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ORIGINAL RESEARCH COMMUNICATION

Glutaredoxin Participates in the Reduction of Peroxides by the Mitochondrial 1-CYS Peroxiredoxin in *Saccharomyces cerevisiae*

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Abstract

The mechanism for regeneration of the active-site "peroxidatic" cysteine in 1-Cys peroxiredoxins is a matter of debate. *Saccharomyces cerevisiae* Prx1 is a mitochondrial enzyme belonging to the 1-Cys Prx, whereas Grx2 is involved in antioxidant defense and localizes at the mitochondria, so we hypothesized that it could be a perfect candidate to resolve the sulfenate in Prx1 with GSH. *In vitro* experiments with purified Prx1p and Grx2p demonstrate that Grx2p, at concentrations $<1\ \mu M$, coupled to GSH, is a very efficient thiolic intermediary for the reduction of the peroxidatic Cys in Prx1p. Prx1p forms oligomeric aggregates natively, but depolymerizes down to a dimeric state after treatment with GSH. The catalytic cycle involves glutathionylation of dimeric Prx1p and deglutathionylation by Grx2p. Dihydrolipoamide, a genuine mitochondrial dithiol, can efficiently substitute for GSH. The activity is highest at alkaline pH, consistent with the conditions of active respiring mitochondria, and the process is highly specific for 1-Cys Prx because Grx2p is totally inactive with human PRX1, a typical 2-Cys Prx, as opposed to the promiscuity of Trx. Our results suggest that although Trx is the reductant involved in the reduction of peroxides by 2-Cys-Prx, Grx might be the natural resolving partner of 1-Cys Prx through a monothiolic mechanism. *Antioxid. Redox Signal.* 13, 249–258.

Introduction

Peroxiredoxins (Prxs) are a family of ubiquitous non-heme peroxide-scavenging enzymes also involved in redox signalling in mammalian cells (7, 38).They are among the most abundant proteins in most cells, which speaks of their relevance. They can act on a variety of peroxides from hydrogen peroxide to organic peroxides through peroxinitrite (2). In eukaryotic organisms, Prxs are not confined to the cytosol, but also are found within the mitochondria, chloroplasts, nucleus, peroxisomes, and extracellular regions (5, 12). Most organisms produce several Prx isoforms, some monomeric, other dimeric, and even changing to oligomers with chaperone activity with oxidative and heat stress (10).

A great variety of structural and biochemical properties are found among peroxiredoxins. Based on sequence homologies, five subfamilies have been proposed (5), but other classifications, based on catalytic mechanisms, distinguished six groups (38). For the purpose of the research reported herein, we would distinguish just two types of Prx: the so-called 2-Cys Prxs ("typical" or "atypical") all contain two cysteines per monomer that form a disulfide bond necessary for catalysis, and the 1-Cys Prxs that contain just one catalytic Cys residue per monomer. A third group of Prxs are called hBCP (PrxQ in plants); these are monomeric and share very little sequence and structural homology with the rest of the Prxs. All types are represented in *Saccharomyces cerevisiae* (22): Tsa1p and Tsa2p (typical 2-Cys-Prx, cytosolic); Ahp1p (atypical 2-Cys-Prx, cytosolic and peroxisomal); Prx1p (1-Cys-Prx, mitochondrial) and Dot5p (hBCP, nuclear peroxiredoxin).

Mechanistically, all Prxs have a first reaction step in common: the oxidation of the so-called peroxidatic cysteine (Cys-S_PH) to a sulfenic acid (Cys-S_POH) by the substrate peroxide. To close the catalytic cycle, this sulfenic peroxidatic cysteine has to be resolved in a second step. Here is where the different Prxs differ. In the 2-Cys Prxs, the second cysteine is the resolving group (Cys-S_RH), forming an intermolecular disulfide in the dimeric typical 2-Cys Prxs or an intramolecular disulfide in the monomeric atypical 2-Cys Prxs. The final

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catalytic step consists of reduction of this disulfide by one cellular reductant system composed of one flavoprotein disulfide reductase, and another protein containing a thiol or dithiol motif. The thioredoxin system composed by NADPH, thioredoxin reductase, and thioredoxin is the reductant for most peroxiredoxins, the reason that these proteins have also been named "thioredoxin peroxidases" (4). However, other reductants have been proposed in a few cases. For instance, an atypical 2-Cys Prx from poplar, similar to yeast Ahp1p, is additionally reduced by Grx (31).

The resolving mechanism for the 1-Cys Prxs is a matter of debate, as it cannot be explained by an inter- or intramolecular disulfide, because this class of Prxs does not possess a second resolutive cysteine. Yeast contain one single 1-Cys Prx, which is the mitochondrial peroxiredoxin, Prx1, and has been demonstrated to function in protection against oxidative setress (24). Mitochondrial Trx3 has been shown to be effective in resolving the peroxidatic sulfenic acid of Prx1 (24). This was questioned by another study on the basis of the lower efficiency of Trx3 with Prx1 compared with that of Trx with other yeast 2-Cys Prxs (22). Ascorbate, or its yeast counterpart erythroascorbate, has been also proposed as a nonthiolic reductant (19). More recently, another resolving mechanism was proposed (9). According to this proposal, glutathione, coupled to Trr2, but not Trx, is the physiologic electron donor for Prx1, with initial formation of a mixed disulfide between Prx1 and glutathione, followed by direct deglutathionylation of Prx1 by Trr2 (9). This was an unexpected finding, because some kind of specific interaction between GSH and Trr2 would be required, whereas Trx reductase is not a natural partner protein of glutathione, as are Grxs. Involvement of Trx reductase in a glutathionylation process has been demonstrated only for the human mitochondrial TRR2, which is particularly unspecific for disulfide substrates. Even in this unique case, the involvement is not direct, but with the intermediation of Grx (11).

These results prompted us to check whether the homologous mitochondrial Grx2 could act as the natural reductant of yeast Prx1p. The results we report in the present study demonstrate for the first time that Grx2 is actually an excellent catalyst for the recycling of Prx1 with either glutathione or lipoamide as reductants. In the course of the investigation, we also observed that GSH has an additional effect on the aggregation state of Prx1.

Materials and Methods

Materials

HEPES, NADPH, reduced glutathione, glutathione reductase, *tert*-butyl hydroperoxide, human PRX1, lysozyme, DNase I, and human plasma thrombin were purchased from Sigma, St. Louis, MO. Yeast strains CMML235 (WT) and MML44 (Δ*GRX2*) were a kind gift from Prof. E. Herrero, University of Lleida, Spain (29); strain Y13090 bearing a Δ*PRX1* deletion was obtained from Euroscarf collection; strain JR038 carrying a double mutation Δ*GRX2* and Δ*PRX1* was obtained mating MAT*a* and MATα haploid cells from strains MML44 and Y13090, followed by formation of diploids (MAT*a*/α), meiosis induction, ascospore formation, and haploid selection by micromanipulation. After selection by growth in selective medium, genotyope and phenotype were confirmed by using PCR and Western blot, respectively.

