The Yeast Glutaredoxins Are Active as Glutathione Peroxidases*

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The yeast *Saccharomyces cerevisiae* contains two glutaredoxins, encoded by *GRX1* and *GRX2*, which are active as glutathione-dependent oxidoreductases. Our studies show that changes in the levels of glutaredoxins affect the resistance of yeast cells to oxidative stress induced by hydroperoxides. Elevating the gene dosage of *GRX1* or *GRX2* increases resistance to hydroperoxides including hydrogen peroxide, tert-butyl hydroperoxide, and cumene hydroperoxide. The glutaredoxin-mediated resistance to hydroperoxides is dependent on the presence of an intact glutathione system, but does not require the activity of phospholipid hydroperoxide glutathione peroxidases (*GPX1–3*). Rather, the mechanism appears to be mediated via glutathione conjugation and removal from the cell because it is absent in strains lacking glutathione-S-transferases (*GTT1, GTT2*) or the GS-X pump (*YCF1*). We show that the yeast glutaredoxins can directly reduce hydroperoxides in a catalytic manner, using reducing power provided by NADPH, GSH, and glutathione reductase. With cumene hydroperoxide, high pressure liquid chromatography analysis confirmed the formation of the corresponding cumyl alcohol. We propose a model in which the glutathione peroxidase activity of glutaredoxins converts hydroperoxides to their corresponding alcohols; these can then be conjugated to GSH by glutathione-S-transferases and transported into the vacuole by Ycf1.

All aerobic organisms are exposed to reactive oxygen species (ROS), such as *H₂O₂*, the superoxide anion, and the hydroxyl radical during the course of normal aerobic metabolism or following exposure to radical-generating compounds. These ROS can cause wide-ranging damage to cells, and an oxidative stress is said to occur when the cellular survival mechanisms are unable to cope with the ROS or the damage they cause (1). Oxidative damage is associated with various diseases such as cancer, vascular, and neurodegenerative disorders, as well as with aging processes (2–4). To protect against damage, cells contain a number of defense mechanisms including enzymes, such as catalase, superoxide dismutase, glutathione peroxidase, and low molecular weight antioxidants such as glutathione (GSH) and vitamins C and E (5, 6). Recent studies have highlighted the key role played by sulfhydryl groups (–SH) in the response to oxidative stress, and in particular, the roles of the GSH/glutaredoxin and thioredoxin systems, which maintain the redox homeostasis of the cell (7–10). In this present study, we examine the role of yeast glutaredoxins in protection against hydroperoxides.

Glutaredoxins are small heat-stable oxidoreductases, first discovered in *Escherichia coli* as GSH-dependent hydrogen donors for ribonucleotide reductase (11). They form part of the glutaredoxin system, comprising NADPH, GSH, and glutathione reductase, which transfers electrons from NADPH to glutaredoxins via GSH (12). The yeast *Saccharomyces cerevisiae* contains two glutaredoxins, designated Grx1 and Grx2, which share 40–52% identity and 61–76% similarity with those from bacterial and mammalian species (13). In common with all classical glutaredoxins, the active sites of Grx1 and Grx2 contain two highly conserved redox-active cysteine residues. Strains deleted for both *GRX1* and *GRX2* are viable, but lack heat-stable oxidoreductase activity in experiments using β-hydroxyethylene disulfide as a model disulfide substrate. A new family of glutaredoxin-related proteins has been identified in yeast (*GRX3–5*) that is conserved throughout evolution from bacterial to mammalian species (14). These glutaredoxin-like proteins differ from classical glutaredoxins in that they contain a single cysteine residue at their putative active site. Thus, they would be unlikely to substitute for glutaredoxins or thioredoxins as oxidoreductases with substrates like ribonucleotide reductase, which require a dithiol mechanism (15). The triple mutant lacking all three isoforms (grx3–5) is inviable, and interestingly, a *grx2 grx5* mutant is also inviable indicating that there may be some overlapping function between the two classes of glutaredoxins.

