Cytoplasmic glutathione redox status determines survival upon exposure to the thiol-oxidant 4,4′-dipyridyl disulfide

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Abstract

Dipyridyl disulfide (DPS) is a highly reactive thiol oxidant that functions as electron acceptor in thiol–disulfide exchange reactions. DPS is very toxic to yeasts, impairing growth at low micromolar concentrations. The genes *TRX2* (thioredoxin), *SOD1* (superoxide dismutase), *GSH1* (γ-glutamyl-cysteine synthetase) and, particularly, *GLR1* (glutathione reductase) are required for survival on DPS. DPS is uniquely thiol-specific, and we found that the cellular mechanisms for DPS detoxification differ substantially from that of the commonly used thiol oxidant diamide. In contrast to this oxidant, the full antioxidant pools of glutathione (GSH) and thioredoxin are required for resistance to DPS. We found that DPS-sensitive mutants display increases in the disulfide form of GSH (GSSG) during DPS exposure that roughly correlate with their more oxidizing GSH redox potential in the cytosol and their degree of DPS sensitivity. DPS seems to induce a specific disulfide stress, where an increase in the cytoplasmic/nuclear GSSG/GSH ratio results in putative DPS target(s) becoming sensitive to DPS.

Introduction

The reduced form of the peptide-like compound glutathione (GSH) is typically found in living cells in millimolar concentrations, whereas its oxidized (disulfide) form (GSSG) is present in much lower amounts. The reduced form is synthesized *de novo* by most organisms, whereas the disulfide form may arise from endogenous GSH-oxidation pathways (Jocelyn, 1970; Cuozzo & Kaiser, 1999). GSSG concentrations can increase during oxidative stress (Sies *et al.*, 1972). The ubiquitous enzyme glutathione reductase (GR; EC 1.6.4.2) uses the reducing equivalents from NADPH and has the capacity to reduce GSSG at very high rates (Conn & Vennesland, 1951). In the model prokaryote *Escherichia coli*, deleted for the gene encoding GR (*gorA*), a drop in the cellular GSH concentration is partially compensated by increased *de novo* GSH synthesis (Alonso-Moraga *et al.*, 1987). In eukaryotes, the situation is more complex, due to the compartmentalization of disulfide bond formation to the secretory pathway, i.e. away from oxidant-sensitive processes and activities. Indeed, GSSG/GSH ratios approximately 100 times higher in the secretory pathway than in the cytoplasm have been suggested (Hwang *et al.*, 1992). In the endoplasmic reticulum, GSH may have a role as reductant, as the most important yeast phenotypes that have so far been associated with GSH depletion are related to the biosynthesis of secretory proteins (Cuozzo & Kaiser, 1999). As a coreactant, GSH is also required for the activity of the glutaredoxins, which are small (around 10–30 kDa) and somewhat redundant redox-active enzymes responsible for a number of important cellular thiol–disulfide exchange reactions. Possible roles of glutaredoxin in reversible protein *S*-(de)glutathionylation with impacts on processes such as redox signal transduction (Daily *et al.*, 2001; Murata *et al.*, 2003; Song & Lee, 2003; Starke *et al.*, 2003; Caplan *et al.*, 2004) and secretory transport (Ivarsson *et al.*, 2005) in

eukaryotes have been suggested. However, these processes do not necessarily mimic physiologic redox regulation, as the commonly used oxidative sources, such as H_2O_2, rarely occur in nature at the high concentrations often used in the laboratory. In yeasts, the main cytosolic glutaredoxins, Grx1p and Grx2p, are dithiol glutaredoxins; that is, they contain a pair of conserved cysteines in their active sites (Holmgren, 1989). These glutaredoxins are necessary for resistance to oxidative stress (Luikenhuis *et al.*, 1998), which is underscored by their GSH-dependent peroxidase activity (Collinson *et al.*, 2002). GSH is synthesized in the cytoplasm of *Saccharomyces cerevisiae* in two steps catalyzed by γ-glutamyl-cysteine synthetase and glutathione synthetase, encoded by *GSH1* and *GSH2* respectively. The expression of these genes is increased under oxidative stress, but the *GSH1*-encoded activity is the rate-limiting one for GSH synthesis (Ohtake & Yabuuchi, 1991).

The theoretical ability of the GSH/GSSG couple to determine the redox states of the protein coreactants can be expressed thermodynamically by the redox potential (Gilbert, 1990). Use of a genetically encoded redox reporter (rxYFP, YFP-based redox sensor for the GSH/GSSG couple) revealed that a Δ*glr1* strain, which lacks GR and consequently contains a much larger intracellular amount of GSSG, only exhibits a minor decrease in the GSH redox potential in the cytosol (Østergaard *et al.*, 2004). Nonetheless, the *GLR1* gene product is needed for resistance to thiol oxidants such as H_2O_2 and diamide (Grant *et al.*, 1996a, b; Muller, 1996). GSH is dispensable for resistance to diamide (Grant *et al.*, 1997) but necessary for resistance to H_2O_2 (Grant *et al.*, 1996a, b). Thus, the sensitivity to diamide in the Δ*glr1* strain is probably caused by GSSG accumulation, whereas the sensitivity to H_2O_2 in this mutant could be caused by either GSH depletion or a more oxidizing GSH/GSSG buffer. Nevertheless, little is known about how different GSSG/GSH ratios are maintained in subcellular compartments. Interestingly, yeast and human cells display GR activities in the cytosol, the mitochondria and the nucleus that are all encoded by a single gene (Mbemba *et al.*, 1985; Outten & Culotta, 2004). In yeast, the minor presence of Glr1p in mitochondria (5–10% of the total Glr1p pool) depends on a 17-residue mitochondrial targeting signal and has been unequivocally associated with resistance to hyperoxia (100% O_2) (Outten *et al.*, 2005).

In studies on thiol redox biochemistry, disulfide-containing organosulfur compounds are particularly interesting because of their usefulness in thiol quantification. Among these compounds, the thiol oxidant 4,4′-dipyridyl disulfide (DPS, also known as 4,4′-dithiodipyridone) is particularly reactive, even at low pH (Brocklehurst & Little, 1972). DPS is a thiol-specific oxidant and reacts with thiol groups in a thiol–disulfide exchange reaction (Fig. 1). In addition, DPS is suitable for studies with intact cells, as it is a membrane-permeable compound (Brocklehurst, 1979). In several distinct mammalian proteins, cell types and lines, DPS has strong effects, such as ion release from membrane receptors (Haarmann *et al.*, 1999; Lang *et al.*, 2000), neuronal apoptosis (Aizenman *et al.*, 2000), and displacement of bound zinc from the Keap1 sensor (Dinkova-Kostova *et al.*, 2005). DPS is a good GSH oxidant, as shown by *in vitro* and *in vivo* studies (Pedersen & Jacobsen, 1980; Wu *et al.*, 1992). Surprisingly, there are no reports of the effects of DPS on microorganisms. In the present article, we describe the toxicity of DPS in the yeast *S. cerevisiae* as an approach to determine the role of the GSH redox potential and its regulation in eukaryotes.