Expression and purification of the recombinant proteins

Cloning into the pET-15b expression vector (Novagen, Darmstadt) of the *TRR2*, *TRX3*, *PRX1,* and *GRX2* genes of *Saccharomyces cerevisiae*, excluding the coding sequences for the N-terminal signal peptide, was performed as described elsewhere (23–25). The resulting constructions were used to transform *Escherichia coli*.

Each transformant was inoculated in 0.5 L of LB medium containing 0.1 mg ampicillin per milliliter and grown at 37°C until $A_{600} = 0.5$. Then the recombinant protein was induced by adding 0.5 m*M* isopropyl-1-thio-β-D-galactopyranoside. Growth was continued either at 25°C for 1 night for the Trx3p, Trr2p, and Prx1p expression or at 37°C for 4 h for Grx2p. Cells were harvested and diluted in 25 ml of 20 m*M* TrisHCl, pH 8.0, containing 0.1 *M* NaCl, 15 mg lysozyme, and 0.3 mg DnaseI. This buffer also contained 10 m*M* β-mercaptoethanol in the case of cells expressing Prx1p. Cells were centrifuged, suspended in PMSF containing buffer, and disrupted by sonication. After centrifugation at 10,000 *g* for 30 min, the cell-free extract was loaded onto an IMAC column (TALON Metal Affinity Resin, Clontech, Madison, WI) equilibrated with 20 m*M* Tris-HCl, pH 8.0, and 100 m*M* NaCl (Buffer A). The column was washed with Buffer A containing 5 m*M* imidazole and the recombinant protein was eluted with Buffer A containing 100 m*M* imidazole. The His-tagged protein was digested with human plasma thrombin (4 units of thrombin/mg eluted protein) for 2 h at room temperature and then dialyzed against buffer A. Finally, the sample was passed through a Talon resin column equilibrated with Buffer A, and the flow-through contained the recombinant protein without His-tag.

The purity of the recombinant proteins Trr2p, Trx3p, Prx1p, and Grx2p, lacking their mitochondrial presequences, was determined with SDS-gel electrophoresis. Protein concentrations were determined from absorbance at 280 nm by using the molar extinction coefficients 10,010, 23,380, 23,710, and 4,470 M/cm for Trx3p, Trr2p, Prx1p, and Grx2p, respectively.

Assay of enzymatic activities

Peroxidase activity of the peroxiredoxins was determined spectrophotometrically by monitoring the disappearance of NADPH at 340 nm. For the thioredoxin-dependent peroxidase activity, the reaction mixture contained 50 m*M* HEPES, pH 7.0, 250 μ*M* NADPH, 0.5 μ*M* Trr2p, 5 μ*M* Trx3p, 5 μ*M* peroxiredoxin, and 100 μ*M* peroxide (H_2O_2 or *t*-BuOOH). For the glutaredoxin-dependent peroxidase activity, the reaction assay was composed of a mixture that contained 50 m*M* Tris-HCl, pH 8.0, 250 μ*M* NADPH, 1 m*M* GSH, 1 U/ml glutathione reductase (mixture named MIX), and also 5 μ*M* glutaredoxin, 5 μ*M* peroxiredoxin, and 100 μ*M* *t*-BuOOH. It should be stressed that although the concentrations of Grx2 and Prx1 in the standard assay are the same (5 μ*M*), this value is limiting for Prx1 but one order of magnitude over the concentration for maximum activity for Grx2 (see Fig. 3C and D). Under these conditions, the limiting factor is the analyzed component, Prx1.

Before the addition of the peroxide, the reaction mixture was incubated at 30°C for 5 min. Just after peroxide addition, the initial rate of NADPH disappearance was determined. One unit of enzymatic activity is defined as the oxidation of 1 μmol of NADPH/min.

Glutaredoxin activity was determined by monitoring spectrophotometrically the disappearance of NADPH at 340 nm due to the glutaredoxin-dependent reduction of 0.5 m*M* 2-hydroxyethyl disulphide (HED) in the presence of 0.5 m*M* GSH and 0.5 units of yeast glutathione reductase (16). One unit is defined as the oxidation of 1 μmol of NADPH/min.

The glutaredoxin-dependent peroxidase activity of Prx1p with lipoamide as electron donor was determined spectrophotometrically by measuring the rate of NADH oxidation according to the following protocol: an aliquote was taken from a 50 m*M* stock solution of lipoamide in 10% acetonitrile to prepare a 0.75 m*M* lipoamide solution together with 1 m*M* NADH, 1 U/ml lipoamide dehydrogenase (Sigma), 2 m*M* EDTA, 0.1 mg/ml BSA, and 100 m*M* Tris-HCl, pH 8; when required, 1 m*M* GSH, 5 μ*M* Grx2p, and 5 μ*M* Prx1p were also included. The absorbance of the reaction mixture was monitored at 340 nm until an equilibrium plateau was observed. Then, 100 m*M* *t*-BuOOH was added to the assay, and the decrease of A340 was registered during 5 min. One unit is defined as the oxidation of 1 μmol of NADH/min.

The concentrations of the coupled flavoenzymes were optimized so that they were not the limiting factors under the assay conditions.

Immunoblotting

To detect glutaredoxin and peroxiredoxin, samples were separated by using a 12% SDS-PAGE gel with the Bio-Rad mini-Protean III system. After electrophoresis, proteins were transferred by using semi-dry blotter onto nitrocellulose membranes. Membranes were blocked for 1 h with 1% BSA in TBS-T and washed 3 x 10 min with TBS-T. The same protocol was used for the detection of glutathionylated proteins in crude extracts. Primary antibodies Prx1 and Grx2 were prepared as described earler (24, 25), and anti-GSH (Virogen, Watertown, MA) was diluted to 1:1,000 and incubated overnight at 4°C. Membranes were washed and incubated with secondary antibodies, goat anti-rabbit (Sigma) for Prx1 and Grx2, and goat anti-mouse (Sigma) for anti-GSH. Chemiluminescent signal was detected by using a LAS-3000 camera (Fujifilm, Tokyo).