Previous studies have shown that the yeast glutaredoxins are active as antioxidants and are required for protection against ROS. Mutants lacking the dithiol glutaredoxins are sensitive to oxidative stress conditions, and the grx1 mutant is hypersensitive to the superoxide anion (13). In contrast, both *grx1* and *grx2* mutants are sensitive to *H₂O₂*, with the *grx2* mutant showing slightly higher sensitivity compared with the *grx1* mutant. In addition, overexpression of *GRX1* or *GRX2* increased resistance to *H₂O₂*. Furthermore, the expression of *GRX1* and *GRX2* is up-regulated in response to various stress conditions, including exposure to oxidants, with both genes regulated by stress-responsive elements (STRE) (16). In this present study, we investigated the antioxidant activity of the yeast glutaredoxins against hydroperoxides and show that they...
have glutathione peroxidase activity. This is the first evidence of glutaredoxins protecting against the damaging effects of ROS by directly reducing hydroperoxides.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth Conditions**—The *S. cerevisiae* strains used in this study are described in Table I. Strains were grown in rich YEPD medium (2% w/v glucose, 2% w/v bactopeptone, 1% w/v yeast extract) or minimal synthetic-defined media (0.17% w/v yeast nitrogen base with amino acids, 5% w/v ammonium sulfate, 2% w/v glucose) supplemented with 2% w/v leucine, 1 mM isoleucine, 1 mM valine, 0.3 mM histidine, 0.4 mM tryptophan, 1 mM lysine, 0.15 mM adenine, 0.2 mM uracil. Media were solidified by the addition of 2% (w/v) agar.

**Sensitivity to Oxidants**—Dose-response curves to H$_2$O$_2$, tert-butyl hydroperoxide (t-BH) and cumene hydroperoxide (CIP) were made by growing cells to exponential phase ($A_{600}$ = 1) in synthetic-defined medium at 30 °C and treating with oxidants for 1 h. Aliquots of cells were diluted in fresh YEPD medium and plated in triplicate on YEPD plates to obtain viable counts after 3 days of growth.

**Plasmids**—Multi-copy plasmids containing *GRX1* and *GRX2* were constructed in the 2-μm yeast high-copy vector YEp24. For *GRX1*, a 1464-bp fragment was cut from the polylinker HI and I sites of YEp24. For *GRX2*, a 1448-bp fragment was cut from the polylinker region of pLa 13 using BamHI and SphI sites and cloned into the BamHI and SalI sites of YEp24. For GRX2, a 1448-bp fragment was cut from the polylinker region of pLa 13 using BamHI and SalI sites and cloned into the BamHI and SalI sites of YEp24.

**Protein Purification and Antibody Production**—Plasmid pBAD-YGRX1 contained a six-histidine residue-tagged version of *GRX1* and was a kind gift from Barry Rosen (17). GRX1 was purified via the His tag using Ni$_2$-charged Ni$_2$-NTA agarose columns as described previously (17). Purified fusion protein was used to generate rabbit anti-yeast glutaredoxin antibody (Biogenesis Ltd., Poole, UK). Recombinant PHGpx2 was purified as described previously (18).

**Western Blot Analysis**—Protein extracts were electrophoresed under reducing conditions on 15% SDS-PAGE mini-gels and electrobotted onto polyvinylidene difluoride membrane (Amersham Biosciences). Blots were incubated in anti-GPx1 antibody (1:1000 dilution). Bound antibody was visualized by chemiluminescence (ECL, Amersham Biosciences) following incubation of the blot in donkey anti-rabbit immunoglobulin-horseradish peroxidase conjugate (Amersham Biosciences).

**Determination of Peroxidase Activity**—The peroxidase activity of Grx1 with H$_2$O$_2$, t-BH, and CHP was measured in vitro with purified Grx1. The components of the reaction mixture were NADPH (0.4 mM), GSH (1 mM), Glr1 (6 μM), Grx1 (5 pmol), and varying concentrations of hydroperoxides in a reaction volume of 40 μl in 0.1 mM Tris HCl, pH 7.4. Reactions were started by the addition of Grx1 and followed by the decrease in absorbance at 340 nm due to the oxidation of NADPH using a microplate reader. In this assay, oxidized glutathione (GSSG) produced by the glutaredoxin catalyzed reduction of hydroperoxides is reduced by Glr1. Reactions are followed by the oxidation of NADPH, which is coupled to the reduction of GSSG to GSH by Glr1. PHGpx2 was assayed for glutathione peroxidase activity using 5 pmol of recombinant protein exactly as described for Grx1.

