FORUM REVIEW ARTICLE

Functions and Cellular Compartmentation of the Thioredoxin and Glutathione Pathways in Yeast

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Abstract

Significance: The thioredoxin (TRX) and glutathione (GSH) pathways are universally conserved thiol-reductase systems that drive an array of cellular functions involving reversible disulfide formation. Here we consider these pathways in *Saccharomyces cerevisiae*, focusing on their cell compartment-specific functions, as well as the mechanisms that explain extreme differences of redox states between compartments. **Recent Advances:** Recent work leads to a model in which the yeast TRX and GSH pathways are not redundant, in contrast to *Escherichia coli*. The cytosol possesses full sets of both pathways, of which the TRX pathway is dominant, while the GSH pathway acts as back up of the former. The mitochondrial matrix also possesses entire sets of both pathways, in which the GSH pathway has major role in redox control. In both compartments, GSH has also nonredox functions in iron metabolism, essential for viability. The endoplasmic reticulum (ER) and mitochondrial inter-membrane space (IMS) are sites of intense thiol oxidation, but except GSH lack thiol-reductase pathways. **Critical Issues:** What are the thiol-redox links between compartments? Mitochondria are totally independent, and insulated from the other compartments. The cytosol is also totally independent, but also provides reducing power to the ER and IMS, possibly by ways of reduced and oxidized GSH entering and exiting these compartments. **Future Directions:** Identifying the mechanisms regulating fluxes of GSH and oxidized glutathione between cytosol and ER, IMS, and possibly also peroxisomes, vacuole is needed to establish the proposed model of eukaryotic thiol-redox homeostasis, which should facilitate exploration of this system in mammals and plants. *Antioxid. Redox Signal.* 18, 1699–1711.

Introduction

THE THIOREDOXIN (TRX) and glutathione (GSH) pathways are universal disulfide-reducing systems. In these pathways, TRX and glutaredoxin (GRX) operate as terminal reductases. They both reduce disulfides by a thiol-disulfide exchange reaction *via* two vicinal (CXXC) active-site cysteine (Cys) residues, which in turn form a disulfide. Oxidized TRX is then reduced back to its dithiol form by the flavin-adenine dinucleotide (FAD)-bound thioredoxin reductase (TRR), whereas oxidized GRX is reduced by the redox tripeptide GSH, which is reduced by the FAD-bound glutathione reductase (GLR). The ultimate electron donor for both pathways is nicotinamide adenine dinucleotide phosphate (NADPH). Since initial discovery of *Escherichia coli* TRX in 1964 as the

natural hydrogen donor for ribonucleotide reductase (RNR), much has been learned about the enzymology and structure-function relationship of the components of these pathways, and their physiological scope. Such knowledge has primarily been acquired in *E. coli*, which constitutes the working model of thiol redox systems in other organisms. However, despite overall similar composition, the *E. coli* system cannot explain the complexity of its eukaryotic counterpart, which stems, at least in part, from the compartmentalized nature of the eukaryotic cell.

Here we consider the thiol redox control pathways of the single-celled eukaryote *Saccharomyces cerevisiae*, focusing on their functions in the different compartments of the cell, and on the known mechanisms that explain the extreme differences of thiol redox states in the environment of the

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mitochondrial matrix, cytosol and mitochondrial intermembrane space (IMS) that is very reducing, and the endoplasmic reticulum (ER) that is very oxidized. We also consider the interplay between the thiol-redox homeostatic controls of each compartment.

Cellular Distribution of Thiol Redox Control Components

Two complete TRX pathways are present in *S. cerevisiae*: one in the cytosol that consists of two apparently redundant TRX isoforms (Trx1 and Trx2) and a TRR (Trr1) (93, 94) (Fig. 1), and one in the mitochondrial matrix that consists of a TRX (Trx3) and a TRR (Trr2) (72) (Fig. 2). Similarly, two complete GSH pathways exist in this organism, one in the cytosol that consists of two dithiol GRXs encoded by *GRX1* and *GRX2* (Grx1, Grx2) (51), and a GLR encoded by *GLR1* (Glr1) (15), and one in mitochondria consisting of activities also encoded by *GLR1* and *GRX2*. Dual cytosolic and mitochondrial localization of both Glr1 and Grx2 is due to the presence of an in-frame translation start site upstream of the canonical one, which produces a *N*-terminal mitochondrial targeting pre-sequence (69, 75, 78). The monothiol Grx6 and Grx7, which are ER and golgi luminal proteins anchored by *N*-terminal transmembrane domains to the membrane of these compartments, possess GSH-dependent oxidoreductase activity (36, 52, 58, 59), in contrast to the other monothiol Grxs, Grx3, Grx4, and Grx5, which are rather involved in iron metabolism and will therefore not be considered here. The dithiol cytosolic enzyme Grx8 carries a low oxidoreductase activity, but will not be further considered, because its function is still unknown (22). GSH is synthesized in the cytosol in yeast, as in mammals (30), by the sequential action of *GSH1*-encoded γ -glutamyl cysteine synthase, and *GSH2*-encoded glutathione synthase. GSH is found in all the yeast cell compartments, but how it distributes to these compartments is unknown. Cellular import of GSH and oxidized glutathione (GSSG) is operated by the plasma membrane proton-coupled oligopeptide transporter Hgt1 (or Opt1) that has a high affinity for GSH (50 μ M) (7). HGT1 expression is stimulated under sulfur-free conditions, as well by all amino acids, except those containing sulfur (102). Degradation of GSH is also an important determinant of its homeostasis. Initially thought to be operated in the vacuole by γ -glutamyl transpeptidase (γ -GT) under conditions of nitrogen starvation (55), recent data indicate that the newly discovered Dug1/Dug2 glutamine amidotransferase, which cleaves GSH at the γ -glutamyl-cysteine bond (38), and Dug3, a Cys-Gly dipeptidase (39), form a complex that is responsible for all GSH-degrading activity of yeast cells under normal and stress conditions, including sulfur and nitrogen starvation and cadmium exposure (4, 23). Lastly, NADPH is the major proton donor for disulfide reduction. NADPH is primarily generated from NADP⁺ by the oxidation of pentose in the pentose phosphate shunt pathway in the cytosol (37, 66), and from nicotinamide adenine dinucleotide (NADH) by the NADH kinase Pos5 in mitochondria (68).

