to the active site cysteine residues in Gto2 and Grx5 are important for determining the activity of the two molecules against HED. A hybrid derivative of Grx5 with the CPWA sequence from the Gto2 active site has some activity. This means that the Gly, Phe and Ser 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 eukaryotes Omega class GSTs 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 representatives 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. These include the N-terminal cysteine essential for hGSTO1-1 (Cys32) and Gto2 (Cys46) thiol oxidoreductase activity. It is remarkable

that while most bacterial and fungal GSTs are structurally separated from mammalian GSTs [17], GSTs with the Omega class basic structure 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 based on a thioredoxin-fold module. It has been suggested that the GSTs classes with GSH-conjugation ability could have evolved from thiol-disulfide 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 *in vitro* measured glutaredoxin activity of Gto1, Gto2 and Gto3 correlates with their *in vivo* expression. Of the total thiol transferase activity detected in cell extracts in our hands about 70% is probably due to the two dithiol glutaredoxins Grx1 and Grx2. The remaining activity in the absence of Grx1 and Grx2 can be increased by about 1.6-1.7 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 GFP-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 (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-region. Asp 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-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 analysed Omega class proteins. 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 Gto2 Asp287 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 hGTO1-1 Asp174, which forms part of the α 6 helix, comes close to the α 1 helix, which is important for the structure of the active site and includes an Arg37 residue conserved in the three Gto proteins (Ref. 6 and Figure 1). This model for hGTO1-1 suggests 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 homology with Ure2, a regulator of nitrogen catabolic gene expression. While inactive as GST [18], the Ure2 protein has glutathione peroxidase activity [46]. Purified Gtt1 is active as a glutathione peroxidase against organic hydroperoxides, although not against hydrogen peroxide. 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 *S. 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 cooperation 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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REFERENCES

- 1 Armstrong, R.N. (1997) Structure, catalytic mechanism, and evolution of the glutathione transferases. *Chem. Res. Toxicol* **10**, 2-18
- 2 Sheehan, D., Meade, G., Foley, V.M. and Dowd, C.A. (2001) Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. *Biochem. J.* **360**, 1-16
- 3 Hayes, J.D., Flanagan, J.U. and Jowsey, I.R. (2005) Glutathione transferases. *Annu. Rev. Pharmacol. Toxicol* **45**, 51-88
- 4 Oakley, A.J. (2005) Glutathione transferases: new functions. *Curr. Opin. Struct. Biol.* **15**, 716-723
- 5 Morel, F., Rauch, C., Petit, E., Piton, A., Theret, N., Coles, B. and Guillouzo, A. (2004) Gene and protein characterization of the human glutathione S-transferase kappa and evidence for a peroxisomal localization. *J. Biol. Chem.* **279**, 16246-16253
- 6 Board, P.G., Coggan, M., Chelvanagayam, G., Eastal, S., Jermin, L.S., Schulte, G.K., Danley, D.E., Hoth, L.R., Griffor, M.C., Kamath, A.V., Rosner, M.H., Chrnyk, B.A., Perregaux, D.E., Gabel, C.A., Geoghegan, K.F. and Pandit, J. (2000) Identification, characterization, and crystal structure of the omega class glutathione transferases. *J. Biol. Chem.* **275**, 24798-24806
- 7 Rouimi, P., Anglade, P., Benzekri, A., Costet, P., Debrauwer, L., Pineau, T. and Tulliez, J. (2001) Purification and characterization of a glutathione S-transferase Omega in pig: evidence for two distinct organ-specific transcripts. *Biochem. J.* **358**, 257-262
- 8 Girardini, J., Amirante, A., Zemzoumi, K. and Serra, E. (2002) Characterization of an omega-class glutathione S-transferase from *Schistosoma mansoni* with glutaredoxin-like dehydroascorbate reductase and thiol transferase activities. *Eur. J. Biochem.* **269**, 5512-5521
- 9 Whitbread, A.K., Masoumi, A., Tetlow, N., Schmuck, E., Coggan, M. and Board, P.G. (2005) Characterization of the Omega-class of glutathione transferases. *Methods Enzymol.* **401**, 78-99
- 10 Hurst, R., Bao, Y., Mannervik, B. and Williamson, G. (1998) Phospholipid hydroperoxide glutathione peroxidase activity of human glutathione transferases. *Biochem. J.* **332**, 97-100
- 11 Zhao, T., Singhal, S.S., Piper, J.T., Cheng, J., Pandya, U., Clark-Wronski, J., Awasthi, S. and Awasthi, Y.C. (1999) The role of human glutathione S-transferases hGSTA1-1 and hGSTA2-2 in protection against oxidative stress. *Arch. Biochem. Biophys.* **367**, 216-224

