Mitochondria of *Saccharomyces cerevisiae* Contain One-conserved Cysteine Type Peroxiredoxin with Thioredoxin Peroxidase Activity*

Received for publication, June 28, 1999, and in revised form, February 1, 2000

José Rafael Pedrajas, Antonio Miranda-Vizuete, Negin Javanmard, Jan-Åke Gustafsson, and Giannis Spyrou‡

From the Department of Biosciences at Novum, Karolinska Institute, S-141 57 Huddinge, Sweden

Peroxiredoxins are ubiquitously expressed proteins that reduce hydroperoxides using disulfur-reducing compounds as electron donors. Peroxiredoxins (Prxs) have been classified in two groups dependent on the presence of either one (1-Cys Prx) or two (2-Cys Prx) conserved cysteine residues. Moreover, 2-Cys Prxs, also named thioredoxin peroxidases, have peroxide reductase activity with the use of thioredoxin as biological electron donor. However, the biological reducing agent for the 1-Cys Prx has not yet been identified. We report here the characterization of a 1-Cys Prx from yeast *Saccharomyces cerevisiae* that we have named Prx1p. Prx1p is located in mitochondria, and it is overexpressed when cells use the respiratory pathway, as well as in response to oxidative stress conditions. We show also that Prx1p has peroxide reductase activity *in vitro* using the yeast mitochondrial thioredoxin system as electron donor. In addition, a mutated form of Prx1p containing the absolutely conserved cysteine as the only cysteine residue also shows thioredoxin-dependent peroxide reductase activity. This is the first example of 1-Cys Prx that has thioredoxin peroxidase activity. Finally, exposure of null Prx1p mutant cells to oxidant conditions reveals an important role of the mitochondrial 1-Cys Prx in protection against oxidative stress.

Reactive oxygen species (ROS)\(^1\) such as the superoxide anion (O\(_2^·\)), hydrogen peroxide (H\(_2\)O\(_2\)), and the hydroxyl radical (HO\(^·\)) are generated in cells as major by-products of respiration and by a wide range of different environmental chemicals, ionizing radiations, or high oxygen pressures. Increased levels of ROS, referred to as oxidative stress, can damage DNA, proteins, and lipid membranes (1, 2). ROS can also induce uncontrolled cellular proliferation, aging, and programmed cell death (3–6). To cope with potentially destructive ROS, all aerobically growing organisms have developed antioxidative enzymes and antioxid-
dant molecules to protect essential cellular components against oxidative modifications such as superoxide dismutases, catalases, peroxidases, thioredoxin, and glutathione.

Peroxiredoxins (Prxs) form a recently discovered large family of antioxidant enzymes that act as peroxidases reducing hydrogen peroxide and alkyl hydroperoxides to water or the corresponding alcohol, respectively (for a review see Ref. 7). The activity of Prxs is dependent upon an absolutely conserved cysteine that is essential for the hydroperoxide reduction step (8, 9). Peroxiredoxins differ from other peroxidases in that they have no redox cofactors such as metals or prosthetic groups (10). The Prx superfamily has been divided into two subgroups, 1-Cys Prx and 2-Cys Prx, according to the presence of one or two conserved cysteine residues. 2-Cys Prxs contain two conserved cysteine residues and use electrons donated by thioredoxin and were thus also named thioredoxin peroxidases (TPx) (8, 11–13). Thioredoxin (Trx) in its turn is reduced by NADPH through the flavoenzyme thioredoxin reductase (Trr), the so-called thioredoxin system (14). The bacterial alkyl hydroperox-
dioxide peroxidase (AhpC) also belongs to the 2-Cys Prx group, but it is reduced by a thioredoxin reductase-like flavoprotein (AhpF) using NADH or NADPH (15). 2-Cys Prxs form homodimers, and substitution of any conserved cysteine residue abolishes the Trx-coupled peroxidase activity (8).

The 1-Cys Prx proteins only contain the absolutely conserved residue. So far only one 1-Cys Prx present in human (hORF6 enzyme) has been characterized (9, 16). In contrast to 2-Cys Prxs, human 1-Cys Prx does not use thioredoxin as electron donor, but it can reduce H\(_2\)O\(_2\) using electrons from dithiothre-
tol (DTT). This human 1-Cys Prx has shown peroxidase activ-
ity *in vivo*, but the physiological electron donor has not been identified (9). The first member of the TPx family was charac-
terized in the yeast *Saccharomyces cerevisiae* as a 25-kDa protein that confers protection against damage by the thiol oxidation system (Fe\(^{3+}\), O\(_2\), and either DTT or 2-mercaptoeth-
anal), and it was named thiol-specific antioxidant (Tsa) (10, 17). Thus far, three proteins with thioredoxin peroxidase activity have been identified in *S. cerevisiae*: two very homologous Tsa isoenzymes named Tsa1p and Tsa2p, also named type Ia TPx and type Ibb TPx, and one alkyl hydroperoxidase Ahp1p, also known as Type II TPx (10, 18–21). For both types of thiore-
doxin peroxidases, the existence of two cysteines (Cys\(_{17}\) and Cys\(_{27}\) for Tsa and Cys\(_{52}\) and Cys\(_{129}\) for Ahp1p) is essential to maintain the Trx-dependent peroxidase activity (8, 17, 20, 21).