Gel-filtration chromatography

A Superose 6 HR 10/30 column coupled to an FPLC instrument (BioRad, Hercules, CA) was equilibrated with 50 m*M* Tris-HCl, pH 8, at a flow rate of 0.5 ml/min. Several standard preparations were run to calibrate the column: Blue Dextran, 200 kDa for void volume determination and alcohol dehydrogenase, 150 kDa. Of the samples to be analyzed, 100 μl was loaded, containing mixtures made up to 20 μ*M* Prx1p $\pm$ 1 m*M* GSH in equilibrating buffer. The mixtures were incubated for 30 min at room temperature before loading onto the column. Absorbance at 280 nm after elution was monitored.

Results

Prx1p accepts electrons from the mitochondrial thioredoxin system Trr2p/Trx3p with low efficiency

Prx1p was incubated with the recombinant proteins of the yeast mitochondrial thioredoxin system and NADPH until a stable reading was obtained at 340 nm. The substrate peroxide, H_2O_2 or *t*-BuOOH, was then added, and the disappearance of NADPH was measured immediately. Figure 1 shows that Prx1 is equally active with both peroxides, and the reaction rate is proportional to the amount of Prx1p concentration. For comparison, recombinant PRX1 from human, a 2-Cys Prx whose accepting electrons from Trx are well known, also was assayed under exactly the same conditions. The activity was three-fold higher than with Prx1p.

We conclude that yeast Prx1p peroxidatic thiol can be resolved by the homologous thioredoxin system, but less efficiently than the peroxidatic thiol of heterologous human PRX1.

Prx1p accepts electrons from mitochondrial glutaredoxin Grx2p

Having observed that Prx1p reduces H_2O_2 or *t*-BuOOH with the same efficiency, we used the latter to check whether Grx2p could also serve the resolving role in the catalytic cycle. H_2O_2 is not a convenient substrate for the *in vitro* reaction assay, because in the presence of GSH and the GR preparation, NADPH is rapidly consumed, thus masking the peroxidase activity. This does not occur with *t*-BuOOH, which was used thereon. Prx1p was incubated with NADPH and the glutathione system plus Grx2p, and then the reaction was started by addition of the peroxide substrate. As shown in Fig. 2, Grx2p is very efficient at reducing yeast Prx1p, but interestingly enough, it does not reduce human Prx1, a 2-Cys Prx. This behavior is contrary to that of Trx3p, as shown in the previous paragraph, and indicates a high degree of specificity in the interaction between Grx2p and Prx1p.

The activity was characterized further to check how the concentration of the different components of the system affects the activity. Figures 3A and B show that the activity is proportional to the concentrations of GSH and peroxide. Too-high concentrations of peroxide inactivate the enzyme, as expected, and the activity is maximal at GSH concentrations below those that prevail in the cell. A strict dependence on the concentration of Prx1p also exists at <4 μ*M* (Fig. 3c), whereas the reaction is maximal at Grx2p concentrations <1 μ*M* (Fig.

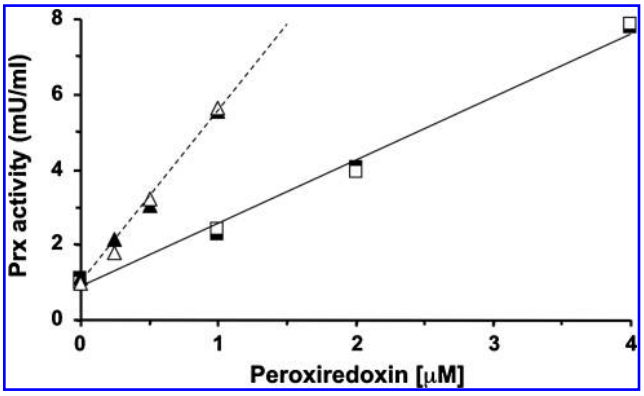

FIG. 1. Thioredoxin-dependent peroxidase activity of Prx1p. NADPH oxidation with different concentrations of either human PRX1 (*triangles* and *dotted line*) or yeast Prx1p (*squares* and *continuous line*) in the presence of 0.5 μ*M* Trr2p, 5 μ*M* Trx3p, and 100 μ*M* of either H_2O_2 (*solid symbols*) or *t*-BuOOH (*open symbols*). mU/ml, nmol oxidized NADPH per minute, and milliliter of assay.

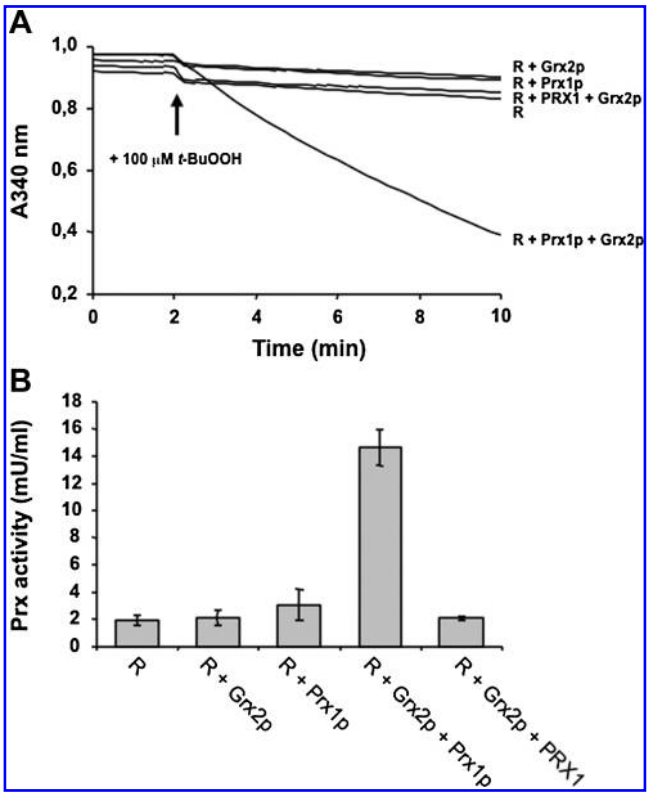

FIG. 2. Glutaredoxin-dependent peroxidase activity of Prx1p. (**A**) Decrease in absorbance due to the oxidation of NADPH in a reaction mixture containing 250 μM NADPH, 1 mM GSH, and 1 U/ml glutathione reductase (reference assay, R), and also containing 5 μM Grx2p and/or 5 μM peroxiredoxin (human PRX1 or yeast Prx1p). The *arrow* indicates the addition of 100 μM *t*-BuOOH to the assay. (**B**) Consumption of NADPH after adding 100 μM *t*-BuOOH to the different reaction mixtures described. Error bars represent the standard deviation (SD) from three different determinations.