Cytosolic Thiol Redox Control

We will first address the respective functions of the TRX and GSH pathways in the cytosol, and then ask in which measure these pathways are functionally redundant.

FIG. 1. Distribution of the components of thiol redox systems in the cytosol, IMS, ER, and vacuole. The cytosol contains entire sets of the TRX and GSH pathways, and its own independent NADPH source, the oxidative part of the pentose phosphate pathway. In the cytosol, the TRX pathway has a major role in thiol redox control and peroxide scavenging, whereas the GSH pathway acts as backup system of the former pathway. GSH carries an essential role in iron metabolism in the cytosol, the exact function of which remains to be elucidated. GSH is synthesized in the cytosol by the action of γ -glutamyl synthase and GSH synthase, and is degraded by the Dug1, Dug2, Dug3-dependent pathway. The ER and IMS do not carry any known thiol redox pathways components, except GSH that originate from the cytosol, and Grx6 and Grx7 in the former compartment, the function of which is not elucidated. These two compartments carry thiol oxidase redox relays that catalyze oxidative folding, Ero1-Pdi1 in the ER, and Ero1-Mia40 in the IMS, and thus generate GSSG. In these compartments GSH might be regenerated by putative GSH ingress and GSSG egress. G-S-X with metals and electrophiles are secreted into the vacuole by the ATP transporter Ycf1 and Ybt1, where GSH is degraded by γ -GT. Are also represented the high affinity GSH plasma membrane transporter that can transport both GSH and GSSG inside the cell, and porin that might transport either GSH into or GSSG out of the IMS. Although totally independent from the cytosol for thiol redox homeostasis, the mitochondria still relies on GSH that is synthesized in the cytosol, and imported by a still unknown mechanism. ER, endoplasmic reticulum; G-S-X, GSH S-conjugate; GLR, glutathione reductase; GRX, glutaredoxin; GSH, glutathione; GSSG, oxidized glutathione; H₂O₂, hydrogen peroxide; IMS, mitochondrial intermembrane space; NADPH, nicotinamide adenine dinucleotide phosphate; TRR, thioredoxin reductase; TRX, thioredoxin; γ -GT, γ -glutamyl transpeptidase. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars.)

The cytosolic functions of the TRX pathway

Functions of the cytosolic TRX pathway have been largely elucidated by genetics, and then refined by biochemical techniques that rely on the differential trapping of reduced versus oxidized Cys residues.

To observe a phenotype linked to cytosolic TRX inactivation, one must delete both *TRX1* and *TRX2* (Δ trx1 Δ trx2), due to the redundant nature of the encoded proteins. The

FIG. 2. The components of thiol redox systems in mitochondria. Mitochondria contain entire sets of the TRX and GSH pathways, and its own independent NADPH source, the NADH kinase Pos5. In the mitochondrial matrix the two pathways are functionally redundant, in contrast to the cytosol. Their known function is to reduce respiratory and exogenous ROS through Prx1. It is not clear what is the contribution of mGpx2 in mitochondrial ROS scavenging, and how would it be reduced. mGrx2 contributes to ROS scavenging, but it is not known through which peroxidase this function is operated. The mitochondrial matrix redox homeostasis is independent and is insulated from redox influences from the other cell compartments, except for an import of GSH from the cytosol through an unknown mechanism. The monothiol glutaredoxin Grx5 has no known redox function but is involved in Fe-S biosynthesis, a function presumably shared with GSH. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars.) GPX, glutathione peroxidase; NADH, nicotinamide adenine dinucleotide; PRX, peroxiredoxin; ROS, reactive oxygen species.

Δtrx1Δtrx2 strain has three remarkable phenotypes. The first one is a protracted cell cycle S phase (64), due to inefficient DNA synthesis by inefficient reduction of RNR (9, 40). Formal proof of the link between this phenotype and defective RNR reduction was provided by the presence in *Δtrx1Δtrx2* of a significant decrease of dNTPs levels (9, 40), an accumulation of RNR in its disulfide form, and relief of these defects upon overexpressing RNR (9). The second phenotype is an auxotrophy for sulfur-containing amino acids (64), due to defective reduction of the catalytic disulfide of 3'-phosphoadenylsulfate (PAPS) reductase (Met16), an enzyme required for inorganic sulfate assimilation. A two-hybrid identification of Met16 using TRX as bait (101) further illustrated the role of TRX in PAPS reductase reduction. The third major phenotype is a profound decrease in tolerance to peroxides (24, 43), a consequence of multiple defects in peroxide metabolic pathways. TRX is indeed the exclusive reductase of the three cytosolic yeast peroxiredoxins (PRXs) Tsa1, Tsa2, and Ahp1 (11, 46, 100), of the three glutathione peroxidase (GPX)-like-enzymes Gpx1, Gpx2, and Gpx3 (19, 92), and of the two methionine sulfoxide reductases Mrx1 and Mrx2 (31, 101). TRX also regulates the Yap1 and Msn2/4 stress transcriptional regulators. In *Δtrx1Δtrx2*, the peroxide-stress regulator Yap1 is constitu-

tively active and accumulates in its active disulfide form, which suggests that TRX negatively regulates this regulator by reduction (10, 18, 35). However, although Yap1 is constitutive in *Δtrx1Δtrx2*, the strain remains highly peroxide-sensitive, due to the concurrent peroxidase defect resulting from the lack of TRX. TRX is also required to activate the general stress regulators Msn2/4 and Maf1 in response to hydrogen peroxide (H_2O_2), through an elusive mechanism that requires TRX in its oxidized form (6). The major role of TRX in peroxide metabolism has been confirmed by the identification of Tsa1, Ahp1, and Met16 in a yeast two-hybrid assay using thioredoxin 2 as bait (101); Ahp1 was also purified from yeast lysates on the basis of its redox association with a plant TRX (100). The inability of *Δtrx1Δtrx2* to use methionine sulfoxide as source of organic sulfate (62) is another proof of the exclusive role of TRX in assisting methionine sulfoxide reductase. Redox proteomics also showed that in *Δtrx1Δtrx2*, Tsa1, Tsa2, Ahp1, Prx1, Gpx2, and Mxr1 along many other proteins have a significant increase in Cys residues oxidation, which demonstrate the importance of TRX in controlling the redox state of these antioxidant proteins (45).