*S. cerevisiae* genome contains a sequence (ORF YBL064C) that codes for a protein homologous to human 1-Cys Prx. We have characterized the product of this open reading frame, which we have named Prx1p. In this paper, we provide evidence showing that this protein is a mitochondrial 1-Cys Prx and uses electron as donor the mitochondrial thioredoxin sys-
tem that we have previously characterized in *S. cerevisiae* (22).
Finally, we show that Prx1p has a protective role in vivo against oxidative stress.

MATERIALS AND METHODS

Strains and Media—The yeast strain FY1679 (MATa ura3-52 his3200) was used for the construction of the Δprx1 mutant. Yeast peptone medium with glucose (YPD) and synthetic complete medium without uracil (SC Ura−) were prepared as described (23).

Cloning of S. cerevisiae PRX1 Gene—To clone the PRX1 gene, two primers (PRX1-NdeI, 5′-AGGAAAGCAACATATGTTATGTCACATTGT-3′, forward, and PRX1-BamHI, 5′-CTCTTGATCCAGGTATTTGCAC-3′, reverse) were designed according with the sequence obtained from the data base (Saccharomyces Genome Data Bank, ORF YBL064C). The forward primer contained the initiation codon (bold letters) and an NdeI site (underlined), and the reverse primer contained the stop codon (bold letters) and a BamHI site (underlined). The primers were used to amplify S. cerevisiae genomic DNA by PCR (30 cycles at 96 °C for 1 min, 60 °C for 1 min, and 68 °C for 2 min) with the Expand Long Template PCR system (Roche Molecular Biochemicals). The PCR product was cloned into the pGEM-T Easy Vector System I (Promega) and sequenced.

Expression and Purification of Recombinant Prx1 Proteins in Escherichia coli—The amplified PRX1 fragment was subcloned into the NdeI/BamHI sites of the pET-15b expression vector (Novagen), fusing the cloned fragment to a sequence that codes for a polypeptide of 20 amino acids at the N terminus, containing histidine residues (His tag) and a thrombin cleavage site. The resulting plasmid was designated pET-PRX1. E. coli BL21 (DE3) was transformed with the pET-PRX1 construct, and a single colony was inoculated in LB medium containing 0.1 mg ampicillin/mL and grown at 37 °C until A600 = 0.5. Then the recombinant protein was induced by addition of 0.1 mM isopropyl-1-thiogalacto-pyranoside, and growth was continued at 37 °C. After 3 h, the cell free extract was filtered and loaded onto a Talon Resin Column (Clontech) equilibrated with 20 mM Tris, pH 8.0, 50 mM NaCl. The column was washed with 20 mM Tris, pH 8.0, 100 mM NaCl (Buffer A) followed by Buffer A containing 20 mM imidazole. The recombinant Prx1 protein was eluted with Buffer A containing 100 mM imidazole and dialyzed against Buffer A. The protein was then digested with human plasma thrombin (Sigma) (14 units of the spiking protein) for 2 h at room temperature. Finally, the sample was passed through a Talon Resin Column buffered with Buffer A. The eluted sample containing the recombinant Prx1 protein without His tag was dialyzed against 50 mM Hepes, pH 7.0. A truncated form of Prx1p (m-Prx1p), lacking the first 20 N-terminal amino acids, was also cloned, expressed in E. coli, and purified. S. cerevisiae DNA was amplified by PCR using a new forward primer (APRX1-NdeI, 5′-CTCTTGATCCAGGTATTTGCAC-3′) and the PRX1-BamHI as the reverse. The cloning, expression, and purification processes were the same as described before.

Purity of both recombinant Prx1 and m-Prx1 proteins was determined by SDS-polyacrylamide gel electrophoresis. Protein concentrations were determined according to the molar extinction coefficients for Prx1p (29,520 m−1 cm−1), m-Prx1p (23,710 m−1 cm−1), and Ser25-m-Prx1p (130,500 m−1 cm−1) [24].