3d). The affinity for Trx3p was about one order of magnitude lower than that for Grx2p (not shown). When rat GRX1 substituted yeast Grx2p, the activity was twofold lower, whereas the activity in the standard glutaredoxin assay with HED was twofold higher (see Table 1, Ref. 27). This further documents the concept of Grx as a general reductant for 1-Cys Prxs.

Optimal connection between Grx2p and Prx1p occurs at conditions similar to those of functional mitochondria

The optimal pH values for the peroxidase reactions catalyzed by Prx1p with Grx2p and with Trx3p/Trr2p were determined and compared (Fig. 4a). The optimal pH with Grx2p was ~8.5 and 6.5 with Trx3p. GR is likely inhibited at high pH, so that the pH profile beyond pH 9 is not likely to be an accurate reflection of the glutaredoxin activity, although Grx2p would be responsible for the steep increase in activity from pH 7 to pH 9.

Grx2p would then be the best reductant in the matrix of actively respiring mitochondria, whereas Trx3p would act optimally as reductant in mitochondria only under fermentative conditions, according to our data.

Conversely, Grx2p had been shown to catalyze the transfer of electrons from the dithiol of lipoamide to GSSG, thus functioning as a real glutathione reductase mechanism independent of NADPH and GR (1, 28). Because lipoamide is a genuine mitochondrial redox-active dithiol, we checked its possible connection to Prx1p. As shown in Fig. 4b, a lipoamide system consisting of NADH, LPD, and lipoamide is an efficient electron source for reduction of peroxide by Prx1p in the presence of Grx2p. The interesting part of this result is that the system can function to a moderate extent in the absence of GSH, which could constitute a mechanism for direct tapping of reducing power from NADH, the most abundant and readily available mitochondrial electron donor in active mitochondria.

In vivo *connection of Grx2p and Prx1p*

Under normal growth conditions, a background production of ROS and protein sulfhydryl groups is oxidized to some extent (13). Mild oxidation to sulfenic acid promotes increased reactivity to GSH (32), so that glutathionylation is indicative of reversible oxidative modification of proteins. Figure 5 shows that when Prx1 is active, glutathionylation is negligible in either the wild type or in the Δ*GRX2* mutant. However, lack of Prx1p induces some degree of glutathionylation, which is highly enhanced by the concomitant absence of Grx2p. Identification of the glutathionylated proteins has not been attempted in this study, but under the conditions used for crude extract preparation, both cytosolic and organellar proteins are represented.

GSSG glutathionylates and GSH induces dissociation of Prx1p

Incubation with GSSG induces glutathionylation of mainly Prx1p dimer, but the monomer is also modified to some extent (Fig. 6). Moreover, the presence of Grx2p seems to enhance the degree of glutathionylation (see, for instance, lane 4 in Fig. 6). Glutathionylation has been shown to occur also in other Prxs (17, 20, 21) by incubation of the enzyme with GSSG. Although this is a demonstration that Prx1p can actually form a mixed disulfide with glutathione, the conditions of the process do not reflect the situation that must take place *in vivo*. Elimination of peroxides by Prxs is a reductive process in which the electrons initially transferred from the enzyme to the substrate are subsequently recovered from a thiol donor, GSH in our case, not GSSG. So we tried to detect the putative Prx1p glutathionylated intermediate under conditions of the peroxidase assay. Peroxidase reaction mixtures lacking a reduction backup system were analyzed with nonreducing SDS-PAGE followed by Western blotting and developing with specific anti-GSH, anti-Grx2, and anti-Prx1 antibodies to detect stable intermediates. Anti-GSH blot did not detect any glutathionylated Prx1p or Grx2p species. No band at molecular mass of ≈36 kDa, or multiples indicative of a heterodimer between Grx2p and Prx1p, could be detected consistently in either Prx1 or Grx2 blots (data not shown); gel-filtration chromatography of these reaction mixtures confirmed the absence of stable mixed disulfide between both proteins (data not shown).

These results seem to rule out the formation of any stable mixed disulfide between GSH and Prx1p under conditions