- 12 Prabhu, K.S., Reddy, P.V., Jones, E.C., Liken, A.D. and Reddy, C.C. (2004) Characterization of a class alpha glutathione *S*-transferase with glutathione peroxidase activity in human liver microsomes. *Arch. Biochem. Biophys.* **424**, 72-80
- 13 Schmuck, E.M., Board, P.G., Whitbread, A.K., Tetlow, N., Blackburn, A.C. and Masoumi, A. (2005) characterization of the monomethylarsonate reductase and dehydroascorbate reductase activities of Omega class glutathione transferase variants. Implications for arsenic metabolism and age-at-onset of Alzheimer's and Parkinson's diseases. *Pharmacogenet. Genomics* **15**, 493-501
- 14 Dulhunty, A., Gage, P., Curtis, S., Chelvanayagam, G. and Board, P. (2001) The glutathione transferase structural family includes a nuclear chloride channel and a ryanodine receptor calcium release channel modulator. *J. Biol. Chem.* **276**, 3319-3323
- 15 Laliberte, R.E., Perregaux, D.G., Hoth, L.R., Rosner, P.J., Jordan, C.K., Peese, K.M., Egger, J.F., Dombroski, M.A., Geoghegan, K.F. and Gabel, C.A. (2003) Glutathione *S*-transferase omega 1-1 is a target of cytokine release inhibitory drugs and may be responsible for their effect on interleukin-1 β posttranslational processing. *J. Biol. Chem.* **278**, 16567-16578
- 16 Li, Y.J., Oliveira, S.A., Xu, P., Martin, E.R., Stenger, J.E., Scherzer, C.R., Hauser, M.A., Scott, W.K., Small, G.W., Nance, M.A. et al Glutathione *S*-transferase omega-1 modifies age-of-onset of Alzheimer disease and Parkinson disease. *Human Mol. Genet.* **12**, 3259-3267
- 17 McGoldrick, S., O'Sullivan, S.M. and Sheehan, D. (2005) Glutathione transferase-like proteins encoded in genomes of yeasts and fungi: insights into evolution of a multifunctional protein superfamily. *FEMS Microbiol. Lett.* **242**, 1-12
- 18 Choi, J.H., Lou, W. and Vancura, A. (1998) A novel membrane-bound glutathione *S*-transferase functions in the stationary phase of the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* **273**, 29915-29922
- 19 Collinson, E.J. and Grant, C.M. (2003) Role of yeast glutaredoxins as glutathione *S*-transferases. *J. Biol. Chem.* **278**, 22492-22497
- 20 Luikenhuis, S., Dawes, I.W. and Grant, C.M. (1998) The yeast *Saccharomyces cerevisiae* contains two glutaredoxin genes that are required for protection against reactive oxygen species. *Mol. Biol. Cell* **9**, 1081-1091
- 21 Veal, E.A., Toone, W.M., Jones, N. and Morgan, B.A. (2002) Distinct roles for glutathione *S*-transferases in the oxidative stress response in *Schizosaccharomyces pombe*. *J. Biol. Chem.* **277**, 35523-35531
- 22 Rodríguez-Manzanque, M.T., Ros, J., Cabisco, E., Sorribas, A. and Herrero, E. (1999) Grx5 glutaredoxin plays a central role in protection against protein oxidative damage in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **19**, 8180-8190

