Review

Redox characteristics of the eukaryotic cytosol

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Abstract

The eukaryotic cytoplasm has long been regarded as a cellular compartment in which the reduced state of protein cysteines is largely favored. Under normal conditions, the cytosolic low-molecular weight redox buffer, comprising primarily of glutathione, is highly reducing and reactive oxygen species (ROS) and glutathionylated proteins are maintained at very low levels. In the present review, recent progress in the understanding of the cytosolic thiol–disulfide redox metabolism and novel analytical approaches to studying cytosolic redox properties are discussed. We will focus on the yeast model organism, *Saccharomyces cerevisiae*, where the combination of genetic and biochemical approaches has brought us furthest in understanding the mechanisms underlying cellular redox regulation. It has been shown in yeast that, in addition to the enzyme glutathione reductase, other mechanisms may exist for restricting the cytosolic glutathione redox potential to a relatively narrow interval. Several mutations in genes involved in cellular redox regulation cause ROS accumulation but only moderate decreases in the cytosolic glutathione reducing power. The redox regulation in the cytosol depends not only on multiple cytosolic factors but also on the redox homeostasis of other compartments like the secretory pathway and the mitochondria. Possibly, the cytosol is not just a reducing compartment surrounding organelles with high oxidative activity but also a milieu for regulation of the redox status of more than one compartment. Although much has been learned about redox homeostasis and oxidative stress response several important aspects of the redox regulation in the yeast cytosol are still unexplained.
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1. Main players in cytosolic redox regulation

In eukaryotic organisms, organelles and insoluble cytoskeletal structures are embedded in an aqueous phase, the cytosol. Redox reactions taking place in the cytosol are essential for the maintenance of the metabolic competence of the cell and the integrity of cellular components. Many of the redox-active enzymes and metabolites participating in the cytosolic redox reactions in eukaryotes have extensively been characterized in the unicellular fungus *Saccharomyces cerevisiae*. This yeast represents the most extensively studied model organism in the field of eukaryotic redox regulation. Most of the following discussion will focus on what we have learned from studying redox metabolism in this organism.

Oxygen-related chemical species, commonly termed reactive oxygen species (ROS), theoretically occur in all oxygen-con-

suming organisms. These include superoxide anion radicals ($^\bullet O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^\bullet OH^-$) [1]. ROS are highly reactive toward cellular components, and are likely to be disruptive to many cellular functions [2]. Glutathione (GSH), which is a tripeptide-like thiol-containing molecule (γ-GluCysGly), is present in large amounts in the cytosol and it is thought to be the main low-molecular weight reducing player. GSH plays pivotal roles in redox homeostasis through thiol–disulfide exchange reactions with cysteine-containing proteins, and also as an electron carrier for many enzymes involved in ROS reduction [3,4]. Under normal growth conditions (i.e. in the presence of oxygen, glucose, and normal growth temperature of 30 °C), steady state levels of ROS in the yeast cytoplasm are extremely low [5]. Under these conditions, the oxidized form of glutathione, glutathione disulfide (GSSG), is also found at very low levels compared to the approximately 3000-fold higher GSH levels [6]. Furthermore, cytosolic protein cysteines are rarely found in oxidized states, such as disulfide-bonded (RS-SR), sulfenic acid (R-SO$^-$) or sulfinic acid (RSOO$^-$) [7–9]. However, the cytoplasm is exposed to

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low levels of endogenous oxidizing compounds arising as byproducts of mitochondrial, peroxisomal, and endoplasmic reticulum-localized oxidative metabolisms. For instance, ROS produced in mitochondria may reach the yeast cytosol as the result of apoptotic and ageing processes [10,11]. There is limited knowledge about specific mechanisms regulating the release of oxidizing compounds (ROS and GSSG) from membrane-bound compartments to the cytosol [12,13]. On the other hand, it is clear that the cell must possess mechanisms for maintaining ROS and cysteine oxidation at very low levels in the cytosol.

Many redox enzymes necessary for removing ROS and products of oxidation from the cytosol have been described. These include: a) non-thiol based enzymes: catalase (Ctt1p[1]) and superoxide dismutases (e.g. Sod1p) and b) thiol-based enzymes: thioredoxin-dependent (Tsa1p, Tsa2p) or GSH-dependent (Gpx1p, Gpx2p) thiol peroxidases, and thioredoxin-dependent alkyl peroxidases (Ahp1p). These enzymes differ considerably in their catalytic mechanisms and in their substrate specificity. The main superoxide dismutase (SOD) in the yeast cytosol is Sod1p which is a copper-and zinc-containing enzyme. Like its mitochondrial counterpart, Sod2p (a manganese-containing SOD), it is responsible for the disproportionation of superoxide anion ($^{\cdot}O_2^-$) to H_2O_2 and oxygen [14]. In turn, two enzymatic activities are able to remove peroxides in cells, catalase and thiol peroxidases. These two activities differ substantially in their substrate specificity. Catalase localizes to both the cytosol (catalase T, Ctt1p) and peroxisomes (catalase A, Cta1p) and can only decompose H_2O_2, whereas thiol-dependent peroxidases can reduce both H_2O_2 and organic hydroperoxides by transferring electrons from GSH or from thioredoxin [15–18]. Moreover, upon peroxide-driven oxidation, reactive oxidized cysteines in peroxidases can mediate the oxidation of protein substrates like the Yap1p thus activating this important transcription factor [19,20]. Table 1 summarizes the gene names used in the present review and what enzymes they encode.

Cytosolic protein thiol oxidation may occur under normal or stress conditions and the cell also needs mechanisms for recovering such thiols. In yeast and in other eukaryotes, two main redox systems are involved in maintaining the reduced state of protein thiols: the thioredoxin (Trx) and the glutaredoxin (Grx) systems. Yeast possesses two Trx and two Grx enzymes in the cytosol [21–23]. Although both systems utilize NADPH as the final electron donor, a fundamental difference between the two systems is that while thioredoxins use reducing equivalents from NADPH through the thioredoxin reductase activity, Grx's are dependent on GSH [24]. This means that there is a direct enzymatic link between the reduction of disulfide bonds by thioredoxin and oxidation of NADPH whereas reducing equivalents in the glutaredoxin pathway must pass through the glutathione pool and that the redox status of this pool ultimately decides the efficiency and direction of the glutaredoxin path-