In yeasts, most of the antioxidant genes are regulated by the transcription factor Yap1p, which is conserved among eukaryotes. Yap1p functions by directly or indirectly sensing diverse stresses by means of its reactive cysteine residues. Yap1p contains two cysteine-rich domains (CRDs), one at the N-terminus and one at the C-terminus (n-CRD and c-CRD, respectively), which are required for the nuclear localization of Yap1p observed during H_2O_2 stress (Kuge *et al.*, 2001). Previous studies have revealed that thiol-reactive compounds such as *N*-ethylmaleimide and diamide activate Yap1p by covalently modifying and/or by inducing disulfide bond formation in the c-CRD (Azevedo *et al.*,

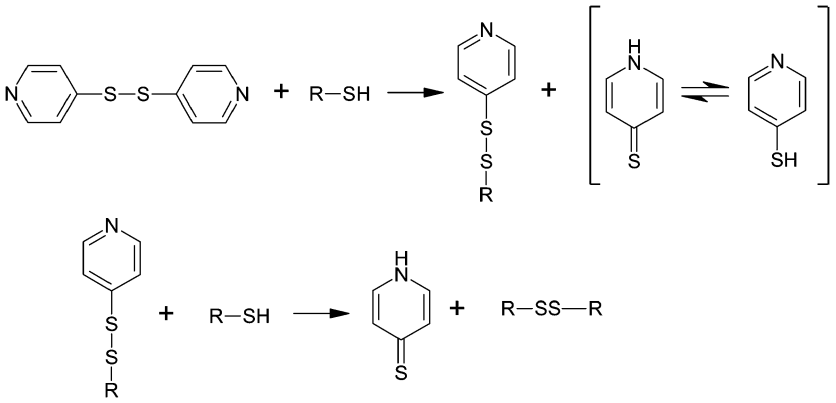

Fig. 1. General mechanism for the reaction between DPS and thiol groups.

2003). The peroxide receptor Orp1p is the primary sensor of H_2O_2, and transduces the signal by mediating disulfide bridge formation between N-terminal and C-terminal cysteines in Yap1p, as previously reviewed (Toledano *et al.*, 2004). We found that Yap1p and several Yap1p-regulated functions that are involved in the regulation of the GSH redox potential are required for tolerance to DPS. Mutants lacking Glr1p and/or Gsh1p stood out by their strong sensitivity to DPS. Therefore, changes in the cellular GSSG/GSH redox status caused by the exposure to DPS may determine the toxicity of this compound.

Materials and methods

Yeast strains and growth conditions

The *S. cerevisiae* strains used in this study are listed in Table 1. Strains were grown in synthetic complete (SC) medium, consisting of 0.67% yeast nitrogen base without amino acids (DIFCO), 2% glucose, and a mixture of succinic acid ($10\,g\,L^{-1}$) and sodium hydroxide ($6\,g\,L^{-1}$) as buffer (pH 5.5). The medium was supplemented with additional L-leucine ($150\,mg\,L^{-1}$) and adenine ($100\,mg\,L^{-1}$) and other nutrients as specified (Grauslund *et al.*, 1995). In some cases, this medium was enriched with 0.1% Casamino Acids (DIFCO) to yield the CASA medium. Whenever the presence of plasmid was required, selection was maintained by omission of the relevant nutrient according to the plasmid-borne selective marker. Complex medium (YPD) contained 1% yeast extract, 2% peptone and 2% glucose. Solid media containing agar, DPS and peroxide were added at the indicated concentrations before solidification but after cooling to 50 °C. A strain lacking both monothiolic glutaredoxins (BY4742 Δ*grx3* Δ*grx4*::*KanMX4*) was generated using a PCR-aided deleting cassette for multiple KanMX4-selectable deletions as previously described (Guldener *et al.*, 1996).

Plasmids

The plasmids utilized are listed in Table 2. Plasmid pMT102 was constructed starting from the tetracycline-regulation cassette from plasmid pCM183 (Garí *et al.*, 1997). A DNA fragment containing this cassette was obtained by digestion with NotI and KpnI, and ligated into pRS306 (Sikorski & Hieter, 1989) to yield plasmid pMT100. A fragment of the *GSH1* gene lacking 400 bp was PCR-amplified from genomic DNA using primers that introduce BglII and EagI sites at the ends. This truncated *GSH1* ORF was then treated with BglII

Table 1. Yeast strains used in this study

Strain	Genotype	Source
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Brachmann *et al.* (1998)
Y12737	BY4742 Δ*glr1*::*KanMX4*	Euroscarf
Y16913	BY4742 Δ*sod1*::*KanMX4*	Euroscarf
Y14839	BY4742 Δ*trx2*::*KanMX4*	Euroscarf
Y10569	BY4742 Δ*yap1*::*KanMX4*	Euroscarf
RM178	BY4742 Δ*gsh1*::pMT102 (*tetO$_7$-GSH1*)	This study
RM179	BY4742 Δ*glr1*::*KanMX4*Δ*gsh1*::pMT102 (*tetO$_7$-GSH1*)	This study
M5060	BY4742 Δ*grx1*::*HIS3*Δ*grx2*::*KanMX4*	Carlsberg laboratory
M5223	BY4742 Δ*glr1*::*URA3*Δ*grx1*::*HIS3*Δ*grx2*::*KanMX4*	Carlsberg laboratory
M5053	BY4742 Δ*trx1*::*KanMX4*Δ*trx2*::*HIS3*	Carlsberg laboratory
RM180	BY4742 Δ*grx3*Δ*grx4*::*KanMX4*	This study
Y252	*MATa ura3-52 lys2-801^{amber} ade2–101^{ochre} trp1-Δ1 leu2-Δ1*	Sikorski & Hieter (1989)
RM105	Y252 *leu2*::pRS305 (*LEU2*)	This study
Y252-yap1	Y252 Δ*yap1*::*TRP1*	Gift from Dr A. Delaunay
Y252-yap1/orp1	Y252 Δ*yap1*::*TRP1*Δ*orp1*::*KanMX4*	Gift from Dr A. Delaunay
ySOG1-1	Y252 Δ*gsh1*::*LEU2 PRO2–1*	Spector *et al.* (2001)

Table 2. Plasmids used in this study

Plasmid	Description	Source
pYAP1	Myc-Yap1 (*CEN URA3*)	Delaunay *et al.* (2000)
pYAP1-c-CRD	Myc-Yap1^{C598T,C620A,C629T} (*CEN URA3*)	Delaunay *et al.* (2000)
pYAP1-n-CRD	Myc-Yap1^{C303A,C310A,C315A} (*CEN URA3*)	Delaunay *et al.* (2000)
pYAP1-NES*	Myc-Yap1^{L619S,L623S} (*CEN URA3*)	Delaunay *et al.* (2000)
pHOJ150	rxYFP under *PGK1* control (*TRP1*, *LEU2*)	Østergaard *et al.* (2004)
pCO114	Glr1^{M17L} (*CEN LEU2*)	Outten & Culotta (2004)
pCO116	Glr1^{M1L} (*CEN LEU2*)	Outten & Culotta (2004)
pMT102	*GSH1* fused to *tetO$_7$* promoter (pRS306 backbone)	This study

and EagI, and inserted between the EagI and BamHI sites behind the tetracycline-regulation cassette of pMT100. Change in the control of chromosomal *GSH1* gene expression from the native promoter to tetracycline regulation was accomplished by transforming yeast with BamHI-digested pMT102. Proofreading polymerases were used in the PCR amplifications, and the DNA sequences of the inserts were checked.