Mutation of Cys38 for Ser in m-Prx1p—The codon TGC that codes for the Cys38 residue of the Prx1p was mutated to AGC (Ser) following the protocol of the QuikChangeTM site-directed mutagenesis kit (Stratagene). We used two mutagenic primers (primer A, 5′-CCACAGACCATTTGCTAAGGCTAATTTACAAAAATCAGT-3′, for the coding strand and primer B, 5′-CATTTCCTTTATGTTGATTCTGATTGAGTGGTATTCGCTGACG-3′, for the complementary strand) and the pET-m-Prx1 as template. The entire plasmid DNA was amplified by PCR, and subsequently the parent chains were digested with DpoI restriction enzyme. The resulting product containing the mutated construction (pETSer25-m-PRX1) was used to transform Epipuran E. coli XLI-Blue cells. The pET-Ser25-m-PRX1 was purified from several transformant colonies, and the mutation was confirmed by sequencing. Finally, E. coli BL21 (DE3) was transformed with the new construction, and the expression and purification of the recombinant Ser25-m-Prx1p was carried out as described above.

Construction of Δprx1 Mutant—The S. cerevisiae PRX1-disrupted strain (Δprx1) and a plasmid containing the PRX1 gene (pRS416-URA3) were a kind gift from B. Scheren (Institut de Recherches Microbiologique J. M. Wiame, Brussels) and were prepared following the proposed methods by the B0 program of the European Functional Analysis Network. To express Prx1p in the Δprx1 strain, the mutant was transformed with pRS416-PRX1, and transformed cells were selected on SC Ura− medium.

Antibodies—For antibody production, female chickens were injected subcutaneously at multiple sites with at least 250 μg of Prx1 recombinant protein, mixed with complete Freund’s adjuvant (Difco), as described (24). Affinity-purified antibodies were prepared using a cyano-gen bromide-activated Sepharose 4B column where 2 mg of Prx1p had been coupled following the procedure recommended by the manufacturer (Amersham Pharmacia Biotech). Immunoblotting was completed as described (22). The intensity of immunostaining in Westerns was evaluated by a computerized image analyzer (GelPro). Preparation of Subcellular Fractions from S. cerevisiae—The subcellular fractions were obtained from cells grown in YPD medium to stationary phase as described (22). For preparing total cell-free extracts, cells were harvested from YPD cultures and diluted to 100 mM Tris-HCl, pH 8.0, containing 1% Nonidet-P40 and 0.5% SDS. 4 g of 425–600 microns glass beads (Sigma/ml of buffer were added, and the samples were vortexed five times for 1 min. After collecting the liquid from the glass beads, the total cell extracts were clarified in a microcentrifuge.

Enzymatic Activity and Sensitivity Assays—Thioredoxin peroxidase activity was assayed in a reaction mixture containing 250 μM NADPH, 0.1 μM thioredoxin reductase, 4 μM thioredoxin, and 0.5 μM Prx1p, considering the Prx1p as homodimeric protein, in 50 mM Tris-HCl, pH 7.0. Recombinant Trx3p and Trr2p were purified as described (22). The reaction was started with the addition of 500 μM hydroperoxide, and the A540 decrease was followed at 37 °C for 30 min. The kinetic values of Prx1p with the different substrates (Trx3p, H2O2, and t-BuOOH) were calculated using the following concentrations: (a) for Trx3p, the reactions contained 0.125 μM Prx1p, 2.5–25 μM Trx3p, and 500 μM H2O2 and (b) for H2O2 or t-BuOOH, 0.125 μM Prx1p, 25 μM Trx3p, and 1–25 μM hydroperoxide. Glutathione peroxidase activity was assayed as described (25), using bovine erythrocyte glutathione peroxidase (Sigma) as standard.

Cells were subjected to oxidative stress by heat exposure as described (26). Cells from overnight cultures were diluted in phosphate saline (14 × 106 cells/ml) and aliquoted in 100-μl samples. Samples were heated at 50 °C for different times, cooled on ice, and, after suitable dilutions, plated on YPD and incubated at 30 °C for 3 days. Sensitivity to oxidant chemicals was also tested using patch assays. 10-μl aliquots containing approximately 106 cells of an overnight culture were spotted on YPD plates containing H2O2 and were incubated at 30 °C for 3 days.

RESULTS

Identification and Cloning of Prx1p—An ORF, YBL064C, coding for Prx1p was identified in the Saccharomyces Genome Database (locus SCYBL064C, accession number Z53825). This ORF was isolated from S. cerevisiae genomic DNA by PCR amplification, cloned, and sequenced, confirming the sequence described in the data base. The ORF YBL064C maps at chromosome II and codes for a protein of 261 amino acids with a predicted mass of 29.5 kDa and pI 8.87 that has been classified as member of the one-conserved cysteine peroxiredoxin family (1-Cys Prx) (7, 9, 18, 21) (Fig. 1). Its amino acid sequence

![Fig. 1. Amino acid sequences of S. cerevisiae Prx1p. Cysteine residues are indicated in black boxes. The arrows indicate the probable mitochondrial targeting signal (MTS).](image-url)
displays a 47% identity with the human 1-Cys Prx, 29% identity with the yeast Tsa1p, and 26% identity with SP-22, a mammalian mitochondrial thioredoxin peroxidase (27, 28).