- 23 Bellí, G., Polaina, J., Tamarit, J., de la Torre, M.A., Rodríguez-Manzanque, M.T., Ros, J. and Herrero, E. (2002) Structure-function analysis of yeast Grx5 monothiol glutaredoxin defines essential amino acids for the function of the protein. *J. Biol. Chem.* **277**, 37590-37596
- 24 Rahlfs, S., Fischer, M. and Becker, K. (2001) *Plasmodium falciparum* possesses a classical glutaredoxin and a second, glutaredoxin-like protein with a PICOT homology domain. *J. Biol. Chem.* **276**, 37133-37140
- 25 Tamarit, J., Bellí, G., Cabisco, E., Herrero, E. and Ros, J. (2003) Biochemical characterization of yeast mitochondrial Grx5 monothiol glutaredoxin. *J. Biol. Chem.* **278**, 25745-25751
- 26 Fernandes, A.P., Fladvad, M., Berndt, C., Andrésen, C., Lillig, C.H., Neubauer, P., Sunnerhagen, M., Holmgren, A. and Vlami-Gardikas, A. (2005) A novel monothiol glutaredoxin (Grx4) from *Escherichia coli* can serve as a substrate for thioredoxin reductase. *J. Biol. Chem.* **280**, 24544-24552
- 27 Collinson, E.J., Wheeler, G.L., Ocón-Garrido, E., Avery, A.M., Avery, S.V. and Grant, C.M. (2002) The yeasts glutaredoxins are active as glutathione peroxidases. *J. Biol. Chem.* **277**, 16712-16717
- 28 Weiner, M.P. and Costa, L. (1995) Rapid PCR site-directed mutagenesis. In *PCR Primer, a Laboratory Manual* (Dieffenbach, C.W. and Dveksler, G.S., eds), pp. 613-621, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- 29 Gietz, R.D. and Sugino, A. (1988) New yeast-*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74**, 3065-3073
- 30 Sherman, F. (2002) Getting started with yeast. *Methods Enzymol* **350**, 3-41
- 31 Habig, W.H., Pabst, M.J. and Jakoby, W.B. (1974) Glutathione S-transferases. *J. Biol. Chem.* **249**, 7130-7139
- 32 Holmgren, A. and Aslund, F. (1995) Glutaredoxin. *Methods Enzymol* **252**, 283-292
- 33 Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673-4680
- 34 Combet, C., Blanchet, C., Geourjon, C. and Deléage, G. (2000) NPS@: network protein sequence analysis. *Trends Biochem. Sci.* **25**, 147-150
- 35 Lussier, M., White, A.M., Sheraton, J., di Paolo, T., Treadwell, J., Southard, S.B., Horenstein, C.I., Chen-Weiner, J., Ram, A.F., Kapteyn, J.C., Roemer, T.W., Vo, D.H., Bondoc, D.C., Hall, J., Zhong, W.W., Sdicu, A.M., Davies, J., Klis, F.M., Robbins, P.W.

- and Bussey, H. (1997) Large scale identification of genes involved in cell surface biosynthesis and architecture in *Saccharomyces cerevisiae*. *Genetics* **147**, 435-450
- 36 Blackwell, J.R. and Horgan, R. (1991) A novel strategy for the production of a highly expressed recombinant protein in an active form. *FEBS Lett.* **295**, 10-12
- 37 Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., Botstein, D. and Brown, P.O. (2000) Genomic expression programs in the response of yeast cells to environmental changes. *Mol. Biol. Cell* **11**, 4241-4257
- 38 Winayanuwattikun, P. and Ketterman, A.J. (2005) A electron-sharing network involved in the catalytic mechanism is functionally conserved in different glutathione transferase classes. *J. Biol. Chem.* **280**, 31776-31782
- 39 Martin J.L. (1995) Thioredoxin--a fold for all reasons. *Structure* **3**, 245-250
- 40 Ferrari, D.M. and Söling, H.D. (1999) The protein disulphide-isomerase family: unravelling a string of folds. *Biochem. J.* **339**, 1-10
- 41 Fladvad, M., Bellanda, M., Fernandes, A.P., Mammi, S., Vlamis-Gardikas, A., Holmgren, A. and Sunnerhagen, M. (2005) Molecular mapping of functionalities in the solution structure of reduced Grx4, a monothiol glutaredoxin from *Escherichia coli*. *J. Biol. Chem.* **280**, 24553-24561
- 42 Caccuri, A.M., Antonioni, G., Allocated, N., Di Ilio, C., De Maria, F., Innocenti, F., Parker, M.W., Masulli, M., Lo Bello, M., Turella, P., Federico, G. and Ricci, G. (2002) GSTB1-1 from *Proteus mirabilis*: a snapshot of an enzyme in the evolutionary pathway from a redox enzyme to a conjugating enzyme. *J. Biol. Chem.* **277**, 18777-18784
- 43 Huh, W.K., Falvo, J.V., Gerke, L.C., Carroll, A.S., Howson, R.W., Weismann, J.S. and O'Shea, E.K. (2003) Global analysis of protein localization in budding yeast. *Nature* **425**, 686-691
- 44 Fernandes, A.P. and Holmgren, A. (2004) Glutaredoxins: glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system. *Antiox. Red. Sign.* **6**, 63-74
- 45 Molina, M.M., Bellí, G., de la Torre, M.A., Rodríguez-Manzaneque, M.T. and Herrero, E. (2004) Nuclear monothiol glutaredoxins of *Saccharomyces cerevisiae* can function as mitochondrial glutaredoxins. *J. Biol. Chem.* **279**, 51923-51930
- 46 Bai, M., Zhou, J.M. and Perrett, S. (2004) The yeast prion protein Ure2 shows glutathione peroxidase activity in both native and fibrillar form. *J. Biol. Chem.* **279**, 50025-50030

LEGENDS TO THE FIGURES

Figure 1. Comparison of Omega class GST 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 Omega class GSTs, 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, O09131; 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 human O1-1 enzyme [6,9].