Spot test for sensitivity to DPS

Yeast cultures were grown to exponential phase ($OD_{600\,nm}$ between 0.5 and 1) and subsequently reconcentrated and serially diluted to different cellular concentrations as indicated. Five microliters of each cell suspension was spotted onto agar plates containing various concentrations of DPS. Growth was registered 2 and 5 days after incubation at 30 °C. All phenotypes described in this work were confirmed by multiple tests.

Examination of rxYFP redox state by fluorescence

The redox state of the fluorescent protein reporter rxYFP was determined essentially as previously described (Østergaard *et al.*, 2004). Strains were grown in SC medium without leucine. The fluorescence intensity was monitored with a Perkin-Elmer Luminescence Spectrometer LS50B equipped with an XF3074 emission filter (Omega Optical Inc.) and thermostatic control for 30 °C.

Determination of GSH and GSSG levels in yeast samples

The intracellular levels of GSH and GSSG were determined by HPLC using derivatization with *N*-(1-pyrenyl)maleimide (NPM) as described elsewhere (Winters *et al.*, 1995) and optimized for yeast (Østergaard *et al.*, 2004). The samples consisted of 20 mL of SC-grown yeast cultures harvested at $OD_{600\,nm}$ of 0.4–0.5. NPM-derivatized thiols were determined by HPLC using a ReliaSil C18-AQ column (5 mm, 250 × 4.6 mm; Column Engineering Inc.) and fluorescence detection with excitation at 330 nm and emission at 375 nm.

Results

DPS is very toxic for yeast

On solid SC medium, DPS at concentrations of 10–16 μM provoked appreciable growth impairment in the wild type, whereas 4-thiopyridine had little or no effect at these concentrations. Concentrations of 4-thiopyridine in the order of 200 μM were needed to elicit any effect on growth (data not shown). Thus, a disulfide-related toxicity associated with thiol oxidation can reasonably explain the high toxicity of DPS.

Upon induction, the stress sensor Yap1p is concentrated in the nucleus, where it exerts its transcriptional function (Kuge *et al.*, 1997). Yap1p accumulates in the nucleus of DPS-treated wild-type cells to only a very limited degree, whereas in the Δ*glr1* mutant, DPS induced a stronger, faster and more persistent nuclear sequestration of Yap1p than in the wild type (data not shown). The fact that the nuclear location of Yap1p is induced by DPS does not necessarily mean that this localization increases resistance to DPS treatment. Therefore, we carried out a sensitivity test on solid media to assess the role of Yap1p and its cysteine residues in the cellular response to DPS. A strain lacking Yap1p appeared to be very sensitive to DPS (Fig. 2). The c-CRD seemed to be necessary for conferring resistance to DPS, whereas the three N-terminal cysteines in the n-CRD were clearly less important for this. Consistently, the

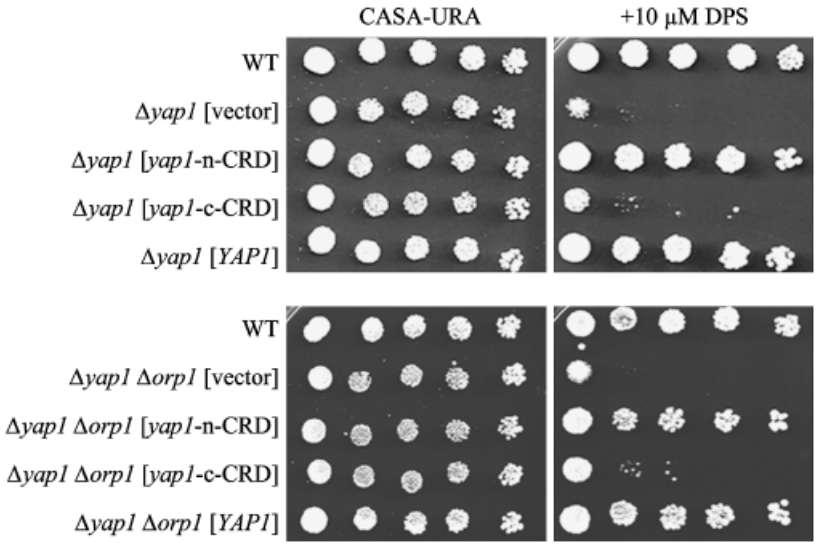

Fig. 2. Substitutions of Yap1p cysteines render yeast sensitive to DPS. The upper panel represents the test in a Δ*yap1* mutant, and the lower panel the test in a Δ*yap1* Δ*orp1* double mutant; the wild type is in both cases Y252. Strains were grown to exponential phase before harvest. Five microliters of cellular suspensions at 10^{-2}, 10^{-3}, 5×10^{-4}, 10^{-4} and 5×10^{-5} $OD_{600\,nm}$ units were spotted per strain. Plates were photographed after 2 days at 30 °C. Mutant *yap*1-n-CRD is the plasmid-encoded Yap1p lacking the N-terminal cysteine-rich domain (n-CRD), and *yap*1-c-CRD is a mutant lacking the C-terminal CRD (c-CRD) (see Table 2 for details).

peroxide sensor Orp1p was not required for cellular resistance to DPS in the wild-type *YAP1* (Fig. 2).

Yeast genes that are important for survival on DPS

We next wanted to know which of the many different antioxidant functions in the cell are important for resistance to DPS. We had noticed that the growth inhibitory effect of DPS has a narrow concentration window in the low micromolar range (5–16 μM in solid SC medium). In addition, cell suspensions must be diluted to no more than 5000 cells per cm^2 of testing surface to avoid a positive effect of crowding on survival. These effects reduce the technical possibilities for wide screenings for DPS-sensitive mutants, and we therefore performed a narrower test of mutants for the most important antioxidant pathways. About 40 mutants, mostly from the Euroscarf strain collection, were tested for their DPS phenotype (Table 3). Most of these mutants are impaired in either Yap1p-regulated redox functions, or in proteins required for Yap1p function. Figure 3a presents a selection of the most sensitive mutants, illustrating that specifically redox-impaired mutants are sensitive to DPS. Deletion of the genes encoding thioredoxin 2 (*TRX2*), cytosolic Cu, Zn-superoxide dismutase (*SOD1*) and GR (*GLR1*) strongly limits the ability of the yeast to grow on DPS-containing SC medium, whereas the double lack of cytosolic glutaredoxins (*GRX1* and *GRX2*) does so to a lesser extent.

The most apparent consequence of Yap1p activation is its concentration in the nucleus (Kuge *et al.*, 1997).