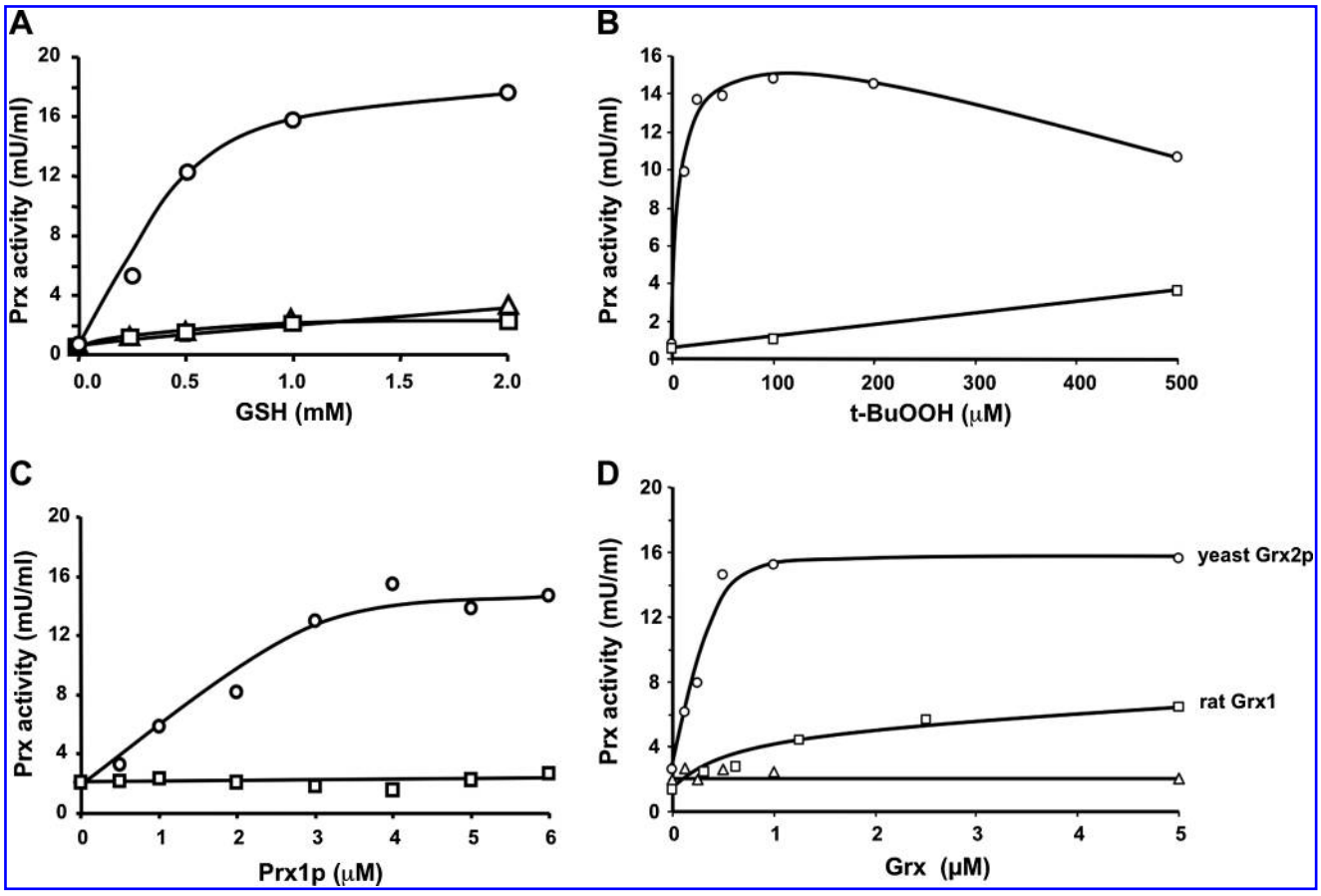

FIG. 3. Characterization of the Grx2p-dependent peroxidase activity of Prx1p. (**A**) Dependence on the concentration of GSH and with 5 μM of either Grx2p (squares), Prx1p (*triangles*), or both (*circles*). (**B**) Dependence on the peroxide concentration: 5 μM Prx1p at different concentrations of *t*-BuOOH in a reaction mixture containing the MIX components with (*circles*) or without (*squares*) 5 μM Grx2p. (**C**) Activity was proportional to the concentration of Prx1p: *circles* correspond to the values of NADPH consumption in assays containing 5 μM Grx2p, and *squares* correspond to assays without glutaredoxin. (**D**) Homologous Grx2p is more efficient than heterologous Grx:NADPH oxidation with different concentrations of Grx2p in assays containing 5 μM Prx1p (*circles*) or without peroxiredoxin (*triangles*). *Squares* correspond to the values of NADPH consumption in assays with Prx1p and rat glutaredoxin.

that resemble those of the standard assay. However, a transient short-lived glutathionylated Prx1 species cannot be excluded, because a trace of it could be detected with MALDI-TOF mass spectrometry on incubation of Prx1p with 0.5 mM H_2O_2 and 1 mM GSH for 4 h, followed by trypsin digestion (9).

In Western-blot analyses, Prx1p was consistently detected, mostly as high-order oligomers of different size that disappear in the presence of GSH. This was confirmed in a straightforward experiment with FPLC gel-filtration chromatography under milder conditions (Fig. 7). The predominant species seems to have an apparent size of $\sim$150 kDa, which should roughly correspond to a hexamer, whereas after incubation with GSH, the predominant lower-size Prx1p species obtained is the dimer. However, reduction with β-ME or DTT produces essentially the monomeric form. Interestingly enough, the enzyme is not glutathionylated after GSH treatment, and most of the protein remains in the dimeric state after glutathionylation with GSSG (Fig. 6). These results indicate that glutathione has a profound influence on the structure and function of Prx1p, but the effects of the reduced, GSH, and oxidized GSSG forms are markedly distinct.

Discussion

Reduction of peroxides by mitochondrial yeast Prx1, a 1-Cys Prx, is of importance to protect this organelle from oxidative stress (24). During the catalytic cycle, the active-site (peroxidatic) cysteine is oxidized to sulfenic acid by the peroxide substrate. Fast recovery (resolution) of this cysteine back to the sulfhydryl state is critical for the catalytic efficiency of the enzyme. This process requires an electron source and an appropriate thiol-containing mediator. The former is usually fulfilled by NADPH or NADH with the participation of a suitable flavoenzyme, but the nature of the intermediary reductant thiol varies among the different types of Prxs. Thioredoxin (24), glutaredoxin (30), thioredoxin reductase (9), sulfiredoxin (21), or even a dedicated peculiar protein AhpD (3) have been shown to fulfill this role. In the case of 1-Cys Prxs, the nature of the thiol reductant is a matter of debate.

Grx2 is a good partner for Prx1

We show that homologous Grx2p can efficiently support the peroxidase activity of yeast Prx1p *in vitro* in a system

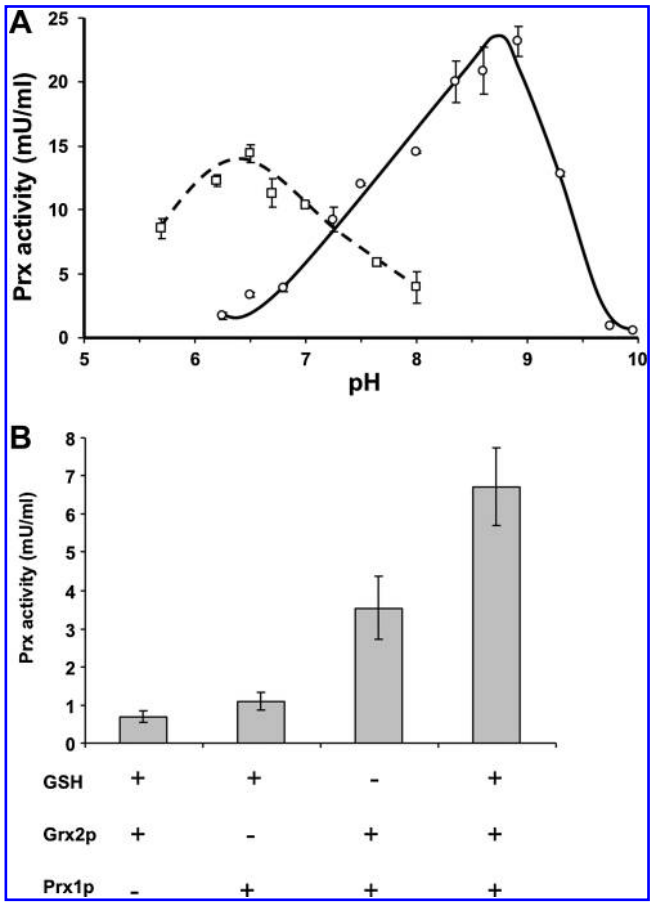

FIG. 4. Peroxidase activity of Prx1p under "mitochondrial" conditions. (**A**) Optima pH of the Trx- and Grx-dependent reaction: glutaredoxin-dependent (*circles and continuous line*) and thioredoxin-dependent (*squares and dotted line*) peroxidase activity in the presence of 100 μM *t*-BuOOH. The pH was adjusted in each assay by adding NaOH or HCl and using an electrochemical sensor (Biotrode, Hamilton, Ontario, Canada). Error bars represent the standard deviation (SD) from three different determinations. (**B**) Lipoamide-dependent reduction of peroxides: NADH oxidation was analyzed in a reaction mixture, described in Materials and Methods, containing 1 mM GSH, 5 μM Grx2p, and 5 μM Prx1p where appropriate, as is indicated. Error bars represent the standard deviation (SD) from three different determinations.