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*, Q8YII9; *Rhizobium meliloti*, Q92N54; *Xanthomonas campestris*, Q8P4Y7; *Escherichia coli*, P42620; *Pseudomonas aeruginosa*, Q9IOM8, *Streptococcus agalactiae*, Q8E608; *Gloebacter violaceus*, Q7NG00; *Halobacterium sp.* Q9HN26; *Arabidopsis thaliana* Omega Q8H121; *A. thaliana* Phi1, P42760; *Brassica juncea* Phi2, Q7X737; *Oryza sativa* Phi1, O65857; *C. elegans* Zeta, Q18938; *Homo sapiens* Zeta1, O43708; *Gallus gallus* Alpha, Q08392; *H. sapiens* Alpha, P09210; *Xaenopus laevis* Sigma, Q8JHA7; *Rattus norvegicus* Sigma, O35543; *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, O94524; *Candida albicans* ECM4, Q5A953; *Neurospora crassa* NCU04368.1, Q7RWR2.

Figure 3. Thiol oxidoreductase activity of Gto proteins as a function of GSH concentration. Thiol oxidoreductase activity of recombinant proteins was assayed according to Ref. 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_{app} , (mM), V_{max} (micrograms/min/mg protein) and Hill coefficient (n_{app}) values are shown for each curve. Data correspond to a representative experiment; differences between three experiments were below 15%.

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 three hours (black bars), while the other half remained untreated (white). Total cell extracts were obtained and glutaredoxin activity was determined in them. Relative values of specific activity are shown over bars; they are the mean of three independent experiments. The unit value corresponds to untreated wild type cells transformed with the vector (absolute specific activity: 3.65 ± 0.16 nmols/min/mg). Error bars: S.D.

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) and hydrogen peroxide (white). Values of specific activity are given as $\mu\text{mols/min/mg}$ protein. Results are the mean of three independent experiments. The requirements for Gtt1 glutathione peroxidase activity against cumene hydroperoxide are shown inside the box, where the percentage of specific activity (relative to the complete assay mixture) is indicated when the mixture lacks the Gtt1 enzyme, GSH or glutathione reductase. Error bars: S.D.

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 this 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 mean of three independent experiments. Proteins were expressed in *E. coli* cultures transformed with the respective plasmids indicated in Table 1. Error bars: S.D.

Figure 7. Intrinsic fluorescence emission spectra analysis of Gto2. Emission spectra of wild type and recombinant Gto2 versions with the indicated amino acid changes were recorded after excitation at 280 nm.

Table 1 **Plasmids employed in this work**

Plasmid	Original vector	Comments
pMM494	pET-21a	<i>GTO2</i> cloned between NdeI-XhoI sites of vector
pMM496	pET-21a	<i>GTO3</i> cloned between NdeI-XhoI sites of vector
pMM498	pET-21a	<i>GTT1</i> cloned between NdeI-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-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-PstI sites of vector
pMM647	pMAL-c2X	pMM646 derivative, the CPWA sequence beginning at position 46 of Gto2 protein substituted by CGFS
pMM652	pMAL-c2X	<i>GTO2</i> sequence coding for aa 1 to 200 cloned between EcoRI-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 the BamHI-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

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

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

	Gto1	Gto2	Gto3	Gtt1
Glutathione transferase activity ^a				
1-Chloro-2,4-dinitrobenzene	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
Dehydro ascorbate reductase ^c	0.23 \pm 0.031	0.11 \pm 0.009	0.16 \pm 0.004	ND
Dimethylarsonate (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 protein

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

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

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

(A)

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YGR154c (GTO1) 1 -----MSVSYKGTISKTHSVFKPKGRYYIYGALGCFPTHRAILARSLKLEPVLGLVLSHWQLDSKGA
YKR076w (GTO2) 1 MSKQWASGTNGAFKRQVSSFRRTISQHPYIKPAKGRYWLYVSLAFCFWAHRLLITRALKGLTSVIGCSVWHHLDEKGN
YMR251w (GTO3) 1 MSEKSASNKAEFKRQSSPFRRTISADHPYIKPAKGRYWLYVSLAFCFWAQRLLITRALKGLAPIIGCSVAHWHLDDKGN

YGR154c (GTO1) 65 RFLPAPH--RPEKYKERFFTATGGIASAKLDES EELGDVNNDSARLFVDSGAFDPVENISRIS ELYLNDPKYFGTKET
YKR076w (GTO2) 80 RFLDMEKQLEDSDFLEHWHDVAGGIRTAKEDESS(SFAEIKNDSQRFMVDATNEP-HYGYKRIS DLYIKSDPPYSA-RFT
YMR251w (GTO3) 80 RFL--EEGDGKTNE--RHWFDIAGGHSVNLNTSIPVANIPNNAHRLLVDGTDDEP-HYGYKRISDFYFKTKPDIYKGR-RFT