Table 3. DPS phenotypes of yeast mutants

Genotype*	Lacking or deficient function	Backgrounds	DPS phenotype†
Δ*glr1*	Glutathione reductase	BY4742, W303, Y252	Very sensitive
Δ*gsh1*‡	Glutathione	BY4742	Very sensitive
Δ*gsh1 PRO2–1*	Glutathione	Y252	Very sensitive
Δ*trx2*	Thioredoxin	BY4742	Sensitive
Δ*trx1* Δ*trx2*	Thioredoxins	BY4742, W303, Y252	Sensitive
Δ*trr1*	Thioredoxin reductase	W303 (hybrid)	Sensitive
Δ*sod1*	Cu, Zn-superoxide dismutase	BY4742	Sensitive
Δ*yap1*	Stress sensor and transcription factor	BY4742, W303, Y252	Sensitive
Δ*grx5*§	Mitochondrial glutaredoxin	BY4742	Sensitive
Δ*grx1*Δ*grx2*	Dithiolic glutaredoxins	BY4742	Moderately sensitive
Δ*lpd1*	Dihydrolipoamide dehydrogenase	BY4742	Moderately sensitive
Δ*grx3* Δ*grx4*	Monothiolic glutaredoxins	BY4742	Slightly sensitive
Δ*gsh2*	Glutathione (contains GSH analog GC)	BY4742	No phenotype
Δ*sod2*	Mn-superoxide dismutase	BY4742	No phenotype
Δ*trx1*	Thioredoxin	BY4742	No phenotype
Δ*tsa1*	Thiol peroxidase	BY4742	No phenotype
Δ*tsa2*	Thiol peroxidase	BY4742	No phenotype
Δ*ahp1*	Alkyl peroxidase	BY4742	No phenotype
Δ*ybp1*	Yap1p-binding protein	BY4742	No phenotype
Δ*gpx1*	Glutathione peroxidase	BY4742	No phenotype
Δ*gpx2*	Glutathione peroxidase	BY4742	No phenotype
Δ*gpx3/orp1*	Peroxide receptor	BY4742	No phenotype
Δ*grx3*	Monothiolic glutaredoxin	BY4742	No phenotype
Δ*grx4*	Monothiolic glutaredoxin	BY4742	No phenotype
Δ*ydl010w*	Putative glutaredoxin	BY4742	No phenotype
Δ*ybr014c*	Putative glutaredoxin	BY4742	No phenotype
Δ*gtt1*	Glutathione-*S*-transferase	BY4742	No phenotype
Δ*gtt2*	Glutathione-*S*-transferase	BY4742	No phenotype
Δ*cbf1*	Transcription factor for *MET* genes	BY4742	No phenotype
Δ*uth1*	Involved in mitochondrial biogenesis	BY4742	No phenotype
Δ*mca1*	Putative caspase	BY4742	No phenotype
Δ*skn7*	Stress transcription factor	BY4742	Slightly resistant

*The detailed genotypes and the respective sources of some of these strains are presented in Table 1. BY4742-derived strains were obtained from the Euroscarf collection.

†Exemplification of the qualitative scale for DPS sensitivities is presented in the following figures. No phenotype: wild type (Fig. 3a). Very sensitive: Δ*glr1* (Fig. 3a). Sensitive: Δ*trx2* (Fig. 3a). Moderately sensitive: Δ*grx1* Δ*grx2* (Fig. 3a). Slightly sensitive: Δ*gsh1*::*tetO*$_7$ − *GSH1* (Fig. 5a).

‡This strain is DPS-sensitive when grown at low GSH levels (< 5 μM).

§This strain grows very slowly in SC medium, which make its DPS sensitivity unreliable.

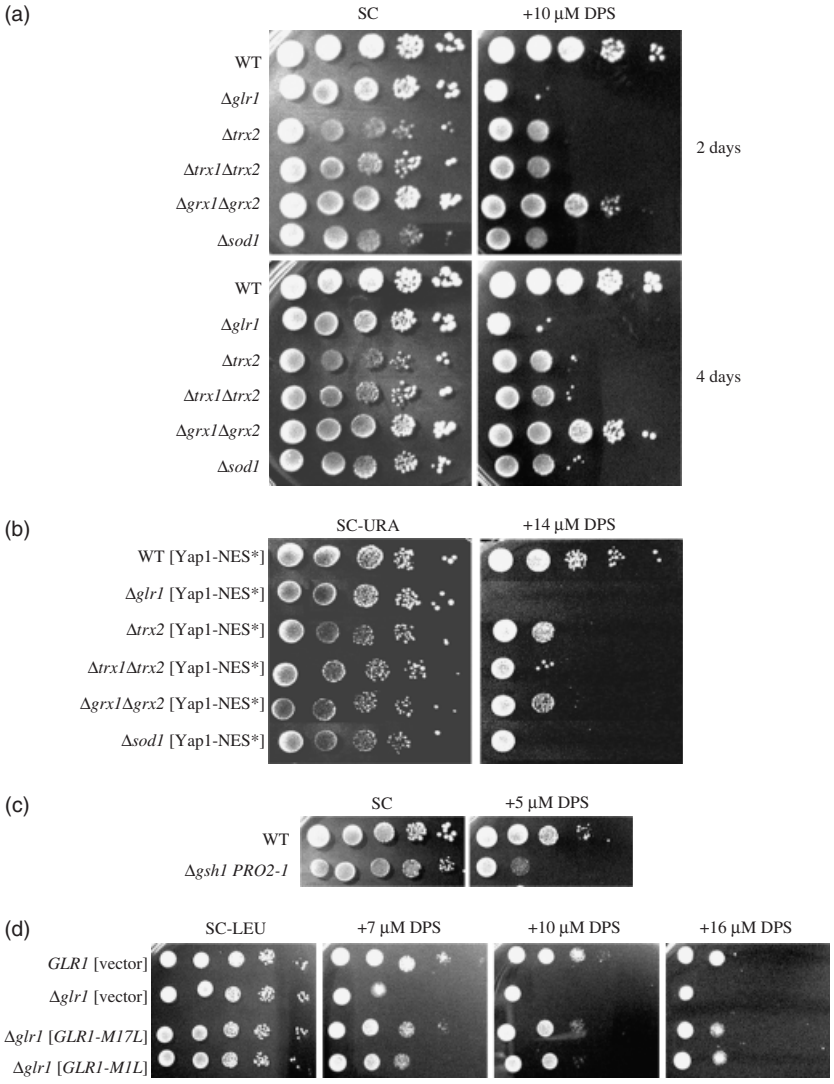

Fig. 3. Redox mutations render yeast sensitive to DPS. For each strain, 5 μL of cellular suspensions at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} $OD_{600\,nm}$ units were spotted. Unless otherwise indicated, plates were photographed after 2 days at 30 °C. (a) BY4742 derivatives. (b) BY4742 derivatives harboring pYap1-NES*. (c) The GSH-nonrequiring Δ*gsh1 PRO2-1* strain. The wild type is Y252 provided with a wild-type *LEU2* gene (strain RM105). (d) BY4742 Δ*glr1* containing the plasmids pCO114 (*GLR1*-M17L) and pCO116 (*GLR1*-M1L).