**HPLC Analysis of Cumene Hydroperoxide and Cumyl Alcohol**—The Reduction of CHP to cumyl alcohol by Grx1 was detected by HPLC analysis using a method described previously (19). The reaction was performed in 50 mM potassium phosphate buffer, pH 7.0, with 0.4 mM NADPH, 1 mM GSH, 6 μM Glr1, Grx1 (5 pmol), and 0.25 mM CHP in a reaction volume of 500 μl. Control reactions were run in parallel either omitting Grx1 or GSH. The reaction mixtures were incubated at room temperature for 1 h and terminated by the addition of 500 μl of HPLC-grade ethyl acetate and 0.2 g of NaCl, followed by vortex mixing and centrifugation. The upper phase containing ethyl acetate, cumene hydroperoxide, and cumyl alcohol was removed and stored on ice prior to analysis. Reaction mixtures were separated using a Dionex Summit$^{	ext{TM}}$ HPLC system with a Spherisorb octadecylsilane (2 μl of HPLC steel column (4.5 × 250 mm). Isocratic elution was performed using 35% acetonitrile in 5 mM KH$_2$PO$_4$, pH 7.0, at a flow rate of 1.5 ml/min. The separation was followed at 254 nm.

**RESULTS**

**Glutaredoxins Are Required for Resistance to Hydroperoxides**—To further investigate the role of glutaredoxins in protection against oxidative stress, we examined the ability of glutaredoxin overexpression to increase resistance to hydroperoxides (Fig. 1). Overexpression of *GRX1* and *GRX2* was achieved using multi-copy plasmids and was confirmed by means of Western blot analysis (Fig. 1D). Resistance to H$_2$O$_2$ (Fig. 1A), tert-butyl hydroperoxide (t-BH, Fig. 1B) and cumene hydroperoxide (CHP, Fig. 1C) was determined using dose-response curves following a 1 h exposure. Elevating the levels of Grx1 or Grx2 was found to increase the resistance of a wild-type strain to H$_2$O$_2$, and CHP compared with the vector alone control. Interestingly, overexpression of *GRX1* increased resistance to t-BH, whereas, overexpression of *GRX2* had little or no effect. Thus, changes in the levels of glutaredoxins were found to affect the resistance of yeast cells to oxidative stress induced by hydroperoxides. To determine the mechanism of this glutaredoxin-mediated hydroperoxide resistance, we next examined its dependence on the components of the glutathione system.

**Glutaredoxin-mediated Protection against Hydroperoxides Is Dependent on Glutathione but Not Mediated by Glutathione Peroxidases**—The effect of overexpressing *GRX1* and *GRX2* on resistance to hydroperoxides was examined in strains deleted for *GSFI* or *GLR1*. *GSFI* encodes γ-glutamylcysteine synthetase, the first enzyme in the GSH biosynthetic pathway (20), and *GLR1* encodes glutathione reductase, which recycles oxidized glutathione (GSSG) to its reduced form (GSH) (21). The *gsh1* and *glr1* mutants were sensitive to CHP compared with the wild-type strain, with the strain lacking GLR1 showing highest sensitivity (Fig. 2). Glutaredoxin-mediated resistance to hydroperoxides was dependent on the glutathione system because neither of the glutathione mutants with mc*GRX1* or mc*GRX2* showed increased resistance to CHP.

One key antioxidant function of GSH is as a cofactor for glutathione peroxidases. Yeast contains three phospholipid hydroperoxide glutathione peroxidases (GPX1–3), which are required for resistance to oxidative stress induced by H$_2$O$_2$ and t-BH (18, 22). We, therefore, tested whether these enzymes are required for the glutaredoxin-mediated resistance to hydroperoxides using a gpox1–3 triple mutant (Fig. 2). The strain deleted for *GPX1–3* did not show increased sensitivity to CHP compared with the wild-type strain. In addition, me*GRX1* and me*GRX2* led to a 1.7-fold increase in resistance to CHP. This was slightly reduced compared with the wild-type strain (2-fold increase in resistance), but these data indicate that glutathione peroxidases are not essential for the glutaredoxin-mediated resistance to hydroperoxides. We next examined the require-
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**Fig. 1.** Overexpression of glutaredoxins increases resistance to hydroperoxides. The wild-type strain containing YEp24, mcGRX1, or mcGRX2 was tested for oxidant sensitivity by growing strains to exponential phase in synthetic-defined medium and treating with 4 mM H$_2$O$_2$ (A), 15 mM t-BH (B), or 2 mM CHP (C). Cells were diluted and plated in triplicate on to YEPD medium to monitor cell viability after 60 min of exposure. Percentage survival is expressed relative to the untreated controls. Overexpression of GRX1 and GRX2 was confirmed by means of Western blot analysis using an anti-glutaredoxin antibody (D).