In addition to these three major phenotypes, *Δtrx1Δtrx2* is paradoxically hypersensitive to the dithiol-reducing agent dithiothreitol (DTT) and resistant to the thiol oxidant diamide (95), which are phenotypes that have not been traced to any specific molecular defects. TRX was also biochemically identified as a factor required for vacuole inheritance, but this function is independent of the TRX Cys residues, and therefore not a redox one (104).

The phenotypes of the *Δtrr1* strain should match those of *Δtrx1Δtrx2*, because of the epistatic relationship between *TRR1*, *TRX1*, and *TRX2*, but this is not so. *Δtrr1* has a unique slow aerobic growth (44, 53, 96) that is significantly improved by anaerobiosis (94, 105), and a much poorer peroxide tolerance than *Δtrx1Δtrx2*. However, *Δtrr1* does not carry the cell cycle and sulfate assimilation defects of *Δtrx1Δtrx2*. The distinct phenotypes of *Δtrx1Δtrx2* and *Δtrr1* could indicate that TRR has enzymatic functions independent of TRX, as shown for mammalian TRR (2). These differences may also be explained by a *Δtrr1* gain of function caused by toxic accumulation of oxidized TRX leading to disulfide stress, as shown in *E. coli* (89). The latter hypothesis is supported by improvement of both growth and peroxide tolerance phenotypes by further deletion of *TRX1* and *TRX2* (*Δtrr1Δtrx1Δtrx2*) (17, 94, 105). Anyhow, lack of the cell cycle defect and organic sulfur auxotrophy of *Δtrr1* also indicates that enough TRX activity remains in the cell to reduce RNR and PAPS reductase.

The *Δtrr1* strain is also moderately DTT hypersensitive and exhibits constitutive activation of the unfolded protein response (UPR), which is physiologically triggered by ER stress (95, 96), and of the Aft1-dependent iron starvation response (44). The *Δtrr1* constitutive UPR cannot be easily rationalized, but its cure upon GSH depletion (96) suggests that it might be linked to the compensatory increase in GSH levels in this strain (see below). Activation of Aft1 might be a consequence of GSH bearing the redox load increase caused by lack of Trx1, and therefore becoming limiting for its function in iron metabolism (see below). *Δtrr1* has other complex defects in translation accuracy, ribosomal aggregation, and sensitivity to the translational inhibitors hygromycin and puromycin,

which are to some extent shared with $\Delta trx1\Delta trx2$ and $\Delta tsa1$ (79, 98).

The cytosolic functions of the GSH pathway

Study of the yeast GSH pathway has been hampered by the essential requirement of GSH for viability. *GSH1*-null strains are unviable unless given exogenous GSH (26, 103), in contrast to those lacking either cytosolic GRXs ($\Delta grx1\Delta grx2$) or GLR ($\Delta glr1$), which indicates that GSH has functions not shared with the other components of the pathway. $\Delta grx1\Delta grx2$ and $\Delta glr1$ do not have any remarkable phenotypes under nonstress conditions, except for a strongly oxidized GSH pool in the latter mutant (Table 1) (25, 51). $\Delta grx2$, $\Delta glr1$, and cells partially depleted of GSH are slightly peroxide-sensitive, whereas $\Delta grx1$ is sensitive to the superoxide anion-generating drug menadione (25, 51, 65, 88). How can the peroxide phenotypes of GSH pathway mutants be rationalized? In mammals, GSH reduces GPxs, an important peroxide-scavenging family, but there are no *bona fide* GPx enzymes in yeast, and as mentioned above, the related enzymes, Gpx1, Gpx2, and Gpx3, are reduced by TRX and not GSH (19, 92). GSH might reduce protein-sulfenic acids (P-SOH) resulting from the reaction of thiols with H_2O_2 , through formation of S-glutathione protein adducts, hence contributing to H_2O_2 scavenging. Oxidation of GSH and S-glutathionylation of several cytosolic proteins in response to H_2O_2 supports this notion (86). Grx1/2 was shown to possess peroxidase and glutathione S-transferase (GST) activities in the presence of GSH *in vitro* (13, 14), which if verified *in vivo* could also contribute to H_2O_2 scavenging. However, as *GLR1* and *GRX2* encode both the cytosolic and mitochondrial isoforms of these enzymes (see above), a mitochondrial peroxide scavenging defect should also be considered to explain the peroxide phenotypes of the corresponding mutants, which in fact prevails over a cytosolic defect (see below). GSH is also important for the resistance to heavy metals and electrophiles (76), a function relying on the chelating property of its Cys residue. Resistance to some electrophiles might also be linked to the GST-transferase activity of Grx1/2, a function involving GSH (13). $\Delta grx2$, $\Delta glr1$, and GSH-partially depleted cells are paradoxically resistant to diamide, a phenotype shared with TRX mutants (see above) (51, 65).

Henceforth, the most salient phenotype of yeast GSH pathway mutants is the requirement of GSH for viability, which has been an enigma as to its molecular cause. The GSH auxotrophy of the $\Delta gsh1$ mutant cannot be rescued by alternative small thiol-reducing agents such as Cys, DTT, and N-acetyl cysteine, nor by anaerobiosis (47, 84, 88), which indicated that neither a general disulfide reduction defect nor lack of a reactive oxygen species (ROS) protective function can explain the inviability of GSH-depleted cells. Defective reduction of the essential RNR disulfide could not account for the inviability either, as this disulfide is preferentially reduced by TRX in yeast (9). Attempts at identifying yeast extragenic suppressors of GSH auxotrophy failed, only yielding mutations that, by diverting the proline biosynthetic pathway, restored synthesis of very small amounts of GSH (88). This and other studies thus indicate that minute amounts of the tripeptide suffice for full viability (47, 84, 88), excluding the idea that GSH is essential as a redox buffer. In fact, the essential nature of GSH is not linked to thiol reduction, but to a function in iron metabolism, a conclusion supported by the results of an analysis of GSH-depleted cells (44). Genome-wide mRNA profiling showed that the near-unique defect of these cells is an alteration of iron metabolism originating from a defect in extra-mitochondrial iron-sulfur cluster (ISC) maturation, a process that requires GSH (87). This requirement is possibly linked to an undefined role for GSH in iron trafficking and delivery that may, at least in part, involve the two cytosolic monothiol GRXs, Grx3 and Grx4, that require GSH to bind a [2Fe-2S] cluster (63) [for a recent review on this subject (48)]. The accumulation of iron in the mitochondria of GSH-depleted cells (87) is a consequence of the ISC assembly defect, and explains the high mitochondrial genome instability of these cells (3). The observation that both the ISC assembly defect and growth arrest characteristic of GSH-depleted cells are partially corrected by exogenous iron further establishes the link between the yeast GSH requirement for viability and its function in iron metabolism (44).