Interestingly, a C629T mutation in the c-CRD confers a constitutive nuclear localization and hyper-resistance to the thiol oxidant diamide (Wemmie *et al.*, 1997), and the current belief is that C629T affects the function of the neighboring leucine-rich nuclear export sequence (NES). Provided that the effect of the C629T mutation is on the NES, mutating the NES sequence may have a similar effect on resistance to thiol oxidation. In fact, we determined that both the *Yap1*-C629T and *Yap1*-L619S-L623S (*Yap1*-NES*) mutants are hyper-resistant to DPS (data not shown). By taking advantage of the dominant character, a plasmid-encoded *Yap1*-NES* mutation, it was confirmed that redox activities are genuinely required for resistance to DPS but apparently not for DPS sensing. The Δ*glr1*, Δ*trx2*, Δ*sod1* and Δ*grx1* Δ*grx2* mutations prevent the effect of the *Yap1*-NES* mutation in conferring DPS resistance (Fig. 3b). Trx2p, Sod1p and Grx1p/Grx2p are somewhat functionally redundant relative to Trx1p, Sod2p and Grx3p/Grx4p, respectively. These functions have elsewhere been found to have additive or different roles in resistance to peroxides and menadione (Rodriguez-Manzaneque *et al.*, 1999; Pereira *et al.*, 2001; Garrido & Grant, 2002). In the cellular detoxification of DPS, the limited redundancy is apparent despite the fact that some of the functional homologs are upregulated via Yap1p (Lee *et al.*, 1999; Garrido & Grant, 2002), which suggests that they might be upregulated in the *Yap1*-NES*-expressing cells. The differential roles of Sod1p and Trx2p are supported by the finding that even cells that might contain higher levels of the functional homologs are dependent on the presence of Sod1p and Trx2p for withstanding treatment with DPS (Fig. 3b). Furthermore, a mutant form of the mitochondrial Mn-superoxide dismutase (Δ*sod2*) is not sensitive to DPS (Table 3), which suggests that the DPS sensitivity of the Δ*sod1* mutant is not simply linked to

higher cellular superoxide levels but could be ascribed to superoxide accumulation in the cytosol. The double lack of Grx3p and Grx4p, and the deletion of any of the thioredoxin-dependent (Tsa1p, Tsa2p or Ahp1p) and GSH-dependent (Gpx1p, Gpx2p) thiol peroxidases, do not affect the DPS sensitivity of yeasts (Table 3). Thus, some GSH-or thioredoxin-dependent antioxidant functions are not important for resistance to DPS.

The Δ*glr1* mutant was significantly more sensitive than any other single mutant tested in this study. As the function of Glr1p is to reduce GSSG to GSH, we hypothesized that a mutant depleted in GSH would be DPS-sensitive. Such a mutant has been obtained by screening for a mutation allowing the rescue of the GSH auxotrophy of a Δ*gsh1* null mutant (Spector *et al.*, 2001). The Δ*gsh1 PRO2-1* mutant is indeed very sensitive to DPS (Fig. 3c). On the other hand, the slow-growing Δ*grx5* mutant resists DPS better than the isogenic Δ*glr1* mutant (Table 3). This suggests that DPS does not primarily affect the mitochondrial synthesis of iron–sulfur clusters, as Grx5p is involved in this crucial process (Rodriguez-Manzaneque *et al.*, 2002). To determine whether the DPS sensitivity observed for the Δ*glr1* mutant is related to a specific GR pool, we transformed this mutant with a plasmid encoding a GR mutant form (Glr1-M1L) lacking the mitochondrial targeting signal, and another plasmid mutant encoding a GR mutant form (Glr1-M17L) that accumulates mitochondrial Glr1p. Assay of the DPS sensitivity suggests that the mitochondrial Glr1p pool is dispensable for resistance to DPS, whereas presumably higher Glr1p levels in the mitochondria of the Δ*glr1* (Glr1-M17L) do not confer DPS resistance (Fig. 3d). Previous studies showed that the mitochondrial Glr1p pool is necessary for resistance to hyperoxia, which was associated with a more oxidizing redox status of GSH/GSSG in mitochondria (Outten *et al.*, 2005). Thus, as cells lacking mitochondrial GR do not have a DPS phenotype, mitochondrial GSH may have no role under DPS stress.

Relationship between the cellular GSH redox status and tolerance to DPS

The remarkable DPS sensitivities of the Δ*glr1* mutant and theΔ*gsh1 PRO2-1* mutant suggest that GSH may be a limiting factor in DPS-stressed cells. As the DPS-sensitive mutations (Δ*trx2*, Δ*glr1*, Δ*sod1*, Δ*grx1* Δ*grx2*) cause a lack of mostly cytoplasmic enzymes, we wished to determine whether the cytoplasmic redox potential of GSH is altered by the DPS-sensitive genotypes. To do so, we made use of the rxYFP reporter, a redox sensor for the GSH/GSSG couple (Østergaard *et al.*, 2004). As summarized in Table 4, the cytosolic GSH redox potentials of the DPS-sensitive mutants are affected to different degrees, roughly correlating with their sensitivity to DPS. However, the levels of oxidized rxYFP do not reflect the more oxidized whole-cell status of GSH in *glr1* and Δ*trx1* Δ*trx2* cells (Fig. 4); this has been previously rationalized by the existence of alternative mechanisms for controlling the cytosolic GSH redox status (Østergaard *et al.*, 2004).

Table 4. Levels of oxidized rxYFP reporter and relative DPS sensitivity of yeast strains

Genotype*	% rxYFP oxidized	DPS sensitivity (μM)†
BY4742	16 ± 1	12–16
BY4742 Δ*gsh1*::*tetO*$_7$-*GSH1*	21 ± 2	5–12
BY4742 (pYap1-NES*)	10 ± 2	< 16
BY4742 Δ*glr1*	61 ± 2	< 5–7
BY4742 Δ*glr1* Δ*gsh1*::*tetO*$_7$-*GSH1*	75 ± 2	< 5–5
BY4742 Δ*glr1* (pYap1-NES*)	54 ± 5	5–7
BY4742 Δ*sod1*	31 ± 1	7–10
BY4742 Δ*trx1*Δ*trx2*	65 ± 1	7–10

The percentage of oxidized rxYFP reflects the redox potential of GSH in the cytosol.

*All strains were made *TRP1*::pHOJ150 for rxYFP expression as previously described (Østergaard *et al.*, 2004).

†DPS-sensitivity range is defined as the concentration range where the spot corresponding to 10^{-3} $OD_{600\,nm}$ units displays sensitivity to DPS. The low-limit concentration reflects the beginning of the effect, and the high-limit concentration indicates disappearance from DPS-containing plates.

As the cellular GSSG/GSH ratio may be an indicator of the effects of DPS *in vivo*, we determined the cellular whole-cell GSH and GSSG levels in the DPS-sensitive mutants before and after a 1-h exposure to 25 μM DPS (Fig. 4). Here, we observed that: (1) the DPS-sensitive mutants were indeed more oxidized in the absence of DPS than was the wild type; (2) DPS increased the total GSSG levels and the GSSG/GSH ratios in the wild type and the mutants; and (3) in the Δ*glr1* mutant, the GSSG/GSH ratio increased much more than in the other strains tested. This increase in the GSSG/GSH ratio in the Δ*glr1* mutant reflects the fact that GSH levels decrease five-fold upon 25 μM DPS treatment, as GSSG levels in this mutant were similar under DPS-stressed and nonstressing conditions. DPS also had a modest effect on the GSH levels (1.3-fold increase) in the wild-type *GLR1*. By a coupled liquid chromatography and mass spectrometry approach (LC-MS), we found that DPS had effects on the levels of GSH precursors. For instance, treatment with DPS increased the levels of cysteine and γ-glutamyl-cysteine *c.* two-fold (data not shown). This is consistent with what we observed in relation to the Yap1p-mediated antioxidant response during exposure to DPS (see above).