Table 1
Gene names used and their translation products

Gene name	Protein	Generic abbreviation
AHP1	Thioredoxin-dependent alkyl peroxidase 1	
CTA1	Catalase A	
CTT1	Catalase T	
ERO1	Endoplasmic reticulum oxidoreductase	
GLR1	Glutathione reductase	GR
GRX1	Glutaredoxin 1	
GRX2	Glutaredoxin 2	Grx
GSA1	Glutathione dependent thiol peroxidase 1	
GSA2	Glutathione dependent thiol peroxidase 2	
GSH1	γ-glutamyl-cysteine synthetases	
GSH2	Glutathione synthetases	
ORP1	Thiol peroxidase, peroxide receptor for Yap1p	
SOD1	Superoxide dismutase 1	SOD
SOD2	Superoxide dismutase 2	
TRR1	Thioredoxin reductase	
TRX1	Thioredoxin 1	
TRX2	Thioredoxin 2	Trx
TSA1	Thioredoxin-dependent thiol peroxidase 1	
TSA2	Thioredoxin-dependent thiol peroxidase 2	
SRX1	Sulfiredoxin	
YAP1	Yeast AP1 protein (transcription factor)	

way. Yeast cytosolic Trx and Grx are similar in many ways: they are small proteins (around 10–15 kDa) built over the same fold and their catalytic sites contain a Cys-X-X-Cys sequence motif [25–27]. Although some specific substrates for Trx and Grx have been identified, understanding why there are two separated classes of thiol/disulfide oxidoreductases in the same compartment is not trivial. The Δ*trx1* Δ*trx2* double mutant deleted for both thioredoxins[2] displays slow DNA replication [28], cell cycle defects [23,29], and impaired sulfate assimilation [28], but only mutants lacking both the Trx and Grx cytosolic isozymes are inviable [25]. Thus, Grx and Trx appear to be redundant to some extent for the reduction of critical substrates that have not yet been identified.

2. Role of glutathione in the regulation of the thiol–disulfide redox status

The GSH–GSSG redox pair is usually considered a biological redox buffer since glutathione is found in high concentrations, and since it can potentially influence the intracellular protein thiol redox state [8]. The yeast cytosol contains several enzymes that are specialized in reactions involving proteins and the GSH–GSSG redox couple.

Reduced glutathione (GSH) is converted to its oxidized form (GSSG) under normal growth conditions [30,31] and during

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[1] We have used the common yeast nomenclature to denote proteins derived from specific genes as the gene name followed by a "p". Thus, the enzyme Ctt1p is the catalase encoded by the gene *CTT1*).

[2] In the yeast nomenclature genes are indicated by a three-letter code followed by a numeral. Upper case letters indicate the dominant allele (usually the wild type gene), whereas lowercase letters indicate the recessive allele (usually the mutant form). Genes disrupted by targeted deletion are typically attributed with a "Δ", while point mutations are followed by a dash and a number, xxx1-1. Concatenated gene names without comma delineation denote combinations of genes in the same cell. See also Table 1 for gene names and corresponding proteins.

stress [32–36]. However, the GSSG formation during stress is far better characterized than the pathways leading to GSSG formation during normal growth conditions. Indeed very little is known about the GSH–GSSG redox flux under normal growth conditions, i.e. how rapid is GSH–GSSG interconversion.

Glutathione reductase (GR) is a key enzyme in the conversion of GSSG to GSH in both prokaryotes and eukaryotes [37–39]. The yeast GR is a FAD-bound homodimer that reduces GSSG to GSH at high rates in a NADPH-dependent fashion with a K_m for GSSG of 55 μM [40]. Its function is required for maintaining the normal GSH redox potentials in the cytosol [6] and in the mitochondria [41]. In the *S. cerevisiae* Δ*glr1* strain (i.e. a strain lacking GR), the whole-cell GSSG levels are dramatically elevated while GSH levels are similar to those of the *GLR1* wild type strain [6,42]. Thus, the synthesis of GSH in the cytoplasm may to some extent compensate for impaired GSSG reduction under these conditions. Studies in vitro showed that reduction of GSSG by the thioredoxin/thioredoxin-reductase systems from *Plasmodium falciparum*, *Drosophila melanogaster*, *Escherichia coli*, and humans is possible [43] although it is not known how relevant this pathway is in vivo. However, GR deficiency causes lethality in some organisms like *Schizosaccharomyces pombe* [44], and congenital diseases in humans [45,46]. These phenotypes might suggest a toxic effect of GSSG in vivo, since GSSG in principle can react with many thiol-containing proteins by thiol–disulfide exchange, thereby giving rise to protein disulfides (RS-SR) or mixed glutathione-protein disulfides (RS-GS) [8]. Surprisingly, although an *S. cerevisiae* Δ*glr1* strain accumulates large amounts of GSSG intracellularly [6,42] it grows indistinguishably from the *GLR1* wild type [47]. Furthermore, deleting either one of the two cytosolic thioredoxins in the Δ*glr1* mutant results in a modest increase in the overall cellular GSSG/GSH, while deletion of both thioredoxins is lethal in this background (i.e. Δ*glr1* Δ*trx1* Δ*trx2* triple mutant is inviable) even under anaerobic conditions [42]. The finding that the Δ*glr1* mutant only has a slightly more oxidizing cytosolic GSH redox potential than the wild type [6], in spite of the increased overall cellular levels of GSSG, suggest that other pathways than the GR-pathway are at play and that high GSSG levels may indeed be toxic in the yeast cytosol. However, this remains a possibility that is difficult to address experimentally, and alternative non-GR related mechanisms which might control the cytosolic GSSG levels remain unknown.

3. Cytosolic glutathione redox potential

Because of its abundance and the many enzymes catalyzing redox reactions between glutathione and protein, the GSH redox potential is an obvious choice as a main indicator of the cytosolic redox environment [8,48]. Since two molecules of GSH are involved in thiol–disulfide exchange mechanisms with cellular dithiol proteins, the GSH redox potential depends on both GSSG/GSH ratio and GSH concentration. While the overall cellular GSSG/GSH ratio can be determined by several methods, the actual intracellular GSSG/GSH ratio and concentration is difficult to estimate in vivo. The GSSG/GSH ratio in the cytosol had widely been considered as comparable or proportional to the

overall cellular GSSG/GSH ratio obtained by invasive methods. In general, the GSH–GSSG levels measured by these methods are normalized for the number of disrupted cells. However, the most commonly cited values for the cytosol redox status predict more reducing conditions compared to other cellular compartments [49]. Significant progress in the estimation of cytosolic GSH redox potential and the cytosolic GSH–GSSG concentrations has been made by the implementation of the redox sensor rxYFP [6,50,51]. rxYFP is a Green Fluorescent Protein (GFP)-based protein whose intrinsic fluorescence depends on the redox state of a pair of genetically engineered cysteines on the surface of the protein. When targeted to the yeast cytosol/nucleus, the rxYFP redox state specifically equilibrates with the redox potential of the intracellular GSH–GSSG buffer in a reaction catalyzed by endogenous glutaredoxin [6]. The rxYFP-based procedure proved to be suitable for estimating the GSH redox potential in the wild type yeast. It is very important to note that the redox potential measured for the GSH–GSSG couple using this method is much lower than previous estimates for the cytosol. In practical terms it means that at an estimated glutathione concentration of 13 mM in the yeast cytosol only ∼4 μM will be on the GSSG form. This gives a GSSG/GSH ratio of ∼1/3000, which is 1–2 orders of magnitude more reducing than other estimates [6,48]. This result also suggests that a significant compartmentalization of GSSG must take place in order to account for the GSSG/GSH ratio of around 2–3% found in whole-cell extracts [6].