What are the other cytosolic functions of the GSH pathway (see Fig. 3)? Although this pathway, and GSH in particular, are classically described as important in cytosol thiol-redox maintenance, data obtained in yeast do not support this notion. It is first instructive to consider that no specific cytosolic thiol reduction functions have ever been attributed to GSH or

TABLE 1. ESTIMATES OF THE TOTAL CELLULAR GLUTATHIONE AND OXIDIZED GLUTATHIONE CONTENT IN *SACCHAROMYCES CEREVISIAE* WILD-TYPE CELLS, AND IN CELLS OVEREXPRESSING HGT1, OR LACKING *GSH1* OR *TRR1*

Strain	GSH added ^a	GSH content	GSSG content	2GSH/GSSG	E_{GSH} (mv) ^b
WT	0	7.2 ± 0.04	0.21 ± 0.02	31.0 ± 1.3	-221.8 ± 0.7
HGT1	0	7.59 ± 0.27	0.19 ± 0.02	38.9 ± 4.9	-224.0 ± 2.0
HGT1	100 μ M	54.53 ± 4.61	1.42 ± 0.06	38.9 ± 3.8	-249.6 ± 2.9
<i>gsh1</i>	100 mM	2.17 ± 0.07	0.08	26.7 ± 0.4	-202.8 ± 0.5
<i>gsh1</i>	1 mM	0.43 ± 0.05	0.03	16.5 ± 1.2	-175.3 ± 2.6
<i>gsh1</i>	0	0.06 ± 0.002	0.02	2.9 ± 0.1	-125.4 ± 0.5
<i>trr1</i>	0	11.46 ± 1.2	1.34 ± 0.04	8.6 ± 1.1	-209.7 ± 0.6
<i>glr1</i>	0	6.58 ± 0.44	4.75 ± 0.24	1.9 ± 0.2	-204.2 ± 0.9

From Ref. (44).

^aIn the case of HGT1, measures were performed 1 h after adding the indicated amount of GSH. In the case of *dgsh1*, growth was initiated in the presence of the indicated amount of GSH, and measures were performed in the late exponential phase. In the case where GSH was not added, measures were performed after six divisions in medium not containing GSH.

^b E_{GSH} was calculated according to the Nernst equation, assuming a pH=7.

GSH, glutathione; GSSG, oxidized glutathione.

FIG. 3. The purported functions of the redox tripeptide GSH in the different cell compartments of *Saccharomyces cerevisiae*. In the ER GSH is presumed to contribute to oxidative protein folding by providing reducing equivalents to Pdi1 for non-native disulfide isomerization; GSH might also provide reducing equivalents for reduction of terminally misfolded proteins that are substrates of the ER quality control. In the IMS, GSH also provides reducing equivalents for disulfide isomerization on substrates that are imported in the IMS by the Erv1-Mia40 redox relay system. G-S-X with endogenous and xenobiotic electrophiles and metals are secreted into the vacuole as a mean of detoxification. See also text. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars.)

to the GSH pathway in yeast, at least when TRX is present, and aside from the possible role of GRXs as peroxidases and/or GSH transferases (see above). In addition, the genome-wide mRNA profiles of GSH-depleted cells did not provide any hint of a thiol-redox maintenance defect (44), which is consistent with a redox proteomics study that also indicated that the redox state of protein-thiols is not altered in cells simultaneously depleted of both Glr1 and GSH, in contrast to those lacking both TRXs and TRR (see above) (45). When present at very high cellular levels up to 10-fold its wild-type concentration, GSH totally inhibits growth and causes cell death, a toxicity caused by a major ER reductive stress (see below) and impairment of ISC assembly, but not attributed to a defect in cytosolic thiol redox control (44).

The TRX pathway has the major contribution in cytosolic thiol redox control

It is clear from the data discussed above that the TRX pathway has a prominent, if not exclusive, role in cytosolic thiol redox control, in contrast to the GSH pathway that has only a limited role (see Figs. 1 and 3). Such a functional configuration would thus imply that these pathways do not overlap functionally, in contrast to their *E. coli* counterparts, either of which is able to support the thiol-redox duties of the cell in the absence of the other (94). However, although yeast thiol redox pathways are not redundant functionally, the GSH pathway can act as backup system when the TRX pathway is absent. RNR is essential as it provides the deoxyribonucleotides required for DNA synthesis and repair. RNR reduction, which thus constitutes a vital target for thiol redox control, is

accomplished by the TRX pathway (see above). However, the viability of $\Delta trx1\Delta trx2$ indicates that the GSH pathway can substitute for the TRX pathway in this function, but only very inefficiently as evidenced by the cell cycle defects of this mutant. The synthetic lethality resulting from the simultaneous deletion of components in both pathways [e.g., $\Delta glr1\Delta trx1\Delta trx2$ (65), $\Delta trx1\Delta trx2\Delta grx1\Delta grx2$ (21), $\Delta glr1\Delta trr1$, $\Delta gsh1\Delta trx1\Delta trx2$ (96), or $\Delta gsh1\Delta trr1$ (44)] is probably caused by defective RNR reduction, which also supports the notion that GSH operates as a backup system in RNR reduction when TRX is absent. Another example of the inefficiency of the GSH pathway as a thiol-redox backup system involves redox control of PAPS reductase (64). The organic sulfur auxotrophy of $\Delta trx1\Delta trx2$ can be relieved by growth in liquid culture under low oxygen tension (21), which might indicate that Grx1 and/or Grx2 can substitute for TRX in PAPS reduction, but only when the cellular oxidative load is decreased. A putative alternate PAPS reductase other than GRX was also alluded to, as overexpressing *GRX1* or *GRX2* did not rescue the sulfur auxotrophy of $\Delta trx1\Delta trx2$ (21). The threefold increase of total cellular GSH in $\Delta trr1$ and $\Delta trx1\Delta trx2$ (see Table 1) (96) probably compensates for the inefficiency of the GSH pathway in replacing the TRX pathway. Still, the very slow growth of $\Delta trr1$ can be significantly improved by exogenous GSH, which indicates that, despite its compensatory increase, GSH remains extremely limiting in this strain (44). The activation of the iron starvation response observed in $\Delta trr1$ is a probable consequence of the limiting amount of GSH in this strain, as it is also corrected by exogenous GSH.