**Fig. 2.** Glutaredoxin-mediated protection against hydroperoxides is dependent on glutathione but is not mediated by glutathione peroxidases. Wild-type cells (CY4) and goh1, gmr1, and gpx1–3 mutants were transformed with YEp24, mcGRX1, or mcGRX2 and tested for sensitivity to 2 mM CHP as described for Fig. 1A.

**Fig. 3.** Glutaredoxin-mediated protection against hydroperoxides is dependent on Gtt1, Gtt2, and Ycf1. Wild-type cells (BY4742) and gtt1, gtt2, and ycf1 mutants were transformed with YEp24, mcGRX1, or mcGRX2 and tested for sensitivity to 2 mM CHP as described for Fig. 1A.

**Fig. 4.** Reduction of CHP catalyzed by Grx1. A, the peroxidase activity of Grx1 with CHP was measured in vitro with purified Grx1. The components of the complete reaction mixture were NADPH (0.4 mM), GSH (1 mM), Glr1 (6 μg/ml), and CHP (0.25 mM) in 0.1 mM Tris-HCl, pH 7.4. Reactions were started by the addition of 5 pmol of Grx1 and followed by the decrease in A$_{410}$ attributable to the oxidation of NADPH. Peroxidase activity was dependent on the presence of GSH, Glr1, and NADPH because minimal activity was detected in reactions where each component was omitted individually. B, the reduction of CHP was found to show strict linearity with Grx1 at concentrations ranging from 5–25 pmol.

*Glutaredoxin-mediated Protection against Hydroperoxides Is Dependent on Gtt1, Gtt2, and Ycf1—*Glutathione-S-transferases from mammalian systems can act as general hydroperoxidases catalyzing the breakdown of alkyl hydroperoxides to their corresponding alcohols (23). Two genes encoding functional glutathione-S-transferases, designated GTT1 and GTT2, have been identified in yeast (24). These enzymes form part of a xenobiotic detoxification pathway that catalyzes the formation of glutathione conjugates, which are removed to the vacuole via an ATP-dependent GS-X pump, encoded by YCF1 (25).

The role of this system in hydroperoxide protection was tested by overexpressing GRX1 and GRX2 in strains deleted for GTT1, GTT2, or YCF1 (Fig. 3). Loss of GTT1 or GTT2 did not affect the basal level of resistance to CHP. However, the increased resistance to CHP mediated by GRX1 and GRX2 was completely abrogated in the gtt1 and gtt2 mutants. The ycf1 mutant was slightly more sensitive to CHP compared with the wild-type strain. Interestingly, overexpression of GRX1 or GRX2 in the ycf1 mutant resulted in increased sensitivity to CHP compared with the vector alone control. This indicates that glutaredoxins may convert CHP to a product that is toxic in the absence of YCF1 (see “Discussion”). Overexpression of GRX1 and GRX2 in the gtt1, gtt2, and ycf1 mutants was confirmed by Western blot analysis (data not shown). The requirement for GTT1, GTT2, and YCF1 indicates that glutaredoxin-mediated detoxification may involve the conjugation of hydroperoxides to GSH prior to their removal from the cell. However, given that Gtt1 does not contain a cysteine residue (24), it is unlikely that oxidoreductases such as Grx1 or Grx2 can directly affect the activity of the glutathione-S-transferases. We therefore tested whether the yeast glutaredoxins could directly reduce hydroperoxides prior to their removal by the GSH conjugation system.

*Glutaredoxins Have Glutathione Peroxidase Activity—*The ability of glutaredoxins to reduce hydroperoxides was tested in vitro using purified Grx1. Reaction mixtures contained GSH,
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Glutaredoxins (Grx) have been shown to play a role in protecting cells against the damaging effects of hydroperoxides, including H$_2$O$_2$, t-BH, and CHP. In this study, we investigated the peroxidase activity of Grx1 and compared it with the yeast GRX2 isoforms.