Table 2 shows a summary of cytoplasmic redox parameters compared with sensitivity to thiol oxidants. Measuring the rxYFP redox states in different yeast strains allowed the estimation of the cytosolic GSH redox potentials. Yeast strains containing mutations in important redox enzymes like glutathione reductase (GR), thioredoxin (Trx), and superoxide dismutase (SOD) [6,34] are all affected in cytosolic glutathione redox potential. For the inspected strains, values for the GSH redox potential lay approximately within the interval −240 and −290 mV. K_{ox} values are perhaps more easily interpretable as they are inversely proportional to [GSSG] assuming constant [GSH]. Interestingly, mutants lacking important redox functions like Trx and SOD, which are impaired in growth, still maintain a highly reducing cytosol. It should be noted that the disulfide bridge in rxYFP is structurally stabilized, so this protein becomes oxidized even under conditions where the GSH redox potential is likely to maintain most cytosolic proteins in the reduced state [8].

Ero1p is included in Table 2 because it functions in transferring oxidizing equivalents for disulfide bond formation in the endoplasmic reticulum (ER) and at the same time gives rise to hydrogen peroxide [57]. We found that strains with either impaired or abnormally high Ero1p activity display increased GSH redox potentials in the cytosol.

A less reducing glutathione redox potential in the cytosol does not correlate with increased protein glutathionylation levels but is somewhat correlated with elevated ROS levels and sensitivity to thiol oxidants (discussed below). The GSH redox potential also changes with the growth phase in most strains (Lopez-Mirabal and Winther, unpublished result). The latter represents an interesting behavior suggesting a possible role for

Table 2
Yeast mutant phenotypes related to thiol/disulfide status in the cytoplasm

Genotype [a]	% rxYFP oxidized	E'$_{GSH}$ [mV]	K_{ox}	ROS	RS-SG levels	DIA	DPS
WT	16±1	−287	36	N	–	wt	wt
WT [Yap1-NES*] [b]	10±2	−294	60	N	?	RR	RR
Δglr1	61±2	−259	4	N	–	SS	SS
Δglr1 [2 μ ERO1] [c]	82±4	−245	1.5	Y [d]	?	SS	SS
Δtrx1 Δtrx2	65±1	−257	4	Y	+	R	S
Δsod1	31±1	−275	15	Y	?	S	S
Ero1-1	37±3	−272	12	Y	?	SS	S

The rxYFP redox state data have been published previously [34,52]. The cytosolic GSH redox potentials (at T=30 °C, assuming intracellular pH=7.0, and using E°'$_{GSH}$=−265 mV as the standard redox potential for GSH) were calculated using the Nernst equation as described previously [6]. K_{ox} represents a more intuitive picture of redox conditions as [GSH]^2/[GSSG] for the cytosolic glutathione buffer in the given strain. Intracellular ROS levels and RS-SG (protein glutathionylation levels) have been described elsewhere [52–54]. Sensitivity to diamide (DIA) and dipyridyl disulfide (DPS) have been described previously [34,42,52,55] or derive from our unpublished results. N (wild type ROS levels), Y (increased ROS levels).+ (increased PS-SG) levels over wild type), – (wild type PS-SG levels), ? (data not available). RR (very resistant), R (resistant), wt (wild type level of resistance), S (sensitive), SS (very sensitive).

[a] The data represented in this table correspond in some cases to different strains backgrounds. However, with the exception of the W303-hybrid ero1-1 mutant [34], DPS-sensitivity and rxYFP-related results were obtained in congenial strains (BY4742 background).

[b] The strain contains a plasmid-encoded Yap1p constitutive version (Myc-Yap1^{L619S,L623S}), which is described elsewhere [56].

[c] This strain carries the wild type ERO1 gene on a high-copy plasmid (2 μ) as indicated.

[d] In this strain the ROS distribution is asymmetrical, with some cells displaying high ROS levels and others seeming ROS-devoid. Possibly, this is due to uneven partition of the ERO1-overxpressing plasmid.

the GSH redox potential in the regulation of the cell cycle and/or growth phase-dependent metabolic switches.

4. Influence of ROS on the GSH redox potential: possible roles in redox signaling

There are many possible mechanisms by which Trx, SOD and Ero1p can affect the regulation of the GSH redox potential. It is, however, difficult to discriminate among these mechanisms experimentally. A central problem of the genetic approaches for addressing redox mechanisms is that many redox functions are partially redundant [14,25,42,59,60], but at the same time are not necessarily at equilibrium [51]. In addition, matters are complicated by the fact that mutants lacking redox functions typically display several phenotypes that are not causally related (discussed below) and the kinetic flux between systems may be altered when one or the other system is disrupted. Further research is needed to understand whether the phenotypes ascribed to redox mutations are specifically related to enzyme function, or whether they implicate other factors such as GSH redox potential and ROS levels.

Yap1p is a key transcription factor in redox regulation which functions as a sensor for oxidative stress-derived signals. In the response to H$_2$O$_2$, the specific peroxide-receptor Orp1p becomes oxidized to a reactive sulfenic acid. Orp1p in turn specifically induces disulfide-bridge formation between N-terminal and C-terminal cysteines in Yap1p. This results in nuclear localization of Yap1p where Yap1p is responsible for activation of numerous genes [19,56].

An example of how different players on the redox scene can interact in a highly complex fashion is the constitutive Yap1p activation seen in the Δtrx1 Δtrx2 [54] and the induction of several Yap1p-targets seen in the Δtrr1 mutant [61]. The Δtrr1 mutant accumulates both ROS and oxidized thioredoxins [34,62]. The ROS accumulation in the Δtrr1 mutant may relate to a decrease in the reducing power of thioredoxin to a level that may impair both the reduction of Yap1p and the thioredoxin-dependent thiol peroxidases (Tsa1p and Tsa2p). Thus, both ROS accumulation and deficient Yap1p-reduction contribute to the Yap1p constitutive activation. Consistently, both aerobic conditions and the presence of the peroxide receptor Orp1p are required for the Yap1p constitutive localization in the Δtrx1 Δtrx2 mutant [54,63]. However, increase in the cytosolic glutathione redox potential (like that seen in a Δglr1 mutant or a Δglr1 Δmet15 strain) does not activate Yap1p [34,54]. Thus, although Yap1p can be oxidized by H$_2$O$_2$ via Orp1p and reduced via Trx, it will not change its redox state in a GSH/GSSG-dependent manner. This is perhaps not so surprising since the glutathione redox potential even under these aberrant conditions is still very reducing (as discussed above). One may ask where these ROS arise from in the Δtrr1 and Δtrx1 Δtrx2 strains. Why can't the GSH system (comprising glutaredoxins and GSH-dependent peroxidases) supply the Trx-deficient strains with enough ROS-reducing equivalents?