DPS impairs cytosolic GSH redox homeostasis

To further study the relationship between DPS toxicity and cellular GSH, we substituted the wild-type *GSH1* promoter

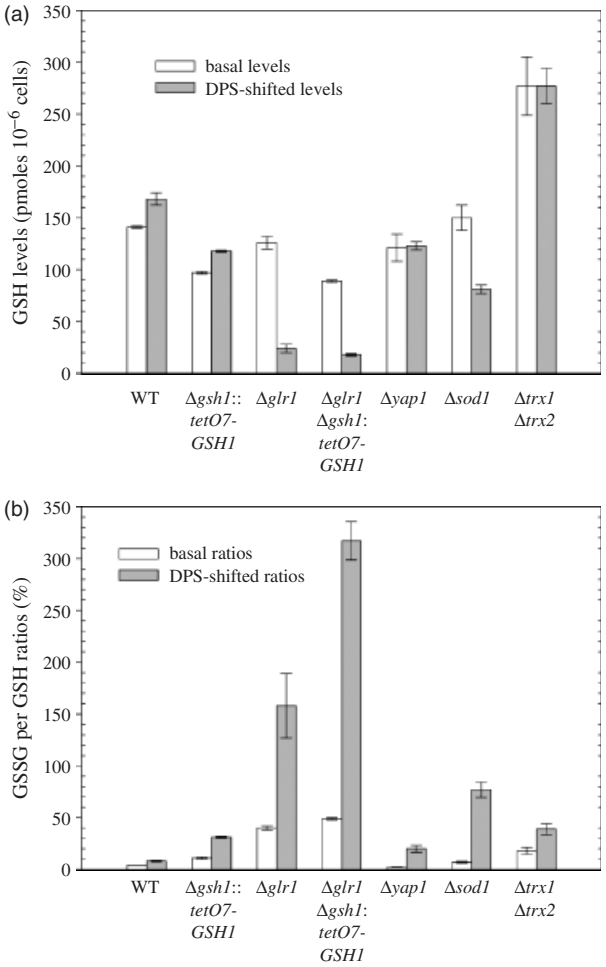

Fig. 4. The whole-cell redox state and levels of GSH are affected by DPS. Cells of the BY4742 background were subjected to a 1-h treatment with 25 μM DPS before harvest. Untreated controls for each strain were analyzed in parallel. White columns indicate measurements with the untreated samples, and gray columns indicate measurements with the DPS-treated samples. Values are the average of three independent experiments. (a) GSH levels decrease in DPS-treated Δ*glr1* and Δ*sod1* mutants. The whole-cell total levels of GSH were calculated as pmoles of GSH per 10^6 cells, considering the number of cells per mL and per $OD_{600\,nm}$ unit as the rough value of 1.5×10^7. (b) The GSSG/GSH ratios increase much more in the DPS-sensitive mutants during exposure to DPS than in the wild type. GSSG/GSH ratios were calculated as percentage of total GSSG levels in relation to total GSH levels.

with a tetracycline-repressible version. The levels of GSH provided by this cassette in the absence of the repressor doxycycline were slightly lower than the levels found in the *GSH1 GLR1* wild type (Fig. 4a). In the *GLR1* wild type, this substitution had no effect on the growth rate and only a minor effect on the cytosolic redox potential (Table 4). Likewise, a Δ*glr1* Δ*gsh1*::*tetO*$_7$ − *GSH1* mutant grew well, although it exhibited a slightly more oxidized cytosolic environment (75% oxidized rxYFP) in comparison to the Δ*glr1* strain. However, the Δ*glr1* Δ*gsh1*: *tetO*$_7$ − *GSH1* strain was significantly more DPS-sensitive than theΔ*glr1 GSH1* strain, whereas the Δ*gsh1*: *tetO*$_7$ − *GSH1* strain was only slightly more sensitive than the *GLR1 GSH1* wild type (Fig. 5a). As β-galactosidase reporter assays showed that *GSH1* was induced *c.* 1.5–2-fold under DPS exposure, and the *tetO*$_7$ promoter did not respond to the presence of DPS in the medium (data not shown), it is likely that the DPS sensitivity seen in the Δ*glr1* Δ*gsh1*: *tetO*$_7$ − *GSH1* strain relates to the absence of *GSH1* induction. Consistent with its exacerbated DPS sensitivity (more sensitive than Δ*glr1*), the Δ*glr1* Δ*gsh1*: *tetO*$_7$ − *GSH1* strain had two-fold more GSSG after DPS exposure than the Δ*glr1* mutant (Fig. 4b). Mitochondrial Glr1 activity was not required for DPS tolerance in a strain that could not induce *GSH1* expression upon DPS stress (data not shown). These results suggest that regulation of the GSH concentration during stress may be of minor importance for DPS tolerance in the *GLR1* wild type, although it is crucial in a strain that lacks cytosolic GR. DPS may affect the cytosolic GSH redox status in a Δ*glr1* mutant, but to a much lesser extent in a *GLR1* wild type.

DPS is a membrane-miscible compound that could exert its toxic effects in subcellular compartments without compromising the cytosolic GSH redox status. We looked for a possible effect of treatment with DPS on the cytoplasmic rxYFP redox reporter, and found that rxYFP became fully oxidized in the Δ*glr1* mutant while staying fairly reduced in the *GLR1* strain (Table 5). We conclude that GR is crucial for maintaining the reduced state of cytosolic GSH under DPS stress. In addition, as the *GLR1* wild type is able to maintain a fairly reduced GSH cytosolic pool, we can infer that the critical oxidized targets during DPS exposure are probably directly oxidized by DPS in the cytosol of the *GLR1* wild type. That glutaredoxins (Grx1, 2p) have no oxidative role in DPS stress is also suggested by the observation that the Δ*glr1* Δ*grx1* Δ*grx2* strain is as sensitive to DPS as the Δ*glr1* single mutant, whereas deleting both *GRX1* and *GRX2* restores wild-type resistance to H_2O_2 in the Δ*glr1* strain (Fig. 5b). Notably, this result recalls the previously documented rescue of the Δ*trx1* Δ*trx2* mutant for growth in the absence of methionine by deletion of either the *GRX1* or the *GRX2* gene (Draculic *et al.*, 2000). The presence of high GSSG levels in the Δ*glr1* mutant caused by H_2O_2 stress may have an effect on the redox state of the enzyme PAPS reductase that is similar to the effect of absence of the thioredoxins. The fact that deleting glutaredoxins does not relieve the DPS sensitivity of a Δ*glr1* mutant can be explained by assuming that the target of DPS is not a glutaredoxin substrate. Nonetheless, the GSSG/GSH ratio in the cytosol may be very high in the DPS-treated Δ*glr1* mutant, so glutaredoxin function may be impaired even if the DPS target is a glutaredoxin substrate. In fact, as shown above, GSH levels drop dramatically in a Δ*glr1* mutant, thereby limiting the reducing capacity of the glutaredoxins.