Prx1p is longer than the human 1-Cys Prx and Tsa1p in part because of an N-terminal extension of 20 amino acid residues that resembles a pr sequence for import into mitochondria. This N-terminal extension shows an absence of acidic amino acid residues, a predominance of basic and hydroxyl-carrying residues (pI 11.1), and a putative amphipatic α-helix, features that most of the mitochondrial targeting sequences share (29).

Mitochondrial targeting sequences are cleaved by proteases when the protein reaches its mitochondrial localization. When the sequence of Prx1p was analyzed with a program for predictions of protein localization sites (PSORT), it displayed a high probability to be located in mitochondria with a predicted cleavage site between amino acids 16 and 17.

**Analysis of the Properties of wt and Mutant Prx1p Proteins by SDS-PAGE**—The prx1 gene was linked to the pET-15b expression vector, and after transforming E. coli cells, the expression of the recombinant protein was induced by the addition of isopropyl-1-thio-β-d-galactopyranoside. The His-tagged recombinant protein was purified by Talon™ metal affinity chromatography, and subsequently the His tag was removed by thrombin digestion.

A recent study has shown that human 1-Cys Prx forms dimers (16). These results led us to examine the dimerization of recombinant Prx1p. Purified recombinant Prx1p was heated at 95 °C for 5 min in 2.5% SDS in the presence or absence of 5 mM H2O2 and subsequently analyzed by SDS-PAGE in a DTT-free gel (PhastGel, Amersham Pharmacia Biotech). Prx1p rendered one band of 58 kDa in the absence of DTT pretreatment that corresponded to the molecular size of monomers, whereas after incubation with DTT, two bands of 32 and 28 kDa were detected that corresponded to the molecular size of monomers (Fig. 2, lanes 1 and 2). This duality in purified peroxiredoxins has been observed in previous works, and it might be due to intramolecular linkage between cysteine residues, modifying the compactness of the protein (9, 21).

Because the N-terminal mitochondrial localization signal should be removed in the mature mitochondrial protein, we expressed and purified a truncated Prx1p starting from the 21st amino acid residue. This recombinant protein (m-Prx1p) showed one 57-kDa band under nonreducing conditions and one 28-kDa band when denatured with DTT prior to SDS-PAGE (Fig. 2, lanes 3 and 4). m-Prx1p contains two cysteine residues (positions 38 and 91). Prx1p Cys38 is homologous to Cys47 of Tsa1p, conserved in all peroxiredoxins. To analyze the function of Cys38 we mutated it to Ser (Ser38-m-Prx1p), as described under “Materials and Methods.” Under nonreducing conditions as well as when pretreated with DTT, Ser38-m-Prx1p showed only one band corresponding to a monomer (Fig. 2, lanes 5 and 6). Thus, it appears that Cys38 participates in the dimerization. On the other hand, both m-Prx1p and Ser38-m-Prx1p at a concentration of 80 μg/ml were subjected to gel filtration in a Superdex 75 column (Amersham Pharmacia Biotech), and both eluted at the same time, according to a 60.0 kDa protein (data not shown). These data suggest that the Cys38 is not essential for dimerization but rather stabilizes the dimer.

**Thioredoxin Peroxidase Activity of Prx1p—**Prx1p was assayed for TPx activity in vitro, measuring the decrease of absorbance at 340 nm because of oxidation of NADPH to NADP+. Prx1p showed peroxide reductase activity in the presence of the mitochondrial thioredoxin system of *S. cerevisiae* (Fig. 3A). The oxidation of NADPH by the thioredoxin system was not significant in the absence of either Prx1p or H2O2 (Fig. 3A, columns a and b). However, when Prx1p was assayed with the complete thioredoxin system and H2O2, the NADPH oxidation increased 20-fold (Fig. 3A, column d), and the diminution of A340 nan/min was constant for at least 30 min. In addition, both thioredoxin and thioredoxin reductase were required for the Prx1p activity, and, in the absence of any of them, the NADPH oxidation was negligible (Fig. 3A, columns c and d not shown). We also measured the TPx activity of Prx1p using *E. coli* and mammalian thioredoxin systems. Prx1p also showed TPx activity with both thioredoxin systems, but the activity was approximately half compared with the mitochondrial yeast system (Fig. 3A, columns d–f).

The m-Prx1 protein, which lacks the putative mitochondrial localization signal, also showed TPx activity. However, the optimum pH for activity of both Prx1p and m-Prx1p was different (Fig. 3B). The highest activity of Prx1p was obtained at pH 7.0, and it significantly decreased at pH values lower than 6.0. In contrast, the truncated protein had an optimum pH around 6.4 and kept most of its activity at even lower pHs. Noteworthy, the recombinant His-tagged Prx1p had very different pH requirements compared with Prx1p, whose His tag has been removed, and it was almost inactive at pH 7.0. The thioredoxin system alone exhibited a very weak H2O2-dependent NADPH oxidation along the tested pH conditions.