Similarly to the Δtrx1 Δtrx2 mutant, the YAP1-NES* mutant, which displays constitutive Yap1p nuclear localization, leads to increases in the whole-cell glutathione levels resulting in GSSG/GSH ratios (around 15–20%) which are higher than that of the wild type strain (4%) [34,60]. Increased GSH synthesis probably results from constitutive Yap1p nuclear localization [64], and it can be hypothesized that a partial oxidation of this GSH surplus may account for the elevated GSSG levels found in the YAP1-NES* mutant. Interestingly, the YAP1-NES* mutant shows a cytosolic GSH redox potential that is slightly lower than that of the wild type strain (Table 2) in spite of its higher whole-cell GSSG/GSH ratio. Thus, similar to the result in the Δglr1 mutant (discussed above), the glutathione redox potential in the cytosol of the YAP1-NES* mutant does not reflect the overall more oxidizing glutathione redox status found in this mutant. From these results it can be inferred that the higher cytosolic glutathione redox potential found in the Δtrx1 Δtrx2 mutant is not related to constitutive Yap1p accumulation in the nucleus but specifically related to the lack of Trx function. The Δtrx1 Δtrx2 mutant displays profound cell cycle, metabolic, and morphological defects [23,28,29,65]. Therefore, it is likely that the lack of cytosolic thioredoxins may indirectly affect the mechanisms necessary for ROS-scavenging and/or redox regulation by the GSH system. Interestingly, mammalian Trx can reduce GSSG [43] and the loss of the mammalian Trx-inhibitor Txnip leads to a decreased GSSG/GSH ratio [66]. The

very low concentrations of GSSG combined with a K_m of 55 μM of Glr1p for GSSG, suggests that a significant flux of GSSG to GSH through the Trx pathway is, however, not likely. We therefore favor the view that yeast Trx is not directly involved in GSSG reduction in the cytosol.

Several mutations affecting the cytosolic GSH redox potential (e.g. Δsod1, Δtrx1 Δtrx2, or ero1-1) also lead to ROS accumulation (Table 2) and glutathione reductase (GR) has been shown to be inhibited by products of lipid oxidation [67]. Thus, cytosolic ROS accumulation in the redox mutants (Δsod1, Δtrx1 Δtrx2, Δtrr1, or ero1-1) may result in an increase in the GSSG levels due to a greater flow of ROS-related oxidative equivalents through GSH-dependent peroxidases and glutaredoxins, and/or by partial inhibition of the GR activity.

As discussed above, the cytosol of the Δglr1 mutant maintains a very reducing cytosol [6] even though the whole-cell GSSG levels increase dramatically in this mutant [6,34,42,68]. This result suggests that additional non-GR related mechanisms controlling the GSH–GSSG concentrations in the cytosol may exist (Fig. 1). These could either be cytosolic GSSG reductive mechanisms not involving GR, or GSSG dissimilation by extrusion to other compartment(s). GSSG degradation would still require GSSG reduction by non-GR reductive systems. In addition, the Δglr1 mutant is very sensitive to thiol oxidants such as H_2O_2, N,N,N',N'–tetramethyl-azodicarboxamide, $((CH_3)_2 NC(O)N=NC(O)N(CH_3)_2$; commonly known as diamide), and dipyridyl disulfide (DPS) [34,42,68]. These results emphasize the key role of GR under conditions of non-physiological GSH oxidation.

Could the inhibition of GR by ROS and ROS-derived compounds have a beneficial role under conditions where GSSG levels are elevated? It is possible that the cytosolic GSH potential affects the oxidative folding process localized in the endoplasmic reticulum since the Δglr1 mutation rescues the temperature-sensitivity of the ero1-1 mutant (Δglr1 Δmet15 ero1-1 mutant grown in GSSG as the sole sulfur source) [58]. Furthermore, manipulating the activity of Ero1p alters the GSH redox potential in the cytosol [52]. Overexpression of ERO1 makes the GSH–GSSG buffer less reducing in the cytosol of a GR-lacking mutant, at the same time the Ero1p-deficient and temperature sensitive ero1-1 mutant also has a less reducing cytosolic GSH potential (Table 2). The ero1-1 mutant also accumulates ROS in the cytosol under semi-permissive conditions (30 °C) [52]. The increase in ROS levels could well account for the less reducing GSH redox potential found in mutants lacking non-cytosolic functions with no direct role in thiol/disulfide homeostasis in the cytosol. Reduction of molecular oxygen by Ero1p generates stoichiometric amounts of H_2O_2 [69]. The phenotype of the ERO1-overexpressing strains suggests that the ROS surplus is produced in the ER and further released to the cytosol, wherein it may affect the GSH redox potential (Fig. 1). Hypothetically, when the Ero1p activity is lowered (ero1-1), homeostatic mechanisms might try to correct the deficiency by increasing the oxidizing power of glutathione in the ER. This putative mechanism could well involve changes in the redox potential of glutathione in both ER and cytosol (Fig. 1 and Table 2).

The intracellular ROS levels can be estimated by several methods, and it is also possible to visualize the presence of ROS
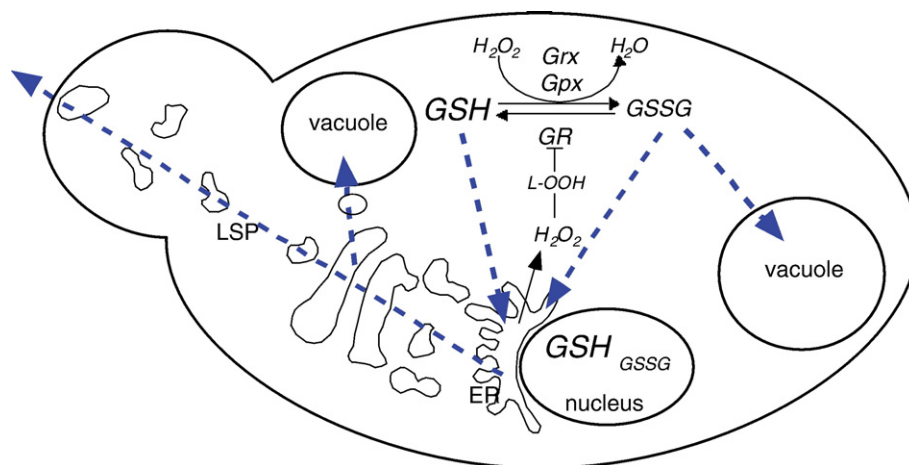

