The Peroxiredoxin Tpx1 Is Essential as a H$_2$O$_2$ Scavenger during Aerobic Growth in Fission Yeast

Mónica Jara,* Ana P. Vivancos,* Isabel A. Calvo, Alberto Moldón, Miriam Sansó, and Elena Hidalgo

Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, E-08003 Barcelona, Spain

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Peroxiredoxins are known to interact with hydrogen peroxide (H$_2$O$_2$) and to participate in oxidant scavenging, redox signal transduction, and heat-shock responses. The two-cysteine peroxiredoxin Tpx1 of Schizosaccharomyces pombe has been characterized as the H$_2$O$_2$ sensor that transduces the redox signal to the transcription factor Pap1. Here, we show that Tpx1 is essential for aerobic, but not anaerobic, growth. We demonstrate that Tpx1 has an exquisite sensitivity for its substrate, which explains its participation in maintaining low steady-state levels of H$_2$O$_2$. We also show in vitro and in vivo that inactivation of Tpx1 by oxidation of its catalytic cysteine to a sulfenic acid is always preceded by a sulfenic acid form in a covalently linked dimer, which may be important for understanding the kinetics of Tpx1 inactivation. Furthermore, we provide evidence that a strain expressing Tpx1.C169S, lacking the resolving cysteine, can sustain aerobic growth, and we show that small reductants can modulate the activity of the mutant protein in vitro, probably by supplying a thiol group to substitute for cysteine 169.

INTRODUCTION

Peroxiredoxins (Prxs) are a family of antioxidant enzymes that reduce hydrogen peroxide (H$_2$O$_2$) and/or alkyl hydroperoxides to yield water and/or alcohol, using reducing equivalents provided principally by thioredoxin. These H$_2$O$_2$ scavengers have been isolated from all kingdoms (Chae et al., 1994b), with six mammalian isoforms distributed in different organelles (Rhee et al., 2005b). Three types of structurally different Prxs have been described: 1-cysteine (Cys), 2-Cys, and atypical 2-Cys (for review, see Wood et al., 2003b). All three types of Prxs are dimers in solution, and they all share the same catalytic mechanism, in which the peroxidatic Cys, located close to the N-terminal domain, is oxidized to a sulfenic acid by either H$_2$O$_2$ or alkyl hydroperoxides. In both types of 2-Cys Prxs, the sulfenic acid then reacts with the C-terminal (or resolving) Cys of the other subunit to form an intermolecular disulfide (classical 2-Cys Prxs), or with the C-terminal Cys of the same monomer to form an intramolecular disulfide (atypical 2-Cys Prxs). In both cases, the disulfides are specifically reduced by the thioredoxin and thioredoxin reductase system, with the exception of some prokaryotic Prxs, such as the Escherichia coli AhpC, which uses another protein, AhpF, for the regeneration of the reduced Prx. In the 1-Cys Prxs, the sulfenic acid is directly reduced to thiol, because there is no nearby Cys available to form a disulfide bond; the source of the reducing equivalents for regenerating this thiol is not known, although glutathione (GSH) has been proposed to serve as the electron donor in this reaction (Kang et al., 1998b).

Prx activity can be regulated by phosphorylation (Chang et al., 2002), and possibly by changes in oligomerization states (Wood et al., 2002, 2003b). Furthermore, some Prxs suffer an oxidation of one of their Cys residues, the peroxidatic Cys, to sulfenic acid upon exposure to high H$_2$O$_2$ doses, which temporarily inactivate these enzymes (Yang et al., 2002; Woo et al., 2003b; Wood et al., 2003a). This hyperoxidation of 2-Cys Prx enzymes is reversible in cells (Woo et al., 2003a,b). The enzymes named sulfiredoxin, found in Saccharomyces cerevisiae (Biteau et al., 2003); Schizosaccharomyces pombe (Bozont et al., 2005; Vivancos et al., 2005), and mammalian cells (Chang et al., 2004), were isolated as the ATP-dependent reductases for Cys-sulfenic acid in Prxs. Another family of enzymes named sestrins has been shown to have a similar role in mammalian cells (Budanov et al., 2004).