We also tested the mutated protein Ser38-m-Prx1 for thioredoxin peroxidase activity. It must be stressed that this mutated
protein only contains one cysteine residue (Cys\(^{91}\)), which is the conserved one. Surprisingly, Ser\(^{38}\)-m-Prx1p still preserves the thioredoxin-dependent peroxide reductase activity with \(\text{H}_2\text{O}_2\) (Fig. 4). In addition, both m-Prx1p and Ser\(^{38}\)-m-Prx1 were able to reduce \(\text{t-BuOOH}\) (Fig. 4). The previously characterized thioredoxin peroxidases require two cysteine residues per monomer for maintaining their thioredoxin-dependent peroxidase activity (8, 21). Thus, this is the first example of one conserved cysteine peroxiredoxin, with only one Cys in its sequence, that is a thioredoxin peroxidase.

Using different concentrations of either \(\text{H}_2\text{O}_2\), \(\text{t-BuOOH}\), or Trx3p, we determined the kinetic parameters for both m-Prx1p and Ser\(^{38}\)-m-Prx1p. The results are shown in Table I. Both m-Prx1p and Ser\(^{38}\)-m-Prx1p showed higher affinity, as well as catalytic efficiency (\(V_{\text{max}}/K_m\)) values for \(\text{H}_2\text{O}_2\) than \(\text{t-BuOOH}\). Comparing with the kinetic parameters of other \(S.\ cerise\)\(\text{viae}\) TPxs, m-Prx1p has similar affinity and catalytic efficiency for \(\text{H}_2\text{O}_2\) and \(\text{t-BuOOH}\) to Tsa1p (21). However, the type II TPx or Ahp1p has 25-fold higher \(K_m\) value than m-Prx1p for \(\text{H}_2\text{O}_2\) and 4-fold higher \(K_m\) for \(\text{t-BuOOH}\) and approximately 2-fold higher \(V_{\text{max}}\) values for both peroxides (21).

We have also investigated whether Prx1p or m-Prx1p could reduce \(\text{H}_2\text{O}_2\) or \(\text{t-BuOOH}\) when the reducing equivalents were provided by glutathione using a reaction mixture containing GSH, glutathione reductase, and NADPH. No NADPH oxidation was observed under these conditions (data not shown).

Subcellular Localization—The localization of Prx1p in the cell was studied by immunoblotting analysis using subcellular yeast fractions. Proteins from cytoplasmic and mitochondrial fractions were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, and developed with affinity purified anti-Prx1p antibodies.

The antibody reacted with a 28-kDa band in the purified mitochondrial sample (Fig. 5, lane c). The molecular mass was similar to that of the recombinant m-Prx1p, in agreement with the fact that the protein contains a presequence that is cleaved off after translocation into mitochondria. A similar but much weaker band was also present in the cytoplasmic fraction, suggesting the breakage of some mitochondria during the purification process (Fig. 5, lane b). As expected, the antibody did not react with any band in the total cell-free extract from a disrupted PRX1 strain (Fig. 5, lane d).

Expression of Prx1p in \(S.\ cerise\)\(\text{viae}\)—We analyzed the expression of Prx1p in fermentation, diauxic shift, and respiration by immunoblotting analysis. Taking as a reference the expression of the protein at initial fermentative phase and the densitometric analysis, Prx1p increased 2.0-fold in cells from medium fermentative phase, 3.2-fold in diauxic shift phase, and 3.7-fold in stationary phase (Fig. 6A). Thus, the expression of Prx1p is paralleled by the cellular oxygen consumption levels and the mitochondrial development.

We also analyzed whether Prx1p is elevated in cells exposed to oxidants. Yeast cells growing in YPD medium at initial fermentative phase were exposed to different concentrations of \(\text{H}_2\text{O}_2\) for 1 h. Cells increased the expression of Prx1p after \(\text{H}_2\text{O}_2\) treatment reaching a maximal induction (4-fold) between 0.25 and 0.5 mM \(\text{H}_2\text{O}_2\) (Fig. 6B). Subsequently the levels of Prx1p decreased even below the control probably because of toxicity (55 and 47% survival at 0.75 and 1.0 mM \(\text{H}_2\text{O}_2\), respectively) at higher doses of \(\text{H}_2\text{O}_2\).