Fig. 1. Model for the regulation of the GSH–GSSG redox state in yeast. Glutathione is distributed along the whole cell in both reduced (GSH) and oxidized (GSSG) forms. GSH and GSSG can readily diffuse across the nuclear envelope, which makes the GSH–GSSG concentrations in the nucleus similar to that of the cytoplasm. The activities of glutaredoxins (Grx) and GSH-dependent peroxidases (Gpx) lead to GSSG formation in the cytosol by using GSH to reduce hydrogen peroxide (H_2O_2) and products of the H_2O_2-driven oxidation. To maintain the cytosolic/nuclear GSH redox potential, the enzyme glutathione reductase (GR) reduces GSSG to GSH. Nevertheless, since the GSH–GSSG buffer remains quite reducing while the whole-cell GSH/GSSG ratio increases in the absence of GR activity, GSSG might be rerouted to another compartment. Hypothetically, GSSG could be transported to the vacuolar compartment by unknown transport systems. Both GSH and GSSG are found in the endoplasmic reticulum (ER), and unknown factors regulate the ER's GSH redox potential, which is more oxidizing than that found in the cytosol. The yeast secretory pathway is a very dynamic membrane-bound array of different subcompartments connected by vesicular transport. Thus, it might be hypothesized that GSH and GSSG can use several routes leading to transport from the ER to the cell surface through the late secretory pathway (LSP). It is also possible that GSH and GSSG recycle between the ER and the vacuole. Possible transport routes for GSSG and GSH are represented with broken arrows. ER-localized enzymes like Ero1p produce H_2O_2, which may be released to the cytosol. GR-inhibition by products of the H_2O_2-driven oxidation like lipidic peroxides (L-OOH) leads to increase in the cytosolic GSSG levels. In a possible regulatory loop, the GSH redox potentials of the cytosol and the ER could influence one another provided redox equivalents in the form of H_2O_2 and GSH–GSSG are exchanged by the two compartments.

in a particular compartment, e.g. by using ROS-reactive fluorescent dyes. However, the most commonly used dyes, 2′, 7′-dichlorodihydrofluorescein (DCFH2) or dihydrorhodamine 123 (DHR), do not discriminate among ROS types and are fairly inaccurate since they are also involved in non-ROS-related reactions within cells [70]. More specific ROS-sensors would be helpful tools to investigate living cells.

5. Role of the cytoplasmic redox regulation during oxidant stress

Two types of oxidant stress affecting the eukaryotic cytosol can be defined: (i) *endogenous* accumulation of byproducts of the intracellular metabolism, (ii) and the stress brought about by treatment of cells with *exogenous* membrane-permeant oxidants. It is generally believed that the exogenous oxidants may mimic the effect of the intracellular accumulation of oxidative compounds resulting from failures in the redox regulation of the cell. However, these two types of oxidative stress differ considerably in their ultimate intracellular effects.

The differential behavior of the endogenous ROS accumulation and the exogenous oxidant supply has several explanations. For example, hydrogen peroxide is specific in activating signaling cascades leading to ageing, apoptosis, and the Yap1p-regulated stress response [5,11,71,72]. Although exogenous oxidants may have similar intracellular effects to those of ROS, they react with cellular components by mechanisms that are considerably different to those of the endogenous oxidants. Furthermore, while ROS are chemically quite unspecific [73–

77], cysteine oxidation [78] appears to be the main mechanism for explaining their toxicity to living cells [2]. When cells are treated with thiol oxidants like hydrogen peroxide (H_2O_2), thiol groups in proteins undergo oxidation by two main pathways (Fig. 2). Protein thiols exposed to H_2O_2 can be oxidized to form sulfenic acid (R-SO$^-$). In the presence of free thiols sulfenic acid is easily converted to disulfide which may be intramolecular (R^1S-SR^1, where R^x is any protein), intermolecular protein–protein (R^1S-SR^2) or mixed disulfides with GSH (RS-SG, where SG is a glutathionyl residue). In a competing reaction sulfenic acid can also be converted to sulfinic acid (RSOO$^-$) by further oxidation [7,9]. For a long time, it was thought that oxidation to sulfinic acid was an irreversible thiol modification. Recently it has been discovered in yeast that sulfinic acid can be rescued by a mechanism involving the ATP-dependent enzyme sulfiredoxin (Srx) [79]. Although sulfinic acids are thought to constitute a minor fraction of the oxidized thiols during H_2O_2 exposure in *S. cerevisiae*, they play an important role in stress signaling in the fission yeast *S. pombe* [78,80].

Better characterized are the thiol/disulfide interconversions during stress, which are regulated by well-known enzymes such as thioredoxin (Trx) and glutaredoxin (Grx). It has been shown (mostly by in vitro studies) that Grx strongly prefers glutathionylated substrates (RS-SG), whereas Trxs are particularly efficient in the reduction of protein disulfides (RS-SR) [81]. GSSG may form upon the action of Grx on oxidized substrates (Fig. 2), and also the GSH-dependent thiol peroxidases contribute to GSSG formation [15].
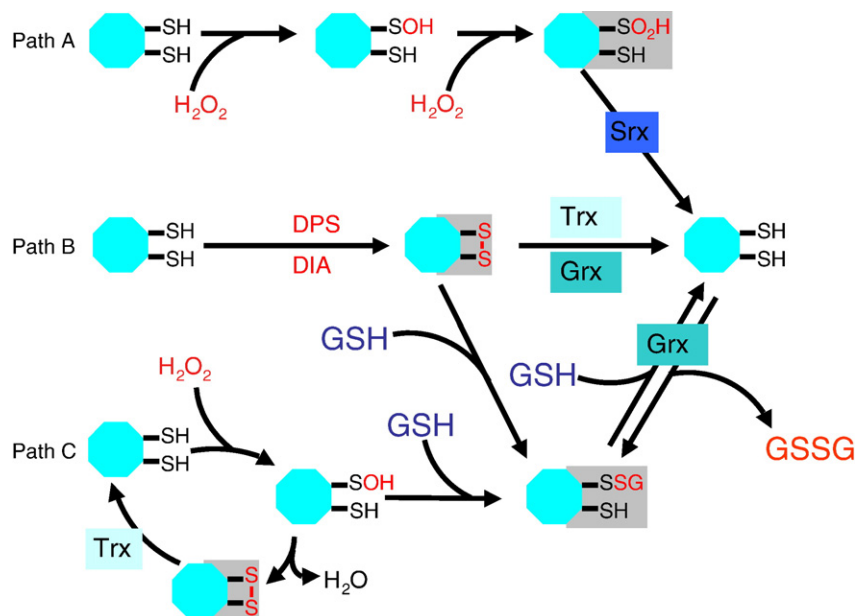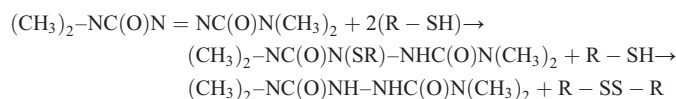

Fig. 2. Protein thiol modifications and reductive pathways during oxidant stress. A hypothetical dithiol protein (blue diamond) may become oxidized by different oxidants in oxidant-specific manner. Path A and Path C represent the protein thiol modifications occurring during exposure to hydrogen peroxide (H_2O_2) starting in both cases with the formation of sulfenic acid groups. In Path A, protein sulfenic acids (R-SOH) are subsequently oxidized by another H_2O_2 molecule to form sulfinic acids (R-SOOH), which can be converted back into reduced thiols by the enzyme sulfiredoxin (Srx). Due to their high reactivity, protein sulfenic acids readily react with free GSH or with a solvent-exposed protein thiol, giving rise to GSH-mixed protein and intramolecular bonds, respectively (Path C). The GSH-mixed protein disulfide bonds are likely reduced by glutaredoxin (Grx), while thioredoxins (Trx) mainly reduce intramolecular disulfide bonds. As schematized in Path B, the oxidants dipyridyl disulfide (DPS) and diamide (DIA) can oxidize thiols to yield mixed protein-glutathionyl bonds and disulfide bonds. Reversible and stable oxidized thiols forms like disulfides (both mixed GSH-protein and protein intramolecular bonds), and sulfinic acids are highlighted with a grey shade.