In conclusion, the data examined here altogether indicate that in the cytosol the TRX pathway has the major, if not exclusive, role in thiol redox control. The GSH pathway in contrast has a very limited contribution to this control, serving as a backup system when the TRX pathway is absent, or when it is overwhelmed by severe redox stresses (44) (see Figs. 1 and 3). The limited contribution of the GSH pathway in cytosolic redox control is further underscored by data of two recent reports, one indicating that reduction of protein-S-glutathione adducts is essentially operated by TRX and not GRX (29), and the other indicating that the TRX pathway can function as an alternate system to reduce GSSG (91).

Mitochondrial Thiol Redox Control

As stated above, the yeast mitochondrion possesses the NADH kinase Pos5 as its own NADPH source and a complete set of both TRX and GSH pathways that are not functionally separated as the cytosolic ones (see Fig. 2). With the exception of $\Delta pos5$, phenotypes resulting from inactivating components of these pathways are only revealed under stress and respiring growth (glycerol) conditions (68, 72, 90, 97). However, it should be noted that due to our incapacity of depleting GSH from mitochondria with currently available experimental tools, the mitochondrial phenotypes of GSH are totally lacking.

The $\Delta pos5$ strain exhibits complex phenotypes, including slow growth in glucose medium that is exacerbated in respiratory medium, a mitochondrial ISC assembly defect and accompanying mitochondrial iron accumulation, and arginine auxotrophy (68, 90). The ISC assembly defect indicates the requirement for NADPH in this process, possibly at the level of the FAD and NADPH-dependent Arh1, an adrenodoxin reductase homolog, or for iron reduction to the

biologically utile form Fe^{++} , or for sulfite reduction into sulfur, or for another unknown process in mitochondrial iron metabolism (71). Decreased protection from respiratory-chain ROS is the other distinct defect underlying the lack of mitochondrial NADPH in Δpos5 (68), which combines with the ISC defect to engender the mitochondrial genome instability and protein carbonylation increase also seen in this mutant (90). The defective mitochondrial ROS protection of Δpos5 is evidenced by hyperoxic hypersensitivity that is partially alleviated by inactivating the respiratory chain *via* deletion of COQ1 encoding a coenzyme Q biosynthetic enzyme (68). The NADPH requirement for respiratory-chain ROS protection is at least in part linked to Glr1-dependent recycling of oxidized GSH, since GSH oxidation is significantly increased in Δpos5 and under hyperoxia, and since inactivation of the mitochondrial form of Glr1 also causes a hyperoxia phenotype (70). Such a ROS protective role for mitochondrial NADPH fits with the identification of POS5 in a screen of peroxide hypersensitive mutants (42).

The only demonstrated function of the mitochondrial TRX and GSH pathways is to assist the mitochondrial PRX Prx1, and possibly also Gpx2 that is also localized in this organelle (99), in protecting mitochondria against respiratory ROS as well as exogenous peroxide (see Fig. 2). GSH is suspected to fulfill important functions in mitochondrial iron metabolism, which should, at least in part, superimpose with those of NADPH (see above), but these functions have not been yet studied, due to the inability of mitochondrial GSH depletion. Inactivation of Trx2 alone causes no or only a very minor peroxide tolerance defect (72, 97), but the combined mutation of Trx2 and Glr1 ($\Delta\text{trr2}\Delta\text{glr1}$) yields a synthetic interaction manifested by a severe respiratory growth defect not seen in either single mutant, and by the exacerbation of the Δglr1 minor peroxide tolerance defect (27, 97). Since as already discussed above GLR1 encodes both cytosolic and mitochondrial Glr1 isoforms (69), the synthetic interaction of GLR1 and TRR2 could either indicate a redox linkage between cytosol and matrix or an overlapping role for Trx2 and Glr1 within the matrix. Accumulation of oxidized Trx3 in

$\Delta\text{trr2}\Delta\text{glr1}$, but not in the corresponding single mutants (97), favors the latter hypothesis. Accumulation of oxidized Prx1 in either Δglr1 or Δtrr2 , and loss in these mutants of the increased peroxide tolerance seen in wild-type cells when Prx1 is overexpressed (27) further support the functional redundancy of Trx2 and Glr1, and indicate that Prx1 is a common downstream substrate of these reductases. The identity of the Trx2 and Glr1 substrates that reduces the 1-Cys Prx Prx1 is differently appreciated. 2-Cys Prxs are almost exclusively reduced by TRX and TRR, but how 1-Cys Prxs are reduced is debated. Oxidation of Prx1 upon GSH depletion (27) indicates that the tripeptide contributes to Prx1 reduction, possibly as shown *in vitro* by formation of an S-glutathione adduct, which is then reduced by Grx2 (74) or Trx3 (28, 73), or less likely by Trx2 (27) or ascorbate (60). Although Δtrx3 is not peroxide hypersensitive, Trx3 clearly contributes to Prx1 reduction as indicated by the change of its redox state from fully oxidized in $\Delta\text{trr2}\Delta\text{glr1}$ to almost fully reduced upon further inactivation of Prx1, and by the identification of a Trx3-Prx1 disulfide-linked complex using a Trx3-Cys trapping mutant (28). Interestingly, the latter study also showed that, similar to deletion of TRR1 that leads to toxic accumulation of oxidized cytosolic TRXs, accumulation of oxidized Trx3 is responsible for the respiratory growth defect of $\Delta\text{trr2}\Delta\text{glr1}$, since further inactivation of either Trx3 or Prx3 in this strain rescues this growth phenotype. Grx2 might also contribute to Prx1 reduction *in vivo*, as suggests the peroxide hypersensitive phenotype of a strain lacking the mitochondrial but not the cytosolic form of the enzyme (77). Grx2 might also be involved in mitochondrial iron metabolism, based on the synthetic lethality of $\Delta\text{grx2}\Delta\text{grx5}$ and the fact that Grx5 plays a role in Fe-S cluster biogenesis (82).