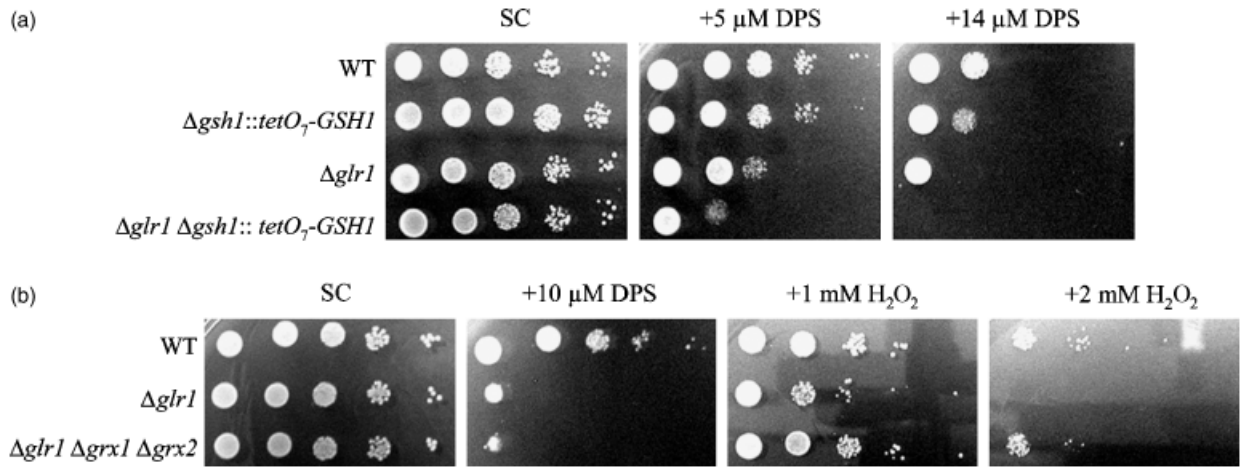

Fig. 5. Exchange of the *GSH1* promoter with a non-DPS-responsive promoter, unlike deletion of Grx1p and Grx2p, has a dramatic effect on DPS tolerance in a Δ*glr1* strain. Spots contain 5 μL of cellular suspensions at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} $OD_{600\,nm}$ units. All the strains are of the BY4742 background. Strains were at standard conditions (see Materials and methods) and plates were photographed after 2 days at 30 °C. (a) DPS phenotypes of strains expressing Gsh1p under the control of the $tetO_7$ promoter. (b) Effect of deleting Grx1p and Grx2p in the Δ*glr1* strain on the sensitivities to DPS and H_2O_2.

Table 5. Fraction of oxidized rxYFP sensor and total cellular GSSG/GSH ratio before and after a 1-h DPS treatment (25 μM)

	+DPS			
Genotype	% rxYFP oxidized		[GSSG]/[GSH]	
BY4742 (wild type)	16 ± 1	34 ± 3	0.04	0.08
BY4742 Δ*glr1*	61 ± 2	100	0.4	1.58

Discussion

Studying cellular redox homeostasis using yeast genetics and DPS

In this study, we describe the correlation between the cytosolic redox state of GSH and the ability of yeast cells to withstand nonphysiologic thiol-oxidation processes. Such processes may occur in all cells, regardless of the kind of habitat, but the number of oxidants found in nature is limited. Yeast is a unicellular eukaryote with a high and versatile metabolic activity. For these reasons, it provides a good example of an organism that has adapted through evolution to withstand and respond quickly to high concentrations of oxidative compounds. Several compounds that cause oxidative damage in yeast have been described, such as inorganic, organic and lipid peroxides, as extensively reviewed (Jamieson, 1998). However, for kinetic reasons, these compounds are not efficient thiol oxidants. On the other hand, the biological reactions involving thiol–disulfide redox conversions are usually reversible and, hence, dependent on the availability of both electron donors and acceptors. To place the particular effects of DPS in perspective, we have compared it with other well-studied thiol oxidants (Table 6). Interestingly, GR mutants are sensitive to DPS, peroxide, and diamide. However, the toxicity ranges for diamide and peroxide in yeast are *c.* 1–2 mM. Furthermore, these oxidants are not nearly as thiol-specific as DPS, as several side-reactions with nonthiol residues are expected (Kosower & Kosower, 1995; Jamieson, 1998). Resistance to DPS depends on Yap1p but is independent of Orp1p (Table 6). This suggests that, as for diamide, yeast senses DPS by a mechanism that differs from peroxide sensing. As Orp1p interacts covalently with Yap1p *in vivo*, it is conceivable that its absence would allow alternative mechanisms for Yap1p activation. As shown in Fig. 2, the effects of mutating cysteine triads in Yap1p are not dependent on Orp1p. Although DPS could in principle induce disulfide bond formation between C-terminal and N-terminal cysteines on Yap1p, our observations suggest that oxidation of the C-terminal cysteines is sufficient to confer protection to DPS.

Analysis of yeast redox-deficient mutants suggests that thioredoxin and GSH are both important in DPS detoxification and cellular protection. However, as thioredoxin-lacking mutants exhibit a more oxidized glutathione pool, and as GSH-lacking mutants contain a more oxidized thioredoxin pool (Trotter & Grant, 2003), it is difficult to distinguish the importance of these two redox systems in detoxification or protection against DPS. GSH is more abundant than the thioredoxins and is less costly for the yeast to synthesize, and its concentration is augmented in a strain that totally lacks thioredoxin activity (Fig. 4a). By analyzing the particular activities required for DPS tolerance, we found that some aspects of enzyme function could account for the phenotypes of their respective null mutants. The Δ*glr1* mutant is defective in maintenance of the reduced state of the GSH/GSSG buffer during DPS exposure (Fig. 4 and Table 5). The putative DPS targets are likely to be thiol-

Table 6. Comparative behavior of different thiol oxidants in yeast mutants

	Yeast mutant					
Oxidant	*Δglr1*	*Δtrx1 Δtrx2*	*Δgsh1*	*Δorp1*	*Δyap1*	-S–S- content
H_2O_2	S	SS	S	SS	SS	No
Diamide	SS	R	RR	wt	SS	No
DPS	SS	S	SS	wt	SS	Yes

The DPS data come from our results; the diamide and the H_2O_2 data have been described before (Delaunay, 2002; Lee *et al.*, 1999; Muller, 1996; Spector, 2001) and confirmed by us in the relevant strain backgrounds. RR, very resistant; R, resistant; wt, similar to the wild-type resistance; S, sensitive; SS, very sensitive.