Exogenous oxidants like diamide and dipyridyl disulfide (DPS) trigger cellular responses like the Yap1p-regulated anti-oxidant response, although the mechanisms for Yap1p activation by these oxidants differ substantially to those for Yap1p activation in presence of ROS [34,82]. Compared to ROS, diamide and DPS are far more specific toward thiol groups and do not induce radical formation [34,83]. Therefore, studying the effects of diamide and DPS has revealed the specific mechanisms of thiol oxidation separately from the non-thiol related effects of oxidants. It is worth noticing that the two oxidants work by quite different mechanisms. Diamide reacts with proteins and GSH by the following two-step reaction:

$$(CH_3)_2-NC(O)N = NC(O)N(CH_3)_2 + 2(R - SH) \rightarrow$$
$$(CH_3)_2-NC(O)N(SR)-NHC(O)N(CH_3)_2 + R - SH \rightarrow$$
$$(CH_3)_2-NC(O)NH-NHC(O)N(CH_3)_2 + R - SS - R$$

A possible competing reaction in the second step is the addition of water:

$$(CH_3)_2-NC(O)N(SR)-NHC(O)N(CH_3)_2 + H_2O \rightarrow$$
$$(CH_3)_2-NC(O)NH-NHC(O)N(CH_3)_2 + R - SOH$$
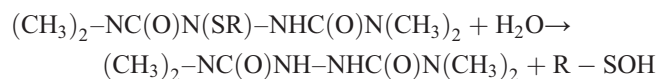
This yields a sulfenic acid which can either be further oxidized to sulfinic acid or react with another thiol to generate a disulfide bond. The reaction with DPS on the other hand is a pure thiol–disulfide exchange reaction [34] which is exceedingly specific.

The intrinsic preference of the two thiol oxidants determines the degree to which various thiols are disulfide-bonded. Proteins oxidized by DPS can be recycled back into reduced forms by similar pathways to those operating during peroxide stress (Fig. 2, Path B).

Different thiol oxidants may affect different protein targets in different compartments. The role of GSH in the cellular redox regulation and the survival capacity during oxidant stress has mainly been deduced from the phenotypes of the GSH-depleted mutants and mutants unable to induce the GSH synthesis upon stress. GSH is synthesized in the cytosol in two steps catalyzed by γ-glutamyl-cysteine synthetase and glutathione synthetase, encoded by GSH1 and GSH2, respectively. The γ-glutamyl-cysteine synthetase activity is the rate-limiting one for GSH synthesis [84], and is up-regulated during oxidative stress [85] and during exposure to heavy metals [86,87]. It has been demonstrated that increase in GSH synthesis in response to stress is specifically required for resistance to cadmium [88] and DPS [34]. The Δgsh1 mutant is unable to grow in the absence of extracellular glutathione, but grows fairly well in presence of low extracellular concentrations of GSH or GSSG [84,88,89]. The essential role of GSH is still unclear in yeast, but it has been suggested that it is related to the mitochondrial redox regulation as well as to the maturation of cytosolic iron–sulfur clusters [11,90].

Although GR is required for resistance to both diamide and DPS, these two oxidants differ dramatically in their effects in yeast. For example, GSH and Trx are dispensable for the resistance to diamide [42,55] but required for the resistance to DPS [34]. In a scenario where thiol-containing proteins may become oxidized directly by oxidants (DPS and diamide), it is expected that removing most of the cellular GSH (Δgsh1 mutant) would result in sensitivity to oxidant treatment. However, the striking diamide-resistance of the GSH-depleted strain emphasizes the quite specific role of GSH in the tolerance to DPS and goes against the view of a general role of GSH as reductant under conditions in which both protein and GSH become oxidized. Most likely, the toxic effect of diamide in yeast may be mediated by GSSG build-up.

The Δgsh1 mutation rescues mutants deficient in the essential ER-localized oxidase Ero1p [32]. However, folding is normal in Δgsh1 strains growing in very low GSH levels, but becomes impaired in the presence of diamide [32]. This diamide effect on ER folding seems not to be critical since the Δgsh1 mutant is surprisingly diamide-resistant [55,62]. This, in combination with the observation that GR function is required for the wild type level of resistance to diamide [42], suggests that the deleterious effect of diamide is mediated by GSSG accumulation. However, the ca. 50-fold increase in GSSG levels (compared to the wild type strain) obtained in a Δglr1 Δmet15 mutant neither affects folding nor viability [58]. Thus, the increased GSSG formation seems to have detrimental effects when provoked by exogenous thiol oxidants capable of penetrating subcellular compartments (e.g. mitochondria and ER) but not when the GSSG surplus is transported from the medium to the cytosol. Nonetheless, depending on the compartment and the relevant critical target(s), the lethal effect of GSH oxidation during oxidant exposure may have to do with GSSG accumulation (like in a diamide-treated GLR1 wild type), GSH depletion (DPS-treated Δglr1 mutant), or changes in the cytosolic GSH redox potential (peroxide-treated Δglr1 mutant and DPS-treated GLR1 wild type), as previously proposed [34].

Oxidants may also specifically affect the regulation of GSH–GSSG levels. For instance, DPS provokes a strong GSH depletion in the Δglr1 mutant and peroxide treatment leads to a considerable decrease in GSH levels in the GLR1 wild type, but none of these oxidants elevate the GSSG levels considerably under the mentioned conditions [34,91]. Thus, DPS may induce GSSG dissimilation in the DPS-treated Δglr1 mutant, and peroxide may have a similar effect in the GLR1 wild type. By contrast, diamide does provoke a significant increase in GSSG levels by oxidizing most of the cellular GSH [62,91].

6. Factors influencing the protein redox state in the cytosol

With rare exceptions, cytosolic proteins are found in their reduced state under normal conditions due to the strongly reducing conditions in this compartment and the absence of structurally stabilized disulfides. However, several cytosolic proteins may become oxidized in mutants lacking important redox functions and/or under exogenous oxidative stress [77,92,93]. Protein thiol oxidation is now beginning to be assessed at the proteome level in yeast by means of in vivo thiol-trapping methods.