The main role of Prxs has classically been considered to be related to their peroxidase activity. Their affinity for H$_2$O$_2$, with published $K_m$ values $\sim$20 $\mu$M, is higher than that of other peroxide scavengers such as catalase or GSH peroxidase (Chae et al., 1999; Rhee et al., 2001), which suggests that Prxs could be efficient at removing low concentrations of H$_2$O$_2$. However, their temporary redox inactivation seems to suggest that Prx enzymes might have a role in signal transduction, with their inactivation shunt causing a temporary or local increase in H$_2$O$_2$ concentration that could then trigger antioxidant cascades (Rhee et al., 2005a).

The first evidence for the participation of a Prx in signal transduction pathways came from studies of fission yeast antistress responses. In S. pombe, the Pap1 and Sty1 pathways constitute the key protective responses to oxidative stress. It has been recently reported that the only 2-Cys Prx in fission yeast, Tpx1, is the H$_2$O$_2$ sensor of the Pap1 pathway, its presence being essential for activation of the tran-
scription factor and its specific gene response. The Pap1 pathway is more sensitive to H$_2$O$_2$ than the mitogen-activated protein kinase Sty1 pathway: maximal activation of Sty1 requires a H$_2$O$_2$ concentration at least fivefold higher than that described to fully activate Pap1 (Quinn et al., 2002; Vivancos et al., 2004). As described above, 2-Cys Prxs such as Tpx1 undergo substrate-mediated inactivation, so high concentrations of H$_2$O$_2$ will temporarily inactivate Tpx1, post-p pioneering Pap1 activation; meanwhile, the Sty1 pathway will be fully functional under these conditions. Activated Sty1 is then required for reactivation of Tpx1, because synthesis of the Tpx1-reductase Srx1 during severe H$_2$O$_2$ stress is dependent on Sty1 (Bozobt et al., 2005; Vivancos et al., 2005).

Thus, Prxs have aroused a great deal of interest during recent years, and their emerging roles other than H$_2$O$_2$ scavenging—as redox signal transducers and molecular chaperones (Jang et al., 2004; Chiang et al., 2006)—is a very interesting field of study. Indeed, Tpx1 was initially identified on the basis of its role in signal transduction to Pap1 in fission yeast. However, evidence from deletion mutants points to additional actions by this Prx. A strain lacking the tpx1 gene is not viable (Vivancos et al., 2005), whereas deletion of either pap1 or sty1 genes does not compromise cell viability, and cells lacking any of the oxidative stress signaling components only display phenotypes in the presence of extracellular stress (for review, see Ikner and Shiozaki, 2005). In this work, we demonstrate that Tpx1 is an essential H$_2$O$_2$ scavenger in S. pombe, with an extremely high affinity for its substrate and that this peroxidase activity is required only for aerobic growth. A mutant Tpx1 protein lacking the resolving Cys169 (Tpx1.C169S) is also able to support aerobic growth of cells expressing only this isoform, and small thiols such as dithiothreitol and GSH can provide the reducing equivalents to recycle oxidized Tpx1 in vivo in the absence of its resolving Cys, in a manner similar to 1-Cys Prx enzymes.

**MATERIALS AND METHODS**

**Yeast Strains**

We used the wild-type strains 972 (h-), PNS13 (h-leu1 ura1), HM123 (h-lea1), AV29 (h-leu1 lea1 ura1 ura4 ade6-M210 ade6-M276; our laboratory stocks) as well as other published strains such as TP108 (h-leu1 his2 ura4 pap1::ura4; our laboratory stocks), NT224 (h-leu1 ura1 sty1::1) (Millar et al., 1995), and S. pombe strains with specific loci deleted, we transformed some of the above-mentioned strains with linear fragments containing ORF:kanMX6, obtained by polymerase chain reaction (PCR) amplification with open reading frame (ORF)-specific primers and plasmid pFA6a-kanMX6 as a template. Following that strategy, we obtained strains AV25 (h- pap1::kanMX6), AV18 (h-sty1::kanMX6) and AM42 (h-leu1 his2 ura4 pap1::ura4 sty1::kanMX6). To isolate cells deleted in sod1, we transformed the wild-type strain PNS13 with a PCR-amplified sod1::lacZ-kanMX6 fragment, obtained by PCR amplification with ORF-specific primers and plasmid pAAYO17 as a template (plasmid AY017 is a pREP3X [Maundrell, 1993] derivative containing a lacZ-kanMX6 insert). Transformed cells were grown under anaerobic conditions and selected by their ability to grow in Kanamyacin-containing plates. The strain generated was named MS1 (h-leu1 ura1 sod1::lacZ-kanMX6). To isolate haploid cells deleted in tpx1, we transformed the diploid strain AV29 with a PCR-amplified tpx1::kanMX6 fragment, yielding strain AV360p (h-leu1 leu1 ura1 ura4 ade6-M210 ade6-M276 tpx1::kanMX6 p Napoleon). Haploid cells were isolated by standard genetic techniques (Moreno et al., 1991), spread in Kanamyacin-containing plates and grown anaerobically (see below). This yielded strain AV42 (h-leu1 ura1 ade6-M210 tpx1::kanMX6). Plasmids p145 (pym1::T; tp1), p145.485 (pym1::ORF), p145.416 (tp1::tp1.C169S) containing the ORF-specific primers for the gene and containing BamH1 and Smal restriction sites. The fragment was cloned into a modified glutathione S-transferase (GST)-tagging fusion vector pGEX-2T-TEV that encoded a TEV protease cleavage site between the tag and the cloned ORF, digested with the same restriction enzymes, and then it was cloned into pGEX-2T-TEV cleaved with BamH1 and SmaI restriction sites, and then it was cloned into the pGEX-2T-TEV expression vector digested with the same restriction enzymes, yielding ORF amplified. The ORF was amplified using specific primers for the gene and containing BamH1 and Smal restriction sites. The fragment was cloned into pGEX-2T-TEV cleaved with BamH1 and Smal, yielding plasmid p206. Once PCR amplified, all ORFs in these expression vectors were sequenced.