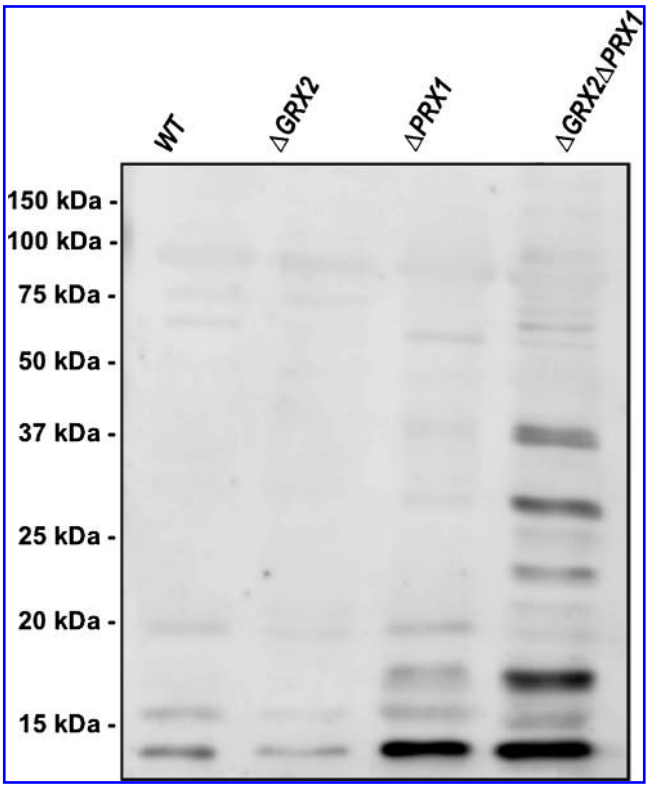

FIG. 5. Analysis of protein glutathionylation in crude extracts of mitochondrial redoxin mutants of *Saccharomyces cerevisiae*. Crude extracts of exponentially growing cells from different yeast strains were subjected to SDS-PAGE and Western blotting with anti-GSH antibodies. WT, wild type; Δ*GRX2* and Δ*PRX1*, cells lacking *GRX2* and *PRX1* genes, respectively; Δ*GRX2*Δ*PRX1*, a double mutant lacking both redoxins.

containing NADPH, GSH, and glutathione reductase. The Prx/Grx connection seems to be specific for 1-Cys Prxs because another Prx of the 2-Cys Prx type, human PRX1, is not a substrate for Grx2p. On the contrary, the thioredoxin system seems to be promiscuous. For the yeast *S. cerevisiae*, it has been taken for granted that all peroxiredoxins are strictly dependent on Trx and are not reduced by Grx *in vitro* (18). Our results clearly demonstrate that this assumption is incorrect.

Despite this newly proven role of Grx2, the connection between Prx1 and the mitochondrial Trx system, composed of Trx3 and Trr2 as the resolving thiol donor (24), still takes place *in vitro* [see Fig. 4A and also confirmed by (9)]. A similar ambivalence occurs for plant Prx-D, an atypical 2-Cys Prx (31). However, Prx1p is not a good substrate for its homologous Trx3p, because the activity is lower than that with heterologous human *PRX1*. Moreover, Trx3p optimal pH for the transference of electrons to Prx1p is lower than the pH of active mitochondria. At pH that prevails in respiring mitochondria, activity with Trx3 is rather low, whereas Grx2 shows its optimal activity. Hence, Grx2 should be the predominant donor for Prx1 in respiring mitochondria. This may explain why Trx3 mutants are not sensitized toward oxidative stress (36) and why the loss of *TRX3* does not affect the redox state of Prx1 *in vivo* (9).

It has been pointed that *in vitro* studies with pure preparations of the proteins have limitations because of the promiscuity of redox reactions, so that validation with *in vivo* studies is important (9). The activity of Grx2p with Prx1p, demonstrated here *in vitro,* agrees well with *in vivo* results in which deletion of the *GRX2* gene diminishes the resistance of yeast cells to oxidative stress (14) and with the co-localization of Grx2p and Prx1p in the mitochondria (25, 27).

A connection between Grx2p and Prx1p *in vivo* has been observed (Fig. 5). The mere absence of Grx2p does not contribute to accumulation of glutathionylated proteins, as mitochondrial background peroxide production is supposedly counteracted by Prx1p with the help of an alternative electron donor (*e.g.*, Trx3p). When mitochondrial Prx1p is absent, an increase occurs in the number of modified proteins and in the degree of glutathionylation as a consequence of inefficient

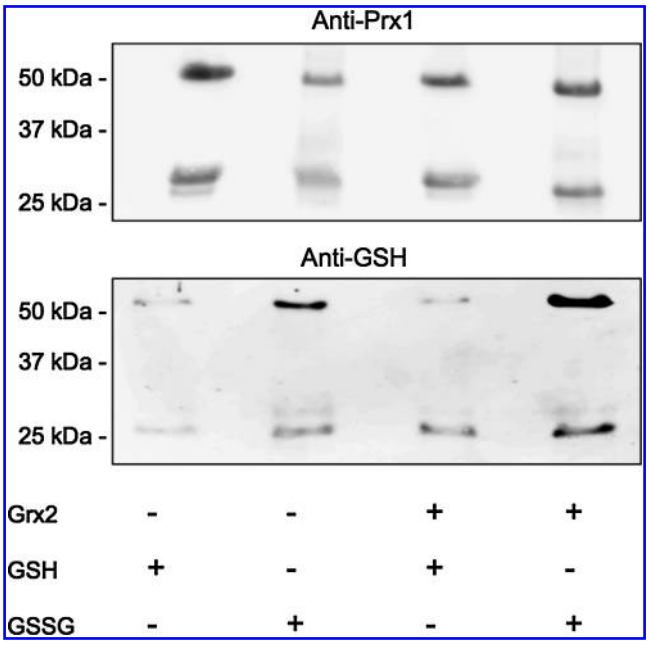

FIG. 6. Glutathionylation of Prx1p demonstrated by Western blotting. (**Upper panel**) Immunoblot with anti-Prx1; (**lower panel**) Immunoblot with anti-GSH. Preparations containing 10 μM Prx1p were incubated with 10 m*M* GSH, 10 m*M* GSSG, or 10 μM Grx2p, or a combination of these, as indicated in the figure. The samples were then subjected to nonreducing SDS-PAGE and Western blot.

elimination of peroxides. Moreover, the simultaneous lack of both mitochondrial redoxins, Grx2p and Prx1p, has a synergistic effect leading to extensive oxidation of protein cysteines. These results speak of the need for Grx2/Prx1 couple to get rid of peroxides efficiently and to avoid its consequences in mitochondria and confirm the involvement of Grx2 in the mitochondrial antioxidative defense systems *in vivo*.