YGR154c (GTO1) 141 VPVLWDSKTRKIVNNESDILIRLINSVGFDEEIQSEETNVIDLVPDHLIDEIDKNIKWVHPKINLGVYKVLGAENKTIY
YKR076w (GTO2) 158 VPVLWDLDTOTIVNNESSEILIRLINSVAFDEEVD-DDHKKTDLVPAQLKTCIDDFNSWVYDSINNGVYKIGFAEKAEVY
YMR251w (GTO3) 154 VPVLWDLDTCTIVNNESDILIGLWNSAAFDEEIVG-EEYRQVRLVPRSLAELTEFNSSWYDKINNGVYKAGFAECAEVY

YGR154c (GTO1) 220 EDEVKTIFENLCKMEVILKENYKRL EEQFSGN-KQKILAKYFVLGDRLTEADIRLYPSIIRFDVYVQHFKCNKNTIR D
YKR076w (GTO2) 236 ESEVNNVEEHLKVEKILSDKYSKLLKAKYGEEDRQKILGEHFTVGDQLTEADIRLYTTVI RFDVYVQHFKCNFTSIRA
YMR251w (GTO3) 232 EREVTSEIYLLKVEKLLDKKYIDLEAEYGNKNDKILLDRYFATGDTLTEADVRLYPTIV RFDVYVQHFKCNLATIR D

YGR154c (GTO1) 298 GFPYLHLWLNLYWNYAEFRITDFTHIKLBYRMEVSRNKINDFSIPLGPKPDISRL
YKR076w (GTO2) 315 GYPFHLWVRNLYWNYDAFRYITDFTHIKLBYR--SHTRIWPLSIPPLGPKPDIRPP
YMR251w (GTO3) 311 DYSRIHTWLNLYWRHEAFRTITDFTHIKLBYR--SOPRVNPISIPPLGPKPDIRPL
    
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(B)

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Gto1          12 THSVFKPEKGRYYIYGALGCFPTHRAILARSLKLEPVLGLVLSHWQLDSKGA RFL
Gto2          27 QHPYIKPAKGRYWLYVSLAFCFWAHRLLITRALKGLTSVIGCSVWHHLDEKGN
Gto3          27 DHPYIKPAKGRYWLYVSLAFCFWAQRLLITRALKGLAPIIGCSVAHWHLDDKGN
C.elegans Omega 15 DAEP-PLSKGSFRVYNMRFCEWAEARALYVAAGKIE--A--EVVNLNVTDKLEWYW
Schistosoma Omega 7 DPKP-LYDPNRLTLIGFRFCFYVDRVKLILSYKVD--Y--DLIDISLAKPEWFL
Mouse Omega O1-1 13 SAPPGPVPEGQIRVYSMRFCEFAQRTLLMVLKAKGIR--H--EVININLKNKPEWFF
Pig Omega O1-1 13 SAPPGPVPEGLIRVYSMRFCEFAQRTLLVNLAKGIR--H--QVININLKNKPEWFF
Human Omega O1-1 13 SAPPGPVPEGSIRIYSMRFCEFAERTRLVNLKAKGIR--H--EVININLKNKPEWFF
Human Omega O2-2 13 SQPPGPVPEGLIRIYSMRFCEYSHRTRLVNLKAKDIR--H--EVVNLNLRNKPEWY
    