Mutation in the PRX1 Gene Sensitizes Cells to \(\text{H}_2\text{O}_2\) and to Lethal Heat Shock—A wild type in the FY16799 background and an isogenic strain with a PRX1 null mutation (Δprx1), carrying or not carrying an episomal plasmid (pPRX1) expressing PRX1, were subjected to phenotype analysis. The expression of Prx1p for every strain was checked by Western blotting (Fig. 7A). The Δprx1 strain did not show any differences either in growth or in cell morphology. We also tested the effect of the PRX1 deletion on the stress tolerance to \(\text{H}_2\text{O}_2\) and to \(\text{t-BuOOH}\) by patch assays. The PRX1 null strain was more sensitive to \(\text{H}_2\text{O}_2\) compared with its isogenic wild type control. The expression of the protein from pPRX1 in the mutant restored the resistance toward \(\text{H}_2\text{O}_2\) (Fig. 7B). The role of PRX1 in the stress tolerance to \(\text{t-BuOOH}\) was also examined. In contrast to its role in the \(\text{H}_2\text{O}_2\) sensitivity, the PRX1 deletion barely affects the tolerance to \(\text{t-BuOOH}\) (data not shown).

Sudden heat shock in yeast generates active oxygen species and induces cell death (26). Therefore, we investigated the possible involvement of Prx1p in the lethal effects of heat exposure. To avoid expression of heat shock genes (26), cells growing in liquid medium at 30 °C were rapidly shifted to a lethal heat shock of 50 °C for different time periods, as described under “Materials and Methods.” Wild type cells showed a survival of \(3.7 \times 10^{-3}\%\) after 60 min compared with the isogenic Prx1p null strain, which shows a survival of \(3.2 \times 10^{-3}\%\) (Fig. 7C). However, the PRX1 null strain, expressing Prx1p from an episomal plasmid, was considerably more resistant to 60 min at 50 °C showing \(8.5 \times 10^{-2}\%\) survival. In addition, these cells showed already higher resistance after 20 min of heat exposure.

DISCUSSION

Peroxiredoxins have been classified into two groups, one containing only one conserved cysteine in the N-terminal region that corresponds to Cys\(^{67}\) of yeast Tsa1p (1-Cys Prx) and another containing an additional conserved cysteine near the C-terminal region (2-Cys Prx). The 2-Cys Prxs referred to as thioredoxin peroxidases reduce peroxides with electrons provided from thioredoxin. 1-Cys Prxs can also reduce peroxides using DTT as electron donor, but the physiological electron donor for the 1-Cys Prxs is not known. When the hORF6 enzyme, a mammalian 1-Cys Prx, was characterized, it reduced peroxides using DTT but, in contrast to its homologous 2-Cys Prxs, did not show thioredoxin-dependent peroxide reductase activity. We were attracted by the existence of a potential mitochondrial thioredoxin-dependent peroxidase because we had previously identified and characterized a mitochondrial thioredoxin system (Trx3p and Trr2p) in \(S.\ cerise\)\(\text{viae}\) (22). The protein coded by the ORF YBL064C showed homology to peroxiredoxins and had a potential mitochondrial targeting signal, and although the product of ORF YBL064C belongs to the 1-Cys Prx group, we decided to test whether it had specific TPx activity with the mitochondrial thioredoxin system. The recombinant protein (Prx1p) had TPx activity, and it could also reduce alkyl hydroperoxides. Prx1p contains three cysteine residues in the positions 6, 38, and 91. The Cys\(^{6}\) would be
removed in the mature protein, considering that the N-terminal domain is a presequence that is cleaved by proteases in mitochondria. Thus, there would be two cysteine residues (Cys38 and Cys91) which might be implicated in the thioredoxin-dependent reduction of peroxides. Cys91, homologous to Cys47 of Tsa1p, shares the conserved position common to all peroxiredoxins. A truncated Prx1p with the Cys38 mutated to serine, thus containing only Cys91, still preserved the thioredoxin-dependent NADPH-oxidation activity coupled to peroxide reduction. Also, Ser38-m-Prx1p did not form dimers upon denaturation in absence of thiol reducing compounds, but it behaved as a dimer when it was subjected to molecular exclusion chromatography, suggesting that other ionic and/or hydrophobic forces would be implicated in the dimerization of Ser38-m-Prx1p. These results suggest that Cys38 is not essential for the activity, although it is implicated in the stabilization of the dimer.

The crystal structure of hORF6, the human 1-Cys Prx, has been solved and suggested that the protein forms a dimer. Prx1p is homologous to the human 1-Cys Prx and most of the amino acids important for dimerization (e.g. Phe43, Thr44, Pro45, Val46, Thr48, Thr49, Glu50, Leu145, and Tyr149) in human 1-Cys Prx (16) are also conserved in Prx1p (Phe87, Thr88, Pro89, Val90, Thr92, Val93, Glu94, Leu98, and Tyr192). In hORF6 the conserved cysteines are too far apart for interacting with each other. However, Prx1 forms a dimer, and our results suggest that there is a disulfide bond, Cys38–Cys38, between the two subunits. This difference could be explained by the location of Cys38 in a 24-amino acid N-terminal extension not present in hORF6.