Mechanism of Grx2-supported peroxidase activity

According to the involvement of Grx2p in the catalytic cycle of Prx1p, the mechanism of peroxide reduction by Prx1 should follow the sequence of reactions depicted in Fig. 8. The formation of a transient mixed disulfide between Prx1p and GSH is highly favored by the reactivity of the sulfenic group (26, 35). We show that Prx1p is glutathionylated by GSSG. Although this is a demonstration that Prx1p can form a stable mixed disulfide with glutathione, GSSG is rather unlikely to be the prevailing species of the glutathione redox pair, even under oxidative conditions, so that *in vivo*, glutathionylation should take place by reaction of GSH with the nascent sulfenic derivative [reactions (1) and (2)].

The equilibrium of reactions (3) + (4) must be highly displaced to the right in the presence of GSH, so that the substrate for Grx2p, glutathionylated Prx1p, is a short-lived transient intermediate. This should explain why the Prx1-S-SG species is difficult to detect *in vitro* under the conditions of the peroxidase reaction, as the relative concentrations of peroxide, reduced enzyme, and glutathione, as well as the reaction time, have to be chosen carefully. The finding of just a trace of the glutathionylated Prx1 in the MALDI-TOF spectrum (9) should be considered a significant result. Glutathionylation of Prx is usually achieved experimentally to a measurable extent by prolonged incubation with 5 m*M* GSSG or higher (9, 17, 20, 21) to force the reaction to the left:

$$(3) + (4) \qquad \text{Prx1-S-SG} + \text{GSH} \rightarrow \text{Prx1-SH} + \text{GSSG}$$

We have not been able to detect any trace of a putative Prx1p-Grx2p covalent binary complex, which, if formed, should be rather stable under non-reducing conditions. This result is coherent with the monothiolic mechanism shown in Fig. 8, in which formation of such a protein–protein covalent interaction is unnecessary. Here Grx2 recognizes and attacks the sulfur atom of the glutathione moiety (reaction 3) consistent with the canonic deglutathionylation monothiolic mechanism of glutaredoxin (6, 8, 15). The sulfur atom of the Prx1p moiety is not attacked, and the peroxidase is released free in the reduced Prx1-SH thiolic form, ready for a new catalytic cycle. Finally, Grx2-(SH)S-SG is recycled to Grx2-$(SH)_2$ by reduction of the glutathione mixed disulfide with another molecule of GSH (reaction 4).

Formation of a covalent heterodimer between Prx1p and Grx2p would be necessary only if a dithiolic mechanism, similar to the typical Trx-dependent mechanism, were operative (reactions 5 to 7 in Fig. 8). GSH and Grx2$(SH)_2$ would compete for the reaction with the nascent sulfenic acid in

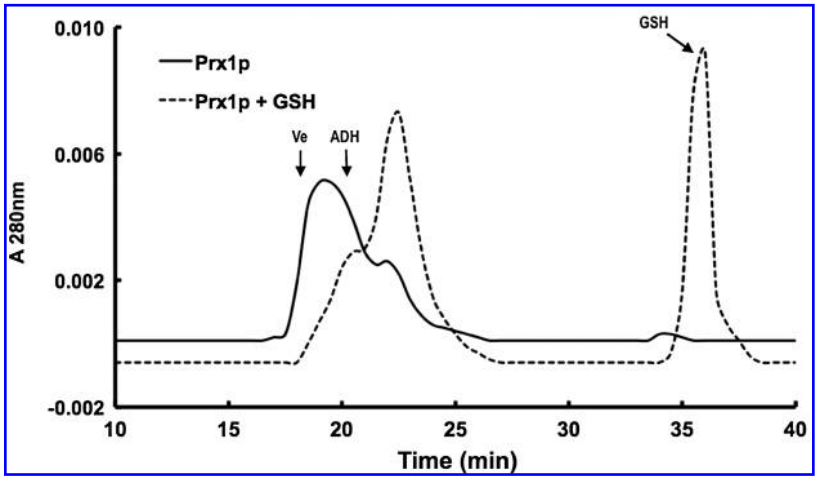

FIG. 7. Aggregation state of Prx1p determined by gel-filtration chromatography. The *arrows* indicate the elution position of standards [Blue dextran for exclusion volume (V_e) and alcohol dehydrogenase (ADH), 150 kDa]. *Continuous line*, purified recombinant Prx1p; *discontinuous line*, Prx1p incubated with 1 m*M* GSH for 30 min at 30°C before the chromatography. Concentration of Prx1p was 20 μM.