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Figure 1

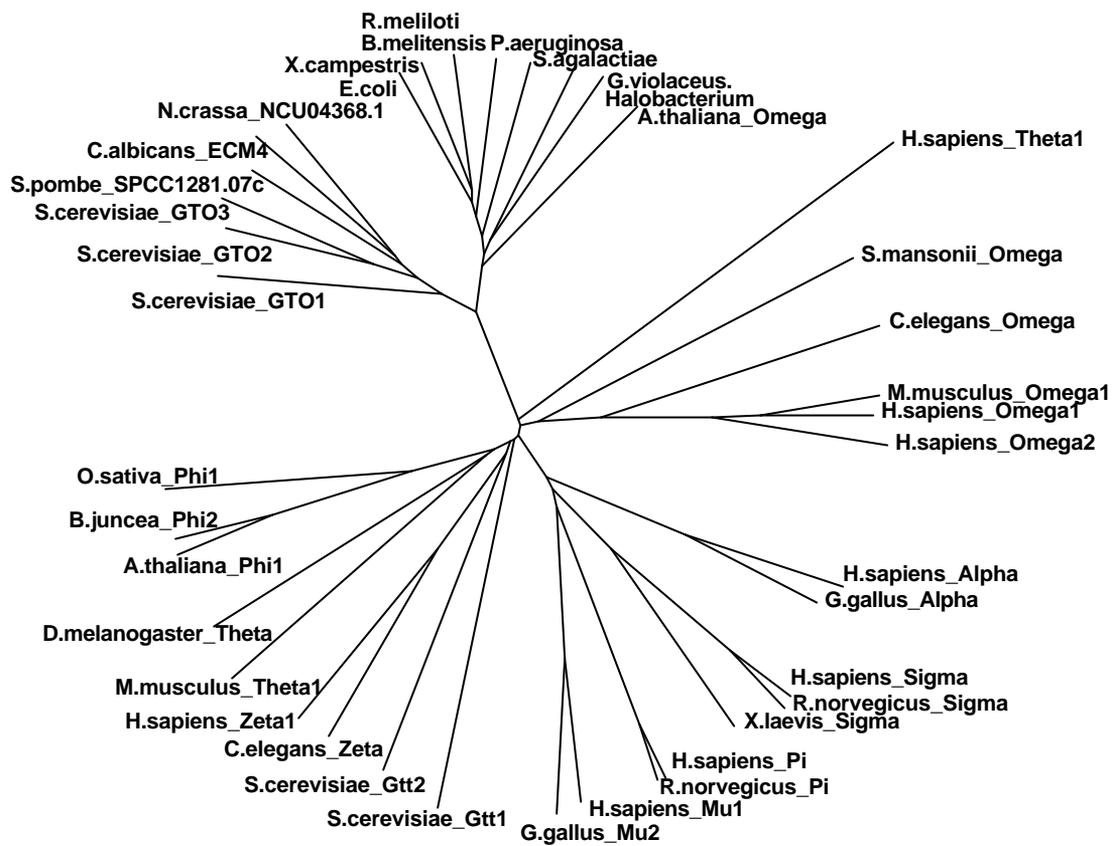


Figure 2