Mitochondria consume approximately 85% of the oxygen demanded by cells because of the oxidative phosphorylation process where molecular oxygen is reduced to water but generate a great deal of ROS as subproducts. Thus, Prx1p with the mitochondrial thioredoxin system would offer a first line of defense.

### Table 1

| Substrates | m-Prx1p | $K_m$ [μM] | $V_{max}$ [μmol min$^{-1}$ mg$^{-1}$] | $V_{max}/K_m$ | Ser38-m-Prx1p | $K_m$ [μM] | $V_{max}$ [μmol min$^{-1}$ mg$^{-1}$] | $V_{max}/K_m$ |
|------------|---------|-------------|-------------------------------------|--------------|--------------|---------|--------------------------------------|--------------|
| H$_2$O$_2$ | 6.2 ± 0.8 | 8.5 ± 0.6 | 1.37 | 3.5 ± 0.6 | 9.8 ± 0.3 | 2.80 |
| $t$-BuOOH | 10.9 ± 0.4 | 7.4 ± 0.5 | 0.68 | 10.1 ± 1.6 | 6.9 ± 0.7 | 0.68 |
| Trx3p     | 8.1 ± 0.9 | 9.5 ± 0.8 | 1.17 | 7.5 ± 1.2 | 10.4 ± 1.3 | 1.39 |
against the main endogenous ROS-generating site in yeast cells. S. cerevisiae cells in a fermentable sugar-containing medium show a rapid, exponential fermentative growth phase with the production of ethanol. However, when the fermentable sugar is used up, cells reset their metabolic capacity from fermentation to respiration and continue growing much more slowly (respiratory phase) using the ethanol derived from the fermentation as a carbon source. The change from fermentation to respiration referred to as diauxic shift is paralleled by changes in mitochondrial development. During the fermentative phase, cells contain only few poorly developed mitochondria, but the exhaustion of glucose induces nearly full mitochondrial development (30). The levels of Prx1p paralleled the development of mitochondria when cells shift from fermentative to respiratory metabolism. These results are in agreement with the results obtained in a DNA microarray study to characterize changes in gene expression accompanying the metabolic shift from fermentation to respiration in which the transcription level of the ORF YBL064C coding for Prx1p was increased 11-fold (31).

In yeast, the transcription factor Yap1 plays a key role in the control of oxidative stress responses. Yap1 is a DNA-binding protein of the AP-1 family that binds to sequences homologous to AP-1 sites termed Yap1 response element (32). In the promoter region of PRX1, there is a potential Yap1-1 site (TTAGTGA) 705 nucleotides upstream from the start ORF codon. Another element, designated as a stress response element with the consensus core sequence AGGGG, is able to mediate transcription induction by different forms of stress (33). Such an AGG element is located 116 nucleotides upstream from the start codon of PRX1. Cells also overexpress Prx1p when exposed to H$_2$O$_2$, thus, the PRX1 gene would have to be included in the battery of H$_2$O$_2$-induced genes or H$_2$O$_2$ stimulon (19).

The sensitivity to oxidant conditions of the Prx1p-null cells also reflected the importance of this peroxidase in the yeast cell protection against heat shocks and exogenous H$_2$O$_2$ exposition. Although Prx1p also reduced t-BuOOH as efficiently as H$_2$O$_2$, the protein did not exert protection against the exogenous t-BuOOH. Similarly, although Ahp1p can reduce H$_2$O$_2$ in vitro, Ahp1p-null mutants did not show increased sensitivity to exogenous H$_2$O$_2$ (20, 21). In this sense, mitochondrial Prx1p could be more efficient in eliminating endogenously generated peroxides.

In bovine mitochondria, a thioredoxin-dependent peroxidase (SP-22) with high homology to yeast Tsa1p has been identified (27, 28). SP-22 has recently been reported to play an important role in the antioxidant defense in the cardiovascular system (34). Mammalian mitochondria also contain a specific thioredoxin system that may provide hydrogens to the mitochondrial TPs (35–37). The presence of a mitochondrial thioredoxin peroxidase/mitochondrial thioredoxin system in both yeast and mammalian cells suggests an important role in the removal of H$_2$O$_2$ and a protective role in defense against oxidative stress and cell death.

Acknowledgments—We thank Ylva Ekendahl for helping us to obtain antibodies, B. Scheren for the generous gift of the yeast strains, and Anthony P. H. Wright and Stefan Herrmann for fruitful discussions.