In summary, apart from the suspected involvement of GSH in mitochondrial iron metabolism, the mitochondrial TRX and GSH pathways have one established and redundant function, which is to assist Prx1 in scavenging respiratory ROS and exogenous H_2O_2 (see Fig. 2). Further, based on phenotypes, and in contrast to the cytosol, the GSH pathway appears as the dominant thiol redox control system in this organelle.

FIG. 4. Is GSH import into the ER required for ER oxidative protein folding? Proteins are translocated in their reduced form and pass electrons to oxidized Pdi1. Reduced Pdi1 is then re-oxidized to the disulfide form by Ero1. Ero1 is reoxidized by FAD, which generates FADH_2 that is oxidized by molecular oxygen, with generation of one molecule of H_2O_2 for each disulfide formed in protein substrates. Ero1 is negatively regulated by formation of a noncatalytic regulatory disulfide when the ER is too oxidizing. GSH is dispensable for ER oxidative protein folding but could limit oxidative folding by acting as an Ero1-Pdi1 substrate. GSH could also contribute to the reduction of the Ero1 regulatory disulfide by maintaining a pool of reduced Pdi1, and to the isomerization of non-native disulfides formed. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars.) FAD, flavin-adenine dinucleotide.

FIG. 5. At high levels, GSH perpetuates Ero1 activation in the ER. When total cellular GSH increases up to 100 mM, GSH enters the ER, possibly by facilitated diffusion, providing both unlimited reduced thiol substrates to the Ero1-Pdi1 redox relay and reducing the negative Ero1 regulatory disulfide, thereby causing Ero1 cycling to perpetuate with generation of excess H_2O_2 and GSSG, competing with substrates for oxidation by Ero1-Pdi1, which eventually blocks secretion. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars.)

Thiol Redox Control of the ER

The ER is the first stage for proteins that are destined for secretion. These proteins are co-translationally translocated into the ER, where they are glycosylated, folded, and acquire their mature conformation, before they are transported, *via* the Golgi, to the plasma membrane and the extracellular space. For many of them, folding requires formation of one or more disulfides [for a review see ref. (54)]. To attain their native arrangement, formed disulfides must be isomerized and reduced. Hence, opposing reactions—the oxidation and reduction of Cys residues—coexist in the ER. Disequilibrium between these two processes impairs oxidative folding and leads to protein misfolding and ER stress.

The yeast machinery catalyzing disulfide bond formation is relatively well understood, involving a redox relay made of the ER oxidase Ero1 that generates disulfides, which are transmitted to protein disulfide isomerase (Pdi1), and then to substrates (8) (see Figs. 4 and 5). This relay is feedback regulated by the inactivation of Ero1 through negative regulatory disulfide formation when the ER becomes hyperoxidizing (83).

The reducing system of the ER is, however, poorly understood (12). Both isomerization and reduction steps are operated by reduced Pdi1, but how this enzyme is recycled to the reduced form is unclear, especially considering the very oxidized GSSG/2GSH ratio of the ER (see Table 2 and Fig. 6) that is unfavorable for thiol reduction. What then is the reducing source for the ER? Newly synthesized proteins entering the ER certainly constitute a net source of reduced thiols. However, whether such reducing source is sufficient to balance the ER oxidative load is questionable, even when considering the decrease of this load by Ero1 feedback inhibition. With the exception of GSH, Grx6, and Grx7, none of the disulfide reduction catalysts of the other cell compartments, or homologues thereof, are present in the yeast ER. Although induced as part of the UPR, Grx6 and Grx7 do not participate, however, to ER oxidative protein folding (36, 59). Grx6 possess both GSH-dependent oxidoreductase and glutathione transferase activity, or alternatively can assemble an iron-sulfur complex together with GSH. This protein could therefore sense the GSH redox state of GSSG/2GSH redox couple or another ER redox signal and keep in the reduced state

FIG. 6. Cellular trafficking routes of GSH establish redox links between compartments. The matrix and cytosol have each entire sets of the two thiol redox pathways. In contrast the ER, IMS, vacuole, and peroxisome are not known to possess thiol oxidoreductases, and are presumably dependent upon GSH emanating from the cytosol for thiol redox control. Estimated values of the 2GSH/GSSG ratio in the different compartments are indicated in gray boxes (for references, see text and Table 2). Values in IMS are taken from Ref. (41), which as discussed in text are more reducing than those from (33). The figure depicts the possible cellular GSH trafficking pathways between the cytosol and the ER, the IMS and the vacuole that may contribute to keeping the indicated values of 2GSH/GSSG in each compartment. According to this hypothetical cartoon, the GSSG generated within the ER and IMS is recycled to GSH by cytosolic Glr1. Although having its own thiol-redox control system, and therefore being independent from the rest of the cell to regulate thiol redox homeostasis, the mitochondrial matrix still requires the import of GSH, which is synthesized in the cytosol. The known cellular GSH transporters, Hgt1, Ycf1, and Por1 are depicted. In the case of Por1, data indicate that it links the IMS redox state of GSH with that of the cytosol, but it is not known which form of GSH, reduced or oxidized, is transported by it. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars.)

TABLE 2. ESTIMATES OF THE REDOX STATE OF GLUTATHIONE IN THE DIFFERENT COMPARTMENTS OF YEAST CELLS

Compartment	2GSH/GSSG measured	E_{GSH} , pH 7 (mV)	2GSH/GSSG calculated rxYFP	$E_{\text{rxYFP/roGFP}}$ (mV)
Cytosol	100:1 ^a (34)	~ -230 (34)	^a 3300:1 (67)	~ -289(67), -306 (41)
ER	1:1–3:1 (34)	~ -170 to -185 (34)	NA	~ -236(20)
IMS	NA	NA	250:1 (33)	~ -255(33), -301 (41)
Matrix	20–40:1 (61, 69, 81, 85)	~ -280 (61, 69, 81, 85)	900:1 (33)	~ -296(33), -301 (41)

^aRefined measures obtained using GSH redox probes explain the important difference from preliminary estimates.