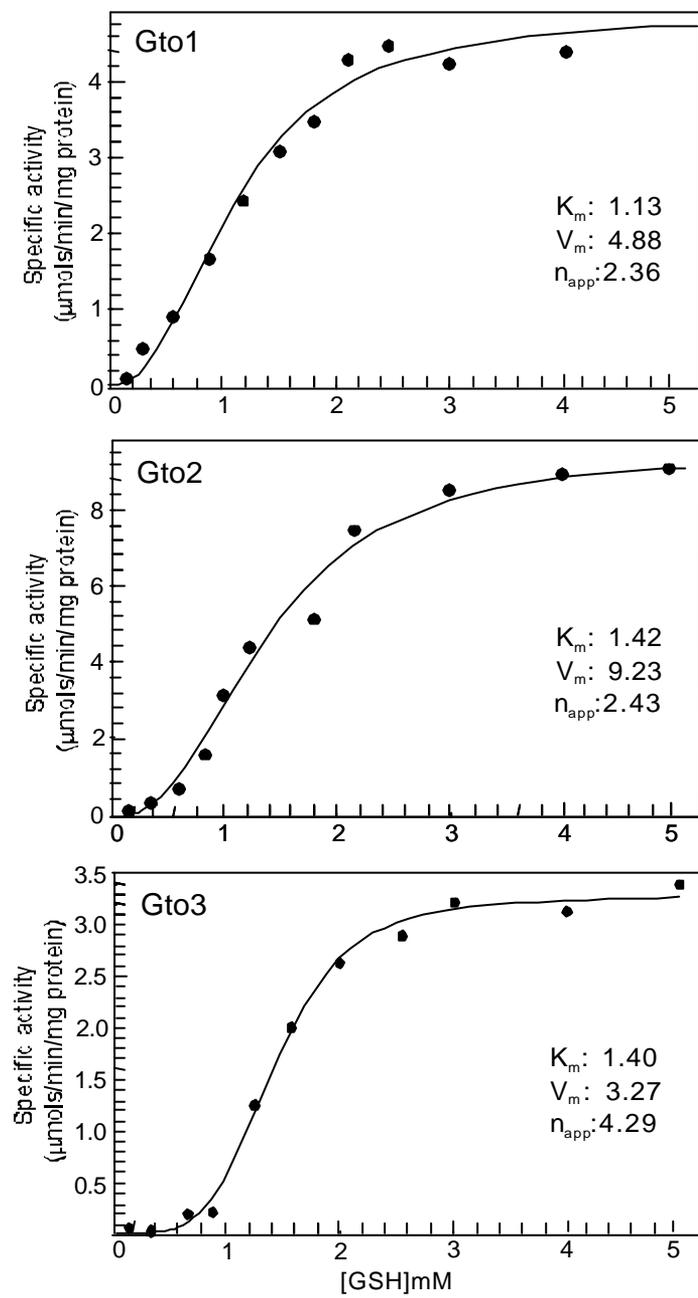


Figure 3

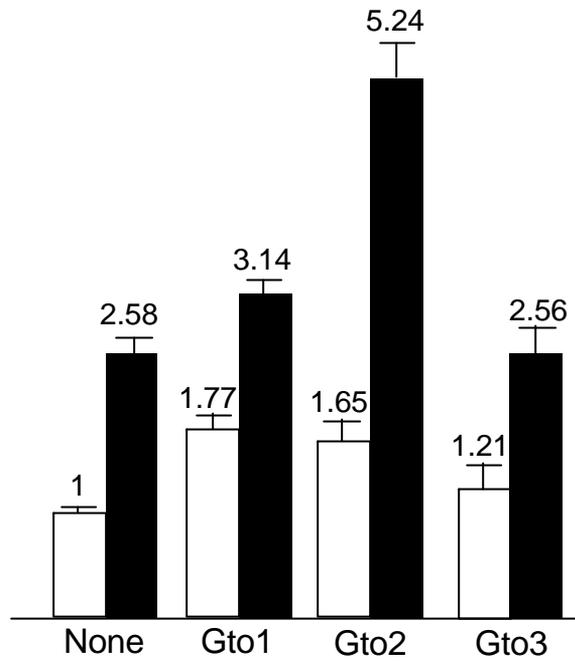


Figure 4

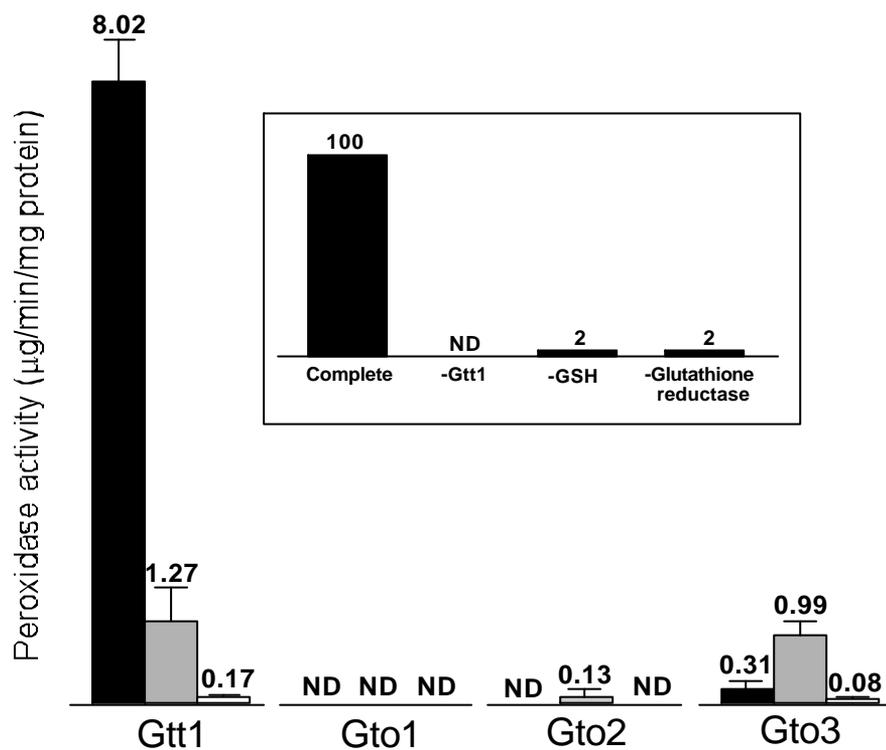