REFERENCES

1. Storz, G., Christian, M. F., Sies, H., and Ames, B. N. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 8917–8921
2. Wolff, S. P., Garner, A., and Dean, R. T. (1986) Trends Biochem. Sci. 11, 21–31
3. Cerutti, P. A. (1985) Science 227, 375–381
4. Harman, D. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 7124–7128
5. Saito, H., Hammond, A. T., and Moses, R. E. (1995) Exp. Cell Res. 217, 272–279
6. Pierce, G. B., Parchment, R. E., and Lewellyn, A. L. (1991) Differentiation 46, 181–186
7. Schörner, E., and Pointing, C. P. (1998) Protein Sci. 7, 2465–2468
8. Chae, H. Z., Chung, S. J., and Rhee, S. G. (1994) J. Biol. Chem. 269, 27670–27678
9. Kang, S. W., Baines, I. C., and Rhee, S. G. (1998) J. Biol. Chem. 273, 6303–6307
10. Kim, K., Kim, I. H., Lee, K. Y., Rhee, S. G., and Stadtman, E. R. (1988) J. Biol. Chem. 263, 4704–4711
11. Cha, M. K., and Kim, I. H. (1995) Biochem. Biophys. Res. Commun. 217, 180–187
12. Kim, K., and Kim, I. H. (1995) Biochem. Biophys. Res. Commun. 217, 180–187
13. Kang, S. W., Chae, H. Z., Seo, M. S., Kim, K., Baines, I. C., and Rhee, S. G. (1998) J. Biol. Chem. 273, 6297–6302
14. Holmgren, A., and Björnstedt, M. (1995) Methods Enzymol. 252, 199–208
15. Tartaglia, L. A., Storz, G., Brodsky, M. H., Lai, A., and Ames, B. N. (1991) J. Biol. Chem. 266, 10535–10540
16. Choi, H. J., Kang, S. W., Yang, C. H., Rhee, S. G., and Ryu, S. E. (1998) Nat. Struct. Biol. 5, 400–406
17. Chae, H. Z., Kim, I. H., Kim, K., and Rhee, S. G. (1993) J. Biol. Chem. 268, 16815–16821
18. Chae, H. Z., Robison, K., Poole, L. B., Church, G., Storz, G., and Rhee, S. G. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 7017–7021
19. Godon, C., Lagniel, G., Lee, L., Buhler, J. M., Kieffer, S., Perrot, M., Boucherie, H., Toledano, M. B., and Labarre, J. (1998) J. Biol. Chem. 273, 22480–22489
20. Lee, J., Spector, D., Godon, C., Labarre, J., and Toledano, M. B. (1999) J. Biol. Chem. 274, 4537–4544
21. Jeong, J. S., Kwon, S. W., Kang, S. W., Rhee, S. G., and Kim, K. (1999) Biochemistry 38, 776–783
22. Pedrajas, J. R., Kosmidou, E., Miranda-Vizuetee, A., Gustafsson, J.-Å., Wright, A. P. H., and Spyrou, G. (1999) J. Biol. Chem. 274, 6366–6373
23. Sherman, F., Fink, G. R., and Hicks, J. B. (1986) Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Miranda-Vizuete, A., Damdimopoulos, A. E., Gustafsson, J.-Å., and Spyrou, G. (1999) J. Biol. Chem. 274, 11761–11764
25. Lawrence, A. R., and Burk, R. F. (1976) Biochem. Biophys. Res. Commun. 71, 350–357
26. Davidson, J. F., Whyte, B., Bissinger, P. H., and Schiestl, R. H. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 5116–5121
27. Watabe, S., Hiroi, T., Yamamoto, Y., Fujisaka, Y., Haasawa, H., Yago, N., and Takahashi, Y. (1997) Eur. J. Biochem. 249, 52–60
28. von Heijne, G. (1986) EMBO J. 5, 1335–1342
29. Pon, L., and Schatz G. (1991) in The Molecular and Cellular Biology of the Yeast Saccharomyces: Genome Dynamics, Protein Synthesis and Energetics (Ibrahaj, J. R., Pringle, J. R., and Jones, E. W., eds) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
30. Dellui, J. L., Iyer, V. R., and Brown, P. O. (1997) Science 278, 680–686
31. Kuge, S., and Jones, J. (1994) EMBO J. 13, 655–664
32. Schuller, C., Brewster, J. L., Alexander, M. R., Gustin, M. C., and Ruis, H. (1994) EMBO J. 13, 4382–4389
33. Araki, M., Nanri, H., Ejima, K., Murasato, Y., Fujiwara, T., Nakashima, Y., and Ikeda, M. (1999) J. Biol. Chem. 274, 2721–2727
34. Spyrou, G., Enmark, E., Miranda-Vizuetee, A., and Gustafsson, J.-Å. (1997) J. Biol. Chem. 272, 2936–2941
35. Miranda-Vizuetee, A., Damdimopoulos, A. E., Pedrajas, J. R., Gustafsson, J.-Å., and Spyrou, G. (1999) Eur. J. Biochem. 261, 405–412
36. Lee, S.-R., Kim, J.-R., Kwon, K.-S., Yoon, H. W., Levine, R. L., Ginsburg, A., and Rhee, S. G. (1999) J. Biol. Chem. 274, 4722–4734