ER, endoplasmic reticulum; IMS, mitochondrial intermembrane space; NA, non-available.

specific Cys residues that in enzymes coordinate prosthetic groups or constitute catalytic centers (52, 58).

Although apparently dispensable for protein secretion in yeast (16), the role of GSH in ER redox homeostasis is, nevertheless, important, but still obscure (Figs. 3, 5, and 6). GSH was initially proposed to act as the ER source of oxidizing equivalents because of the high GSSG/2GSH ratio reported for this compartment (34) (Table 2), but is now thought to influence the ER oxidative machinery by acting in a competing pathway for disulfide reduction (16). Accordingly, the function of GSH in the ER should be to buffer excess Ero1-dependent oxidation. Still, how could GSH act as a redox buffer if no mechanisms are present to recycle its reduced form? This opened question requires further study.

Thiol Redox Control Mechanisms in the IMS

The IMS houses the mitochondrial disulfide relay that is composed of the sulfhydryl oxidase Erv1 and of the oxidoreductase Mia40 (Figs. 1 and 3). This relay couples the oxidative folding of a set of proteins with characteristic twin CX₃C and twin CX₉C motifs to their import into the IMS [for a review see ref. (32)]. Unfolded substrates are translocated, most likely co-translationally, in the reduced form in the IMS where they are recognized by and interact with the oxidized form of the IMS import receptor Mia40, which leads to formation of transient Mia40-substrate mixed disulfides. Mixed disulfides are then transposed into intramolecular substrate disulfides that prevent protein back translocation to the cytosol by promoting protein folding. Released, reduced Mia40 is re-oxidized by Erv1 *via* transfer of electrons to cytochrome *c* and O₂. Except for GSH, the IMS is not known to carry any classical components of thiol redox control pathways (Grxs, Trxs, GSH or Trx reductases). In the IMS, GSH was proposed to increase the efficiency of Mia40-mediated substrate oxidation in an *in vitro* reconstituted system (5). In the absence of GSH, Mia40 accumulated into kinetically trapped mixed disulfide intermediates, which suggested that GSH might favor isomerization of non-native disulfide intermediates by reduction. Initial E_{GSH} measurements using the redox sensor rxYFP expressed in the IMS showed this value to be very oxidizing (~ -255 mV, see Table 2 and Fig. 4) (33). However, a more recent study that used Grx1-roGFP2 indicated the IMS to be much more reducing (E_{GSH} ~ -302 mV), and also provided data suggestive of redox linkage between Mia40 and GSH (41). The discrepancy between these two IMS redox measurements may be due to differences in the rate of equilibration of each sensor with the local GSH:GSSG environment and argues against a native Grx in this compartment to catalyze equilibration of the green fluorescent protein-based sensors with the IMS GSH:GSSG pool. In any case, as already

argued upon discussing ER redox homeostasis, if GSH indeed acts as a reducing buffer in the IMS, what is the mechanism that recycles its reduced form?

Thiol Redox Control in the Remaining Compartments

This survey of the compartment-specific mechanisms that control thiol-redox homeostasis has not yet considered the vacuole, peroxisomes, and the nucleus (see Figs. 1 and 4).

The peroxisome is probably an important organelle with regard to redox homeostasis because of the production of H₂O₂ and other ROS during the β -oxidation of long-chains and polyunsaturated fatty acids and other metabolic processes (1). However, except for the presence of the catalase Cta1 and possibly also of the atypical 2-Cys Prx Ahp1 that carries a peroxisomal targeting sequence (46), it is not yet known whether thiol redox control enzymes and GSH are present in this organelle.

Similarly, the yeast vacuolar compartment hosts an important redox metabolism, principally operated by GSH that has not been yet really studied (see Figs. 1, 3, and 4). The ATP-binding cassette transporter yeast cadmium factor (Ycf1) is present at the vacuolar membrane where it mediates the ATP-dependent transport of GSH conjugates with metals such as cadmium (49, 50), and electrophiles. In the vacuole, these GSH S-conjugates are presumably degraded by γ -GT and a dipeptidase (56). Ycf1 is also proposed to mediate transport of GSH into the vacuole with low affinity (80), and could possibly also transport GSSG.

In the context of thiol redox control, the nucleus and cytosol can be considered a single compartment, based on the lack of a nucleocytoplasmic diffusion barrier for proteins < 40 kDa, and on the knowledge that, with the exception of Glr1, all thiol redox pathways components have masses < 40 kDa. Such an assumption is borne out by the observation that the GSH redox potential (E_{GSH}) is similar in the cytosol and nucleus (17). Only in cells lacking both cytosolic TRXs was E_{GSH} slightly more reduced in the nucleus compared to the cytosol.

Redox Linkage Between Compartments

We have so far considered thiol redox control mechanisms in each of the yeast cell compartments. It is now relevant to evaluate whether and how these mechanisms are linked to each other (Fig. 6).

Independence of cytosolic and mitochondrial matrix redox control

The nucleo-cytosol and mitochondrial matrix should have autonomous thiol redox homeostasis, as each carry entire sets of the TRX and GSH pathways, together with their own NADPH recycling system (see Figs. 1 and 2).

Combined mutations in genes encoding both cytosolic and mitochondrial thiol redox components (97) showed only minimal if any linkage in the redox control of these compartments. The synthetic interaction resulting from the simultaneous mutation of *Trr2* and *Glr1*, as already discussed above, indicated the overlapping role of these enzymes in mitochondrial matrix thiol redox maintenance. In fact, only additional loss of *Trr2* in $\Delta trr1$ produced a synthetic phenotype, manifested by an aggravation of the respiratory growth and peroxide tolerance defects already existing in the latter, which indicates that to some extent mitochondrial and cytosolic TRRs might compensate for each other. Other observations unambiguously indicate the independence of the cytosol and matrix redox controls. The exclusive mitochondrial genome instability phenotype of the *POS5* null mutation is a probable outcome of an exclusive matrix peroxide-scavenging defect (90). Another consequence of the $\Delta pos5$ mutation is an exclusive matrix increase of GSSG; reciprocally, mutation of *ZWF1*, which encode the first enzyme of the oxidative part of the pentose pathway, results in the exclusive cytosolic increase of GSSG (70). Using rxYFP, Hu *et al.* (33) further demonstrated that the GSH redox state of the cytosol and matrix are kept independently at different values (see Table 2). Re-expressing the cytosolic form of *Glr1* in $\Delta glr1$ corrected the GSH redox state of the cytosol but not the matrix, and reciprocally, with the mitochondrial form of *Glr1*. The latter condition also caused some correction of E_{GSH} in the cytosol, due to inefficient targeting of *Glr1* in the matrix. These results have recently been reproduced by another group, also concluding to the independence of the redox control of GSH in the cytosol and matrix (41).