Figure 5

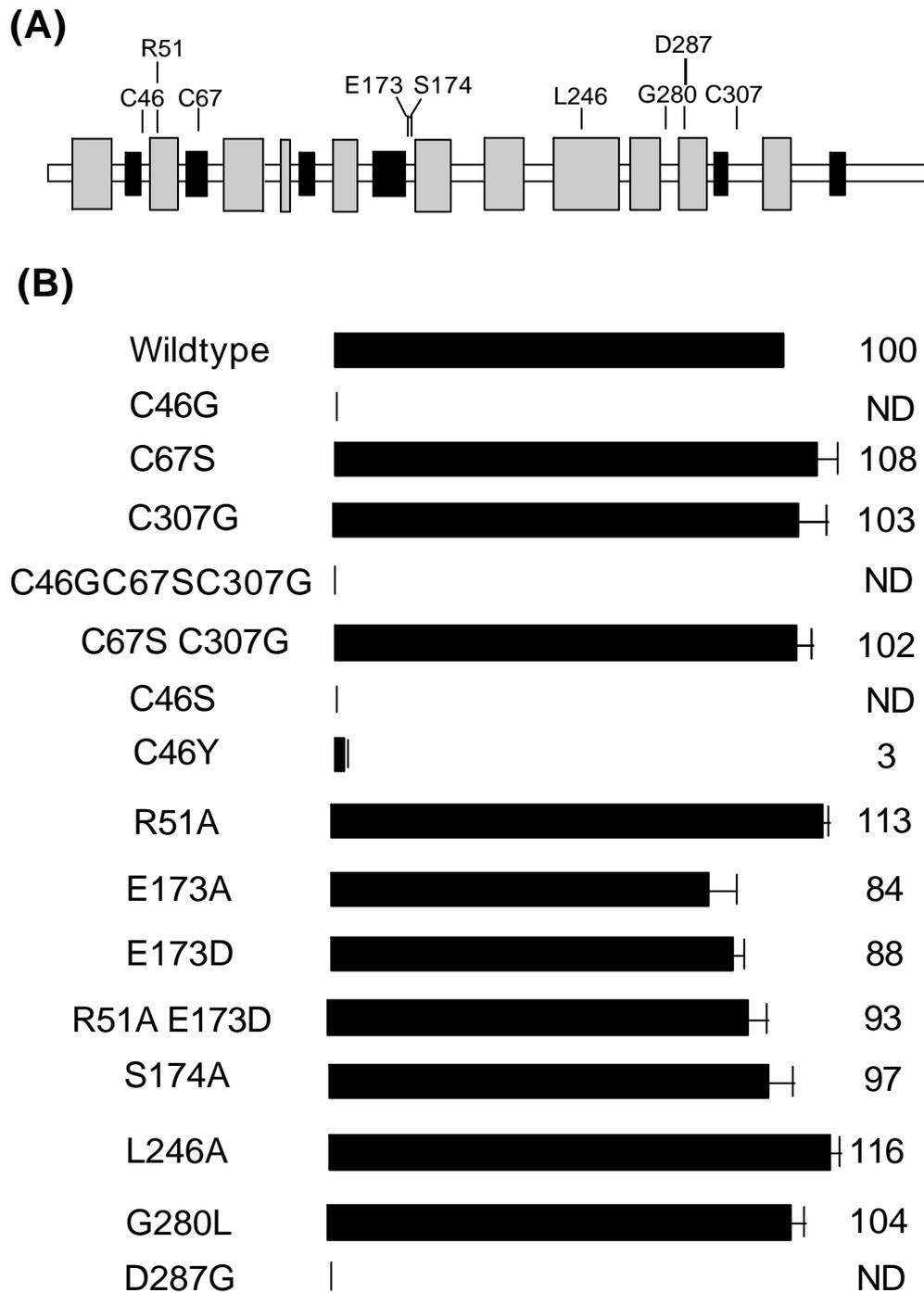


Figure 6

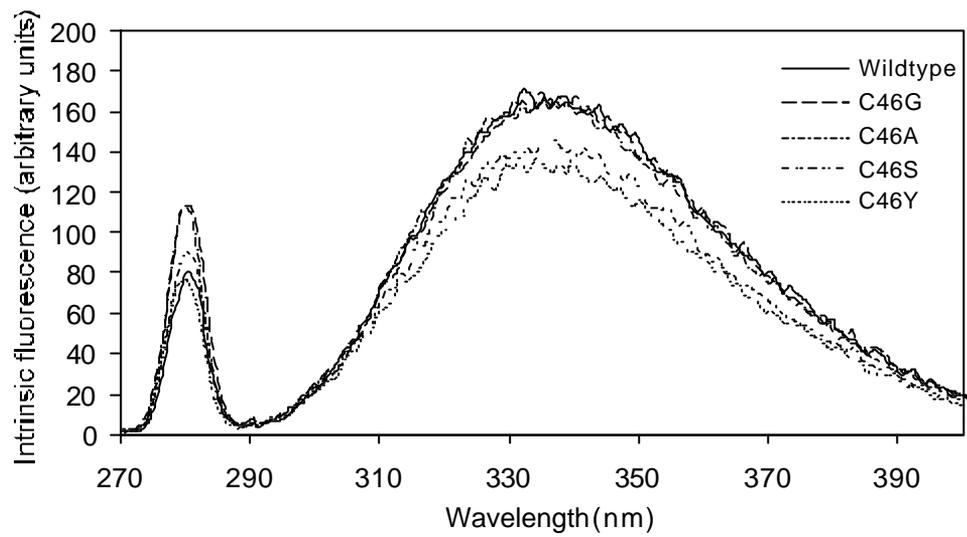


Figure 7