Mechanistic aspects of the thiol/disulfide interconversions have been mainly studied in vitro. Here thiol/disulfide exchanges

between proteins and small compounds are typically reversible and may eventually reach equilibrium, as reviewed [8]. On the other hand, these thiol/disulfide interconversions may serve to control the activity of certain proteins in vivo [94–97]. Although GSSG levels are low in the cytosol, they could induce disulfide formation in proteins, provided that the disulfide bond is sufficiently structurally stabilized (i.e. has a sufficiently low redox potential). Glutaredoxins can catalyze both reduction and oxidation of substrates in vivo [6,91]. As demonstrated experimentally, a protein (rxYFP) with the appropriate redox potential can be found in both reduced and oxidized forms in the yeast cytosol under normal conditions [6]. Despite this, only very few yeast cytosolic proteins contain intramolecular disulfide bonds in vivo under normal conditions [72]. By contrast, some cytoplasmic proteins remain reduced even when containing several reactive solvent-exposed cysteines that are able to form disulfides when treated with non-GSSG oxidants [98]. Thus, there seems to be a selective pressure against cytosolic proteins forming stable disulfides. Protein glutathionylation, also known as GS-thiolation, has been proposed to be involved in homeostatic mechanisms controlling the activity and the stability of proteins [97]. Several proteins involved in glycolysis and protein synthesis are reversibly glutathionylated during peroxide treatment, and it was hypothesized that these modifications may protect protein cysteines from oxidation to sulfinic ($RSOO^-$) and sulfonic acids ($RSOOO^-$) during stress [93]. However, it is not clear whether glutathionylation could also occur during growth in the absence of peroxide. This would require a strong protein-specific stabilization of the GS-thiolated species [99] as the very low cytosolic GSSG/GSH ratio even under the most extreme of physiologically relevant conditions would otherwise be incompatible with the formation of such species. This view is supported by the observation that changing the GSH redox potential by the $\Delta glr1$ mutation or by diamide treatment did not alter the levels of protein glutathionylation in yeast [54,91,92].

Grx is particularly efficient in catalyzing protein glutathionylation and deglutathionylation [81,100–102]. However, removing cytosolic/nuclear glutaredoxins ($\Delta grx1$, $\Delta grx2$, $\Delta grx3$, and $\Delta grx4$ mutations), does not affect the levels of glutathionylated proteins (RS-SG) in yeast [54,91]. This observation contrasts the results in mammalian cells, in which Grx-inactivation (by antisense RNA-mediated interference (RNAi) or by cadmium treatment) led to increased protein glutathionylation [103–105]. In mammalian cells protein glutathionylation is also significant in diamide-treated cells (R.E. Hansen and J.R.Winther, unpublished results). On the other hand, deleting both cytosolic thioredoxins or thioredoxin reductase, or treatment with H_2O_2 significantly increases protein glutathionylation levels in yeast [54,91,106]. It is possible that the $\Delta trr1$ mutant accumulates ROS in the cytosol [52], which per se influences the redox state of protein cysteines. Furthermore, the GSH redox potential is only modestly affected by the $\Delta glr1$ mutation [6], and the $\Delta trr1$ mutant has a cytosolic GSH redox potential comparable to that of the $\Delta glr1$ strain (H.R. Lopez-Mirabal and J.R. Winther, unpublished results). Based on this it seems likely that GSSG accumulation is not the cause for the increased protein glutathionylation in the Trx-lacking strain. This is also consistent with the notion that protein glutathionyla-

tion in yeast can occur without an increase in GSSG levels [91]. Although some Trx isoforms in other organisms can catalyze deglutathionylations [81,107], the aforementioned results raise the question as to whether protein glutathionylation could depend on the GSH redox potential in the yeast cytosol or on homeostatic mechanisms activated exclusively when thioredoxins are unable to perform their reductive function.

It is well-known that proteins with solvent-exposed thiols are susceptible to oxidation to sulfenic acid in the presence of ROS like H_2O_2. Thus, an alternative mechanism to explain the in vivo glutathionylation may involve sulfenic acid (R-SO–) attack to GSH (Fig. 2, Path C) rather than thiol–disulfide exchange between GSSG and the thiol-containing proteins. A mechanism involving sulfenic acid formation in Grx could explain the GSH peroxidase activity displayed by yeast glutaredoxins [108]. Protein sulfenic acids can form RS-SG upon reaction with GSH, or RS-SR by intramolecular thiol-attack. Grx cannot catalyze these transformations, however, these reactions proceed rapidly due to the intrinsic instability of the sulfenic acid. Although deleting both cytosolic glutaredoxins does not have a dramatic effect on the basal levels of protein glutathionylation [54], it is not yet clear whether cytosolic glutaredoxins play a role in promoting GS-thiolation during peroxide stress. This is another important missing link in the complex puzzle of the yeast redox homeostasis.

Deletion of both *GRX1* and *GRX2* in yeast completely relieved the peroxide-sensitivity associated to the lack of GR [34]. Accordingly, the peroxide-sensitivity of the $\Delta glr1$ mutant can be explained by a possible oxidizing action of glutaredoxins on essential protein targets. Which mechanism would support Grx's oxidizing action in the peroxide-treated $\Delta glr1$ mutant? Clearly, this is related to increased GSSG formation and changes in the cytosolic GSH redox potential caused by the hydrogen peroxide stress in this mutant. However, it is unknown why Grx would be oxidizing in a life-compromising manner upon exogenous peroxide stress [34] or in ROS accumulating strains like $\Delta trx1$ $\Delta trx2$ [25], but not in the $\Delta glr1$ $\Delta met15$ strain which contains 50-fold more GSSG than the wild type and still grows well [6].

There are several possible mechanisms to explain the GR-dependence of the glutaredoxin toxicity in peroxide-treated cells (Fig. 3). There is good evidence showing that human Grx can catalyze GS-thiolation with a higher efficiency using glutathione-thyl radical (GS$^\cdot$) than with GSSG [109]. The GS$^\cdot$ radicals are formed upon the reaction of the ROS-produced hydroxyl radicals (OH$^\cdot$) with GSH. It could be hypothesized that glutaredoxins are better at stabilizing GS$^\cdot$ (by forming a mixed disulfide anion radical) when the GSH reducing potential decreases (Fig. 3, Mechanism 1), i.e. less GSH is available to reduce the Grx–glutathione-thyl radical (Grx–GS$^\cdot$).