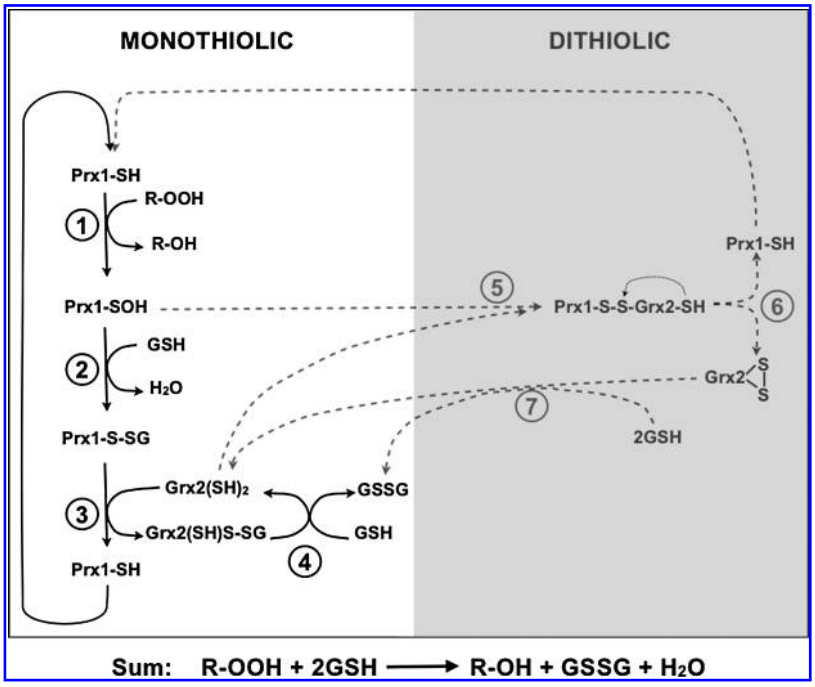

FIG. 8. Proposed mechanism for reduction of peroxides by the Prx1/Grx2 system. The substrate peroxide oxidizes the active cysteine of Prx1 to sulfenic (reaction 1). In a monothiolic mechanism, the sulfenate reacts immediately with GSH, resulting in glutathionylation of Prx1 (reaction 2). Then reduced Grx2 deglutathionylates Prx1, retaining the glutathione moiety and setting Prx1 free for a new catalytic round (reaction 3). Finally, a second GSH molecule recovers $Grx2(SH)_2$ (reaction 4). The sum of this sequence of reactions is shown. A hypothetical dithiolic mechanism is depicted at right, according to which the sulfenated Prx1 reacts with reduced Grx2 instead of GSH, with formation of a heterodimeric complex between the proteins (reaction 5). The complex is resolved by the C-terminal cysteine of the Grx2 active-site dithiol, leaving active Prx1 and oxidized Grx2 (reaction 6). Finally, two molecules of GSH recover the reduced form of Grx2 (reaction 7). The sum reaction is the same as in the monothiolic mechanism, although the dithiolic mechanism is rather unlikely under *in vivo* conditions, as discussed in the main text. Glr and NADPH would recycle GSH by the reduction of GSSG.

Prx1p, so that reactions 2 and 5 would exclude each other. In other words, formation of Prx1p-Grx2p heterodimer (dithiolic mechanism) and glutathionylation of Prx1p (monothiolic mechanism) could not occur at the same time. One would expect that, in the presence of ≈ 5 m*M* concentration of glutathione, GSH would be the prevailing reactant, and the dithiolic mechanism would not be operative.

Covalent protein–protein interaction has been put forward as an obligate step for the action of an atypical 2-Cys Prx from poplar, for both the dithiolic and the monothiolic mechanism (30). This proposal is based on the identification of the heterodimeric complexes under forced experimental conditions by using mutated forms of Grx and Prx and in the presence of the oxidant diamide. However, the wild-type Grx was unable to create any stable association with any Prx (30). This experimental evidence supports the concept that a heterodimeric complex between Grx and Prx would be possible under artifactual conditions, but thermodynamic reasoning in the terms stated in the previous paragraph would exclude this possibility under normal GSH/GSSG redox-buffering conditions.

A covalent complex between Prx1p and Trr2p has also been proposed as part of a hypothetical mechanism in which, contrary to the essence of Trx reductase properties and catalytic mechanism (37), the active-site cysteine of Trr2p cannot resolve the disulfide linking both proteins (9). Reductive input by GSH is then required. In the depicted mechanism of this proposal, one molecule of GSH is produced at an intermediate step under the same conditions that three other GSH molecules are consumed together during the very same peroxidase catalytic cycle (9). The reasoning behind this proposal seems thermodynamically unsound, which, together with the arguments described in the previous paragraph, speak against this mechanism.

Lipoamide as electron source for peroxide reduction in mitochondria

Reduction of peroxide by Prx1p also can be accomplished with Grx2p and lipoamide in the absence of glutathione. Reduction of peroxides with NADH as the electron source and lipoamide as the thiol mediator opens a panoply of speculations. Lipoamide is a genuine mitochondrial coenzyme covalently bound to enzyme E2 of the 2-oxoacid dehydrogenase complexes and to protein H of the glycine cleavage system. Both enzymes are abundant in the mitochondria. In both cases, lipoamide is reduced to capture part of the energy liberated from the decarboxylation of 2-oxoacids or glycine and to channel it to the production of reducing power in the form of NADH. We have demonstrated that this reducing power can be diverted by Grx2p to be used directly in the mitochondrial thiolic pool for antioxidant defense or ribonucleotide reduction (1, 28). Direct reduction of peroxide with lipoamide in the presence of Prx1p and Grx2p, as shown here, is more evidence in favor of this contention. In *Mycobacterium tuberculosis*, a specific thioredoxin-like adaptor protein, AhpD links lipoamide in E2 to peroxiredoxin AhpC (3), but the specificity and exact connection between Grx2p and the lipoamide-containing enzymes in yeast is still under investigation.

Oligomerization state of Prx1

When analyzed in crude cell-free extracts, Prx1p is natively oligomerized, but subsequent treatment with GSH promotes dissociation of Prx1p from an oligomeric to a dimeric state. Reduction with DTT or βME, however, produced mostly the monomeric form. Prx1p has been shown to form a disulfide-bonded dimer *in vitro* (24). According to our results, the dimeric form seems to be the natural state of Prx1p in the

presence of GSH, which is not capable of reducing this disulfide fully, even with the help of Grx2p.

Glutathionylation of the peroxidatic Cys in plant D-Prx induces dissociation of the non-covalent dimer to the monomeric state; reversion to the dimeric form can be achieved by reduction of the mixed disulfide with DTT (20). Our results differ from these, in that glutathionylation does not break the interaction that stabilizes the dimer.

Conclusions

It is demonstrated for the first time that Grx is the resolving thiolic intermediary for 1-Cys Prx. Contrary to the general assumption that yeast Prxs are strictly dependent on Trx (18, 34) we show here that Grx is an efficient component of the mitochondrial peroxidase system of the budding yeast *S. cerevisiae*. The only precedent of a Grx-dependent peroxidase is that of a plant atypical 2-Cys Prx (20, 30, 31). In the mechanism presented here, glutathione is involved in peroxidase action, together with its natural partner Grx. A role of Grx similar to the one in the present study has just been described for the regeneration of *Arabidopsis/ thaliana* 1-Cys methionine sulfoxide reductase (33). Trx appears to be specialized in the regeneration of Prx and MSR possessing "resolving" cysteines and whose catalytic cycle involves intra- and intermolecular disulfide bonds, whereas Grx can be envisaged as a key element responsible for the reduction of the catalytic cysteine in 1-Cys Prx and 1-Cys MSR.

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Author Disclosure Statement

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Abbreviations Used

βME = β-mercaptoethanol
Glr = glutathione reductase
Grx or GRX = glutaredoxin
HED = hydroxyethyl disulfide
LPD = lipoamide dehydrogenase
MSR = methionine sulfoxide reductase
Prx or PRX = peroxiredoxin
t-BuOOH = *tert*-butyl hydroperoxide
Trr or TRR = thioredoxin reductase
Trx or TRX = thioredoxin

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