The peroxidase activity of Grx1 was measured using varying concentrations of H$_2$O$_2$, t-BH, and CHP, and the reaction velocities were determined. Grx1 showed the greatest activity with H$_2$O$_2$ ($V_{max} = 110.2$ mol/min/mg), compared with t-BH ($V_{max} = 7.5$ mol/min/mg) and CHP ($V_{max} = 32.5$ mol/min/mg). To address the question of whether Grx1 is a good peroxidase enzyme, the activity of Grx1 was compared with the yeast PHGpx2 enzyme. PHGpx2 was able to reduce all three hydroperoxides and, similarly, showed the greatest activity with H$_2$O$_2$. The in vitro peroxidase activity of Grx1 is comparable with that of the PHGpx2 glutathione peroxidase enzyme.

### DISCUSSION

Oxidative damage poses a very great risk to the survival of cells because it can undermine cellular structures including DNA, proteins, and lipids. In this present study, we have extended our original observation that glutaredoxins are required for protection against oxidative stress. In particular, glutaredoxins were found to play an important role in protecting cells against the damaging effects of hydroperoxides including H$_2$O$_2$, t-BH, and CHP. In addition, Grx1 and Grx2 may have some different functions, at least for the case of t-BH, since overexpression of GRX1 increased resistance to t-BH, whereas overexpression of GRX2 had no effect. Similarly, a strain lacking GRX1 is more sensitive to t-BH than a strain lacking GRX2 (data not shown) indicating that Grx1 may be more important than Grx2 for protection against this oxidant. Previous work has also indicated that the two glutaredoxin isoforms in yeast may play distinct roles.

Glutaredoxins from higher eukaryotes have been shown to act as antioxidants both in the regulation of other antioxidant enzymes and in a more general way as oxidoreductases. For example, in vitro studies have shown that both the glutaredoxin and thioredoxin systems can serve as electron donors for human plasma (selenium-dependent) glutathione peroxidase. Mammalian glutaredoxins can catalyze the GSH-dependent regeneration of ascorbic acid from its oxidized dehydroascorbate form. However, it is unknown whether the yeast glutaredoxins can function in a similar manner, and only the five-carbon analogue, erythrosecorbic acid, has been detected in yeast.

Mammalian glutaredoxins have also been shown to catalyze
the cleavage of mixed disulfides in vitro, which may serve to protect cells by reducing any mixed disulfides formed during exposure to oxidative stress conditions (36). In addition, a correlation between protein-SSG reduction and glutaredoxin activity has been demonstrated in mammalian cells (37), and the reversible S-glutathiolation of HIV-1 protease can be catalyzed by a glutaredoxin in vitro (38). Similarly, the yeast glutaredoxins GxR1 and GxR2 can reduce the model disulfide substrate, β-hydroxyethylene disulfide in vitro. However, they do not affect the levels of protein-GSH mixed disulfides formed in vivo in response to \( \text{H}_2\text{O}_2 \) (13), and recent studies have shown that the yeast thioredoxins regulate the levels of mixed disulfide formation (39).

Glutathione-S-transferases protect cells against the toxic effects of various xenobiotics. In higher eukaryotes, glutathione-S-transferases are known to detoxify lipid hydroperoxides to their corresponding alcohols and water (23). In addition, rat hepatic glutathione-S-transferase has been shown to catalyze the metabolism of ethanol to fatty acid ethyl esters (40). Two genes encoding functional glutathione-S-transferases, designated \( \text{GTT1} \) and \( \text{GTT2} \), have been identified in yeast (24).

The finding that glutaredoxins have peroxidase activity is unexpected and adds to the growing list of antioxidants that can detoxify hydroperoxides. In yeast, these include catalasases (\( \text{CTT1}, \text{CTA1} \)), phospholipid hydroperoxide glutathione peroxidases (\( \text{GPX1}–3 \)), and thiol peroxidases (five isoenzymes) (reviewed in Refs. 6, 43). Interestingly, a glutaredoxin/peroxide-dioxidin homologue has recently been identified in \( \text{Chromatium graci} \), which has similarity to both the glutaredoxin and peroxide-dioxidin families of antioxidants (44). This enzyme can reduce hydroperoxides including \( \text{H}_2\text{O}_2 \) and alkyl hydroperoxides using reducing power from the glutathione derivative, glutathione amide. Thus, the hydroperoxidase activity of the yeast glutaredoxins, identified in this present study, may represent an evolutionarily conserved activity. It remains to be established whether the glutaredoxins derived from higher eukaryotes similarly possess glutathione-dependent peroxidase activity.

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