The actual levels of cytosolic GSSG might be much higher in the peroxide-treated $\Delta glr1$ mutant than in the GSSG-grown $\Delta glr1$ $\Delta met15$ mutant. Notably, the $\Delta glr1$ $\Delta met15$ mutant contains 50-fold more GSSG than the wild type, but displays an rxYFP redox state that can be calculated to a cytosolic GSSG/GSH ratio of only about 2–3% provided the GSH concentration is similar to that of the wild type strain [6]. If a more oxidizing cytosolic glutathione buffer is generated during peroxide stress,
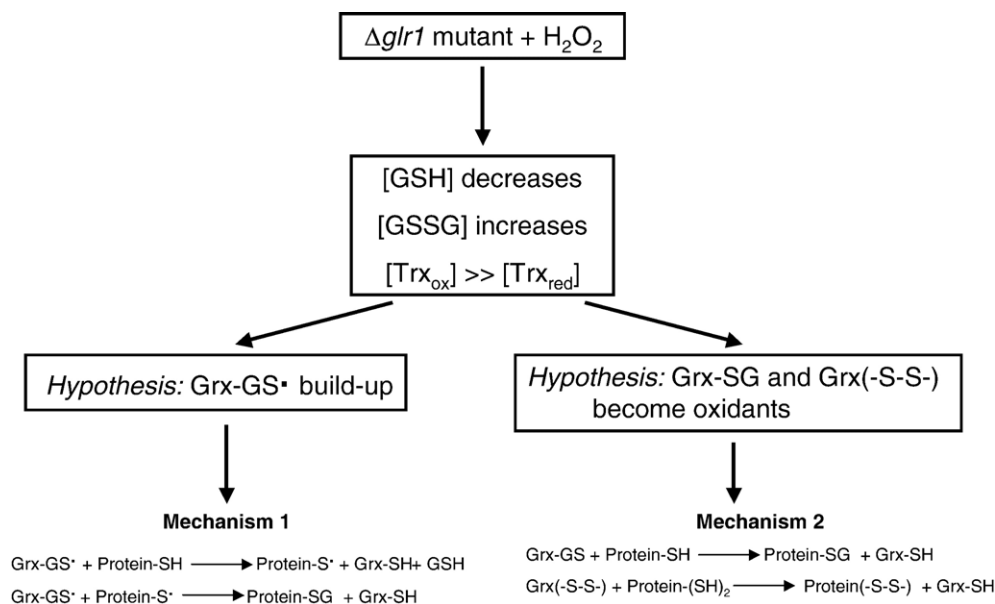
Fig. 3. Possible mechanisms for the toxicity of oxidized glutaredoxins in a peroxide-treated $\Delta glr1$ mutant. Upon exposure to hydrogen peroxide (H_2O_2), the cytosolic concentration of GSSG increases while that of GSH decreases because the GSSG-recycling is impaired in the glutathione reductase lacking strain ($\Delta glr1$). In this mutant, cytosolic thioredoxins (Trx) may become more oxidized than in the *GLR1* wild type. Mechanism 1 is based on the hypothesis that glutaredoxin–glutathione-thyl radicals (Grx–GS) accumulate when the GSH reducing potential decreases. The mechanism explaining Grx–GS-mediated protein glutathionylation for proteins has been proposed by Starke and co-workers [109]. On the other hand oxidized forms of Grx such as glutathionylated Grx (GS–Grx) and disulfide-bonded Grx (Grx(-S-S-)) may accumulate as the GSSG oxidizing potential increases. In this case, protein oxidation through Grx may proceed by thiol–disulfide exchange mechanism yielding glutathionylated or disulfide-bonded proteins (Mechanism 2). Trx oxidation may contribute to Mechanism 2, since putative Trx's protein substrates become unprotected against the Grx-aided formation of intramolecular protein disulfides.

glutathionylated Grx (Grx–GS) and possibly also disulfide-bonded Grx (Grx(-S-S-)) may become powerful oxidants for critical protein thiols. Particularly, the Grx-driven oxidation of essential substrates that are normally the target of Trx may be favored when Trx is impaired by oxidation or deletion (Fig. 3, Mechanism 2). This model is based on several results: (i) the Trx system is more important for resistance to H_2O_2 stress and maintenance of redox homeostasis under normal conditions compared to the GSH–Grx system [25,54]; (ii) cytosolic Trx is highly oxidized during H_2O_2 stress [110]; (iii) the double $\Delta trx1$ $\Delta trx2$ mutant can be rescued for growth in minimal medium by deletion of either one cytosolic Grx [25]; (iv) cytosolic Grx and to a lesser extent Trx display higher oxidized states in a $\Delta glr1$ mutant even in the absence of stress [60], and, (v) Trx and Grx in diverse biological systems can compete for the same protein substrates or participate in the regulation of the same pathway while having quite opposite effects on the protein redox state or the pathway functioning [111–113]. Ablation of the *GRX1* and *GRX2* genes in the $\Delta glr1$ strain did not yield the same effect in DPS as in peroxide, i.e. the DPS-sensitivity of the triple mutant is similar to that of the single $\Delta glr1$ one [34]. This could mean that extremely low GSH levels caused by DPS treatment of the $\Delta glr1$ mutant (less than 10% of GSH remains after a 1-hour treatment) limit the possible deleterious effects of protein GS-thiolation under these conditions. Surprisingly, while the cytosolic GSH redox potential in the $\Delta glr1$ mutant is severely affected (rxYFP fully oxidized) by extensive DPS exposure, that of the *GLR1* wild type is only mildly altered under the same conditions [34]. Together with the fact that deleting both cytosolic glutaredoxins

does not confer resistance to DPS to the $\Delta glr1$ mutant, these results suggest that the effect of DPS may be direct on putative targets rather than GSSG-mediated. It is not clear whether the toxic effect of Grx in the peroxide-treated $\Delta glr1$ mutant deals with RS-SG or RS-SR formation. To address this question, the Grx redox state and the putative oxidant-driven Grx-dependent protein oxidation under exposures to H_2O_2 and DPS could be determined and compared.

Contrary to mixed-glutathionyl protein disulfides, intramolecular protein–protein disulfides appear to have been evolutionarily unfavorable in cytosolic proteins. However, during oxidative stress, intermolecular disulfides and also specific intramolecular ones (e.g. in Yap1p) may arise upon the action of reactive sulfenic residues [19] and perhaps also upon oxidant-caused oxidation of disulfide-reducing enzymes. For instance, oxidized thioredoxins are toxic in $\Delta trr1$ and $\Delta glr1$ $\Delta trr2$ (lacking GR and mitochondrial Trx-reductase) mutants [62,114].

7. Concluding remarks

To solve the problem of how the redox homeostasis works in the eukaryotic cytosol, studies are moving from the basic genetic approaches to the development of accurate analytical methods for the characterization of living cells. Unfortunately, thermodynamic and kinetic aspects determined in vitro cannot always be extrapolated to the intracellular milieu. This is due to the fact that the important conditions of the intracellular environment like the true concentration of metabolites are not known. Particularly, the cytosol poses a great challenge to

bioanalytic methods due to the difficulty in isolating this compartment away from subcellular membrane-bound compartments, which contrasts the feasible isolation of subcellular organelles like the mitochondria. Due to their intrinsic reversibility and unpredictable reaction kinetics, protein–glutathione thiol/disulfide exchange reactions hinge upon the availability of adequate electron donors and acceptors. It is also important to view the system as a dynamic one where we know very little about the redox flux and where redox-active enzymes can kinetically connect the redox state of proteins and glutathione in ways that are difficult to predict. It is important to highlight that the involvement of such thiol–disulfide switches in cytosolic signal transduction processes may entail pathways that remain unknown as yet. The rise of genome-wide methods for identifying redox modifications and interactions in yeast constitute a significant step towards the elucidation of putative regulatory mechanisms. However, the main challenge of the studies dealing with thiol–disulfide interconversions is now to understand the actual molecular mechanisms operating in vivo. In this regard, a complete characterization of the chemical scenario in the cytosol, including GSH redox potential, ROS levels, and the redox state of redox-active enzymes/substrates, may provide clues for a more reliable in vitro mechanistic reconstitution of the thiol–disulfide reactions.

Acknowledgements

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