Are ER and IMS compartments in redox linkage with the cytosol?

The ER and IMS share unique redox homeostatic features not shared with other compartments. In both of them, oxidative protein folding involves thiol oxidation by two similar redox relays (Figs. 3, 4, and 5). Furthermore, thiol reduction that is needed during oxidative protein folding is probably provided by GSH in both cases; however, there are no enzymes to recycle GSSG in either the ER or IMS. The mechanisms by which reduced GSH is replenished in the ER and IMS are still unknown. Whether such replenishment is a consequence of GSH import, and possibly also of GSSG egress, into and out of the lumen of these organelles is highly suspected (12) (Figs. 4–6).

In the ER, real-time E_{GSH} measurements upon tunicamycin-induced ER stress indicated a switch from very oxidizing to very reducing values that lasted hours (57). Could such reduction of the ER GSH pool be linked to a regulated GSH ER import? Existence of ER facilitated GSH transporters is suggested in another study, which examined the consequences of a 10-fold abrupt increase of total cellular GSH (44). A potent and long-lasting UPR was observed at the genome-wide level, along with a major alteration of iron metabolism (discussed above). Secretion of a disulfide-containing folding substrate was eventually arrested. Under these conditions, the ER became profoundly reduced, as manifested by the major and partial reduction of *Ero1* and *Pdi1*, respectively (Fig. 5). These ER manifestations were interpreted as a consequence of an import of GSH into the ER that perpetuated *Ero1* activation by

keeping the enzyme in the reduced, active conformation, and by providing reduced thiol-substrates in unlimited amounts. The fast and enduring reduction of the ER upon increase of GSH levels suggested a facilitated import of GSH into and trapping within this organelle. A regulated ER ingress of GSH is also suggested by the mechanism of *Ero1*^{C150SC295S} unable to form one of the enzyme's negative regulatory disulfides. Overexpression of *Ero1*^{C150SC295S} causes a marked growth inhibition and massive cell death that is explained by constitutive enzyme cycling (83). However, for enzyme cycling to perpetuate, increased supply of reduced-thiol substrates is needed, and the remaining *Ero1*-negative regulatory Cys residues must stay reduced. These two conditions could be fulfilled by continual ER ingress of GSH, an idea fully supported by the finding that GSH depletion both alleviates the growth defect of *Ero1*^{C150SC295S} and switches the enzyme from its fully reduced to its fully oxidized form (83).

E_{GSH} measurement in the IMS showed values that followed those of the cytosol but not the matrix (41). For instance, inactivating the cytosolic form of *Glr1*, which results in oxidation of the cytosolic GSH pool, also resulted in the oxidation of E_{GSH} in the IMS, whereas inactivating *Pos5*, which specifically oxidizes the matrix GSH pool, did not affect E_{GSH} in the IMS. This study also showed that inactivating *POR1*, which encodes the outer mitochondrial membrane channel porin, resulted in oxidation of the IMS GSH pool, suggesting that IMS ingress of reduced GSH through the porin channel is a mechanism ensuring the tripeptide reducing function in the IMS (see Fig. 6).

Conclusions and Perspectives

The TRX and GSH pathways are the two main and universally conserved disulfide reduction systems, and are therefore engaged in a diverse array of cellular functions that involve reversible disulfide formation. Survey of the data on these pathways in *S. cerevisiae* has highlighted several principles of their functional organization and compartmentation that can be now enumerated. In this organism, the TRX and GSH pathways are not functionally redundant, in contrast to *E. coli*. The pathways nonredundant functions differ with the compartment: in the cytosol, the TRX pathway exerts the dominant thiol redox control function, whereas the GSH pathway appears as backup of the former; in mitochondria, the GSH pathway seems to dominate over the TRX pathway. The mitochondrial matrix has an entire set of both pathways that exert a totally independent thiol redox control insulated from influences from other compartments. One main function of the matrix thiol redox system is to assist respiratory ROS scavenging. The ER and IMS are small organelles that lack thiol reductase systems, except for GSH. They host an intense oxidative thiol metabolism and therefore must generate GSSG. How GSH is replenished is unknown, but could involve fluxes of GSH and GSSG between these organelles and the cytosol (see Fig. 6). How these principles apply to higher eukaryotes is unknown, although they should help explore thiol redox control in mammals and plants.

Acknowledgments

This work was supported by grants from ANR (ERRed), FRM, and INCA to M.B.T., and from VLM to A.D.-M.

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Date of first submission to ARS Central, November 8, 2012;
 date of acceptance, December 1, 2012.

Abbreviations Used

Cys = cysteine
 DTT = dithiothreitol
 ER = endoplasmic reticulum
 FAD = flavin-adenine dinucleotide
 GLR = glutathione reductase
 GPX = glutathione peroxidase
 GRX = glutaredoxin
 GSH = glutathione
 GSSG = oxidized glutathione
 GST = glutathione S-transferase
 G-S-X = GSH S-conjugate
 H₂O₂ = hydrogen peroxide
 IMS = mitochondrial intermembrane space
 ISC = iron-sulfur cluster
 NADH = nicotinamide adenine dinucleotide
 NADPH = nicotinamide adenine dinucleotide phosphate
 PAPS = 3'-phosphoadenylylsulfate
 PDI = protein disulfide isomerase
 PRX = peroxiredoxin
 RNR = ribonucleotide reductase
 ROS = reactive oxygen species
 TRR = thioredoxin reductase
 TRX = thioredoxin
 UPR = unfolded protein response
 γ -GT = γ -glutamyl transpeptidase