containing proteins that would either be a substrate of the glutaredoxin–GSH system, or dependent on another redox system that might be sensitive to extreme redox changes in the GSH buffer. As indicated by the Glr1p-dependent drop in GSH levels upon DPS treatment, Glr1p is able to reduce GSSG very efficiently *in vivo* (Fig. 4a). It is puzzling that no significant increase in the GSSG level was found in the DPS-treated Δ*glr1* mutant. Previous studies showed that the drug thiram [bis(dimethylthiocarbamoyl)disulfide] partially inhibits yeast GR *in vivo* and alters the cellular GSH/GSSG ratio (Elskens & Penninckx, 1997). However, unlike the DPS effect in the Δ*glr1* mutant, thiram-treated cells accumulate GSSG. Likewise, a mutant strain that lacks GR activity still can accumulate very high GSSG levels. A possible explanation for this may be that DPS activates GSSG-dissimilatory processes. One might ask why the Δ*sod1* mutant is DPS-sensitive? The Δ*sod1* mutation may affect the GR activity, as recently suggested (Lushchak *et al.*, 2005), as the mutant fails to fully maintain a wild-type GSSG/GSH ratio and wild-type GSH levels during DPS treatment. The reason for these phenotypes could be partial inhibition of the Glr1 enzyme by inorganic thiol oxidants, as suggested by early enzymologic studies of GR showing its sensitivity to sulfhydryl reagents and heavy metals (Mize & Langdon, 1962; Chung & Hurlbert, 1975; Elskens & Penninckx, 1995). Also, toxicologic studies have shown that the products of lipid oxidation could inhibit GR (Remiao *et al.*, 1999). Furthermore, the yeast Δ*sod1* strain is slow-growing and displays hallmarks of oxidative stress (Chang *et al.*, 1991; Pereira *et al.*, 2001). These conditions could also aggravate the consequences of DPS exposure by affecting directly or indirectly the redox state of the putative DPS target(s).

There may be a common reason for the DPS sensitivities of the Δ*trx2*, Δ*trx1* Δ*trx2* and Δ*trr1* mutants (Table 3). Trx2p is a good candidate for the enzyme responsible for maintaining the reduced state of the putative DPS target(s). The two yeast thioredoxins are 78% identical, which raises the question of what gives Trx2p the highest apparent importance. Interestingly, a Δ*trx2* strain is resistant to diamide, whereas Δ*trx1* has no phenotype, and a thioredoxin reductase mutant (Δ*trr1*) is apparently sensitive to diamide (Muller, 1996). This differential behavior could be ascribed to a higher Trx2p contribution to the deactivation of Yap1p and not oxidation of Trx2p, as Trx2p is not oxidized during diamide exposure (Spector, 2001), and the cytosolic GSH redox status in the Δ*trx1* Δ*trx2* mutant is more oxidized than in the wild type (Table 4). Several results suggest that the glutaredoxin and thioredoxin systems have overlapping functions *in vivo*, a redundancy which is required for normal growth (Muller, 1996; Draculic *et al.*, 2000), and may be useful under certain oxidative stress conditions. This view is consistent with the fact that a strain lacking both Glr1p and Trr1p is nonviable (Trotter & Grant, 2003). However, upon exposure to DPS, the increase in GSSG/GSH in the Δ*glr1* mutant is so high that thioredoxins could be oxidized and impaired in performing their role in the DPS stress response. This redundancy hypothesis is also supported by the fact that the Δ*trx1* Δ*trx2* mutants accumulate GSH and GSSG under nonstressing conditions and exhibit a more oxidized environment in the cytosol. By contrast, a Yap1 constitutive mutant, which shows similar GSH/GSSG levels (data not shown) as the Δ*trx1* Δ*trx2* mutants, has a normal GSH redox potential in the cytosol (Table 4). Thus, thioredoxins seem to contribute to homeostasis of the cytosolic GSH redox potential, which has not previously been described. We conclude that the capacity to withstand treatment with DPS is related to the GSH redox status of the cell, a behavior that may be useful in physiologic studies in yeast.

On mechanisms of DPS toxicity

The yeast cytosol can maintain the reducing state of GSH even in cells where GR is absent, although the whole-cell amount of GSSG is augmented by this genotype (Østergaard *et al.*, 2004). The reason why the amount of GSSG in the cytoplasm is only modestly increased in Δ*glr1* cells might be that: (1) GSSG is reduced by another mechanism; (2) GSSG is actively exported from the cytosol; or (3) less GSSG is formed by the cells. The two last possibilities could well involve the existence of some kind of sensor that may signal the cytoplasmic GSSG concentration to the appropriate metabolic pathways or specific transport systems. Studies in *E. coli* have shown that the redox sensor OxyR is capable of sensing the increase in the GSSG/GSH ratio caused by the double *trxA*$^-$ *gorA*$^-$ mutation (Åslund *et al.*, 1999). In physiologic terms, detection of adverse GSSG levels would provide eukaryotic cells with the capability of detecting oxidative stress at different sensitivity levels and in a compartment different from that in which oxidation was initiated. In addition, this kind of mechanism would differentiate exogenous stress from endogenous thiol–disulfide

imbalance. DPS exposure represents an artificial condition under which yeasts are subjected to an abrupt increase in disulfide levels that, perhaps, cannot be fully compensated by the glutathione buffer. We suggest that the effects of DPS are mediated by an increase in GSSG in combination with specific oxidation of one or several target protein(s). The Δ*gsh1 PRO2-1* mutant is very sensitive to DPS, while showing increased resistance to diamide (Spector, 2001). This indicates that GSSG is involved in diamide stress, and that diamide and DPS have different targets, which is also suggested by the fact that some DPS-resistant mutants have no phenotype on diamide (our unpublished results). It is notable that the *GLR1* wild type is capable of maintaining a fairly reducing cytosol (Table 5) at a time when cell growth is has already been stopped by DPS treatment (data not shown). In addition, a strain that accumulates very large amounts of GSSG (46-fold increase compared to the wild type) is viable (Østergaard *et al*., 2004). We therefore favor the view that DPS toxicity in the *GLR1* wild type is not simply the consequence of an increasing amount of GSSG, but rather a result of DPS action on specific target(s) under conditions of an oxidizing GSH/GSSG redox status.

In terms of thiol–disulfide redox potentials, the cell can be subdivided into two main environments, the cytoplasm/nucleus and the secretory pathway. The thiol oxidant diamide reaches several cell compartments, as it is a membrane-miscible chemical (Kosower & Kosower, 1995), and exerts effects on disulfide bond formation of secretory proteins (Frand & Kaiser, 1998). *A priori*, DPS could have toxic effects in the cytoplasm, in the secretory pathway, or both. Our data suggest that the growth-inhibitory effect of DPS may be primarily in the cytosol, as lack of cytosolic proteins with functions in redox homeostasis provokes sensitivity to DPS. Furthermore, a Δ*gsh1* strain is resistant to the endoplasmic reticulum stressor diamide (Grant *et al*., 1997; Spector, 2001) but sensitive to DPS (Fig. 3c) and peroxides (Grant *et al*., 1996a, b). Accordingly, Δ*trx1* Δ*trx2* mutants are resistant to diamide (Muller, 1996), probably because they show constitutive Yap1p nuclear localization and increased GSH levels (Izawa *et al*., 1999). Thus, it is more probable that the DPS-sensitivity phenotype of the thioredoxin mutants is due to an intrinsic redox property of Trx2p rather than being an indirect consequence of its function in dealing with the GSH redox status in the secretory pathway. In conclusion, we propose that cytosolic DPS target(s) may be redox-regulated protein(s). We are currently screening for DPS-resistant yeast mutants, which may help us to understand DPS toxicity.

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