**Preparation of Tpx1, Thioredoxin (Trx1), and Thioredoxin Reductase (Trt1) Proteins**

Protein carbonylation was determined using the Bradford protein assay (Bio-Rad, Hercules, CA). Protein concentration was adjusted to a 4–10 mg/ml range with carbonylation buffer, and a concentrated 12% solution of SDS was added to 10 μg of total protein, to reach final SDS concentration of 6%, and incubated for 2 min at 100°C. One volume of 10 mM DNP buffer in 10% trifluoroacetic acid was added to 1 volume of the sample at 25°C. The reaction was run for 10 min and stopped by the addition of 1 volume of 2 M Tris base, 10% glycerol, and 15% 2-mercaptoethanol. Samples were separated electrophoresis in SDS-polyacrylamide gel electrophoresis (PAGE) gels and carbonylated proteins were immunodetected using a polyclonal anti-2,4-dinitrophenyl (DNP) antibody (Sigma-Aldrich). As a loading control, Sty1 was detected using polyclonal anti-Sty1 antiserum raised against an E. coli fusion protein of GST-Sty1, following standard rabbit immunization procedures. The anti-DNP and anti-Sty1 immunoblots were scanned, and quantification of carbonylated proteins was performed using the ImageQuant 5.2 program (GE healthcare, Little Chalfont, Buckinghamshire, United Kingdom).

**Purification of Recombinant Tpx1, Thioredoxin (Trx1), and Thioredoxin Reductase (Trt1) Proteins for In Vitro Assays**

Bacteria strain FB810 (Benson et al., 1994) transformed with the pGEX-2T-TEV derivatives were inoculated into Luria broth with 100 μg/ml ampicillin and incubated at 37°C for ~16 h with vigorous shaking. The overnight cultures were diluted 25-fold into 400 ml of fresh medium and incubated at 37°C until the culture reached an optical density of 0.8 at 600 nm. Isopropyl-β-thio-o-galactoside (IPTG) was then added to a final concentration of 0.5 mM and shaking continued at 25°C for 4 h. The cells were then harvested, and pellets were resuspended in 100 mM Tris-Cl pH 8.0, 150 mM sodium chloride, 1 mM EDTA, pH 8.0, 1 mM Triton X-100, and 2 mM PMSF and broken by sonication. Debris and unbroken cells were removed by centrifugation. Supernatants containing our GST-tagged fusion proteins were then immobilized to glutathione-Sepharose 4B beads (GE Healthcare) for 1 h at 4°C. The beads were then washed three times with NET-N (20 mM Tris-Cl, pH 8.0, 1 mM EDTA, 100 mM sodium chloride, 0.5% NP-40, and 2 mM PMSF) and once with TEV cleavage buffer (10 mM Tris-Cl, pH 8.0, 150 mM sodium
chloride, 0.1% NP-40, and 0.5 mM EDTA). We could then release the GST-tagged protein with GSH-containing elution buffer (100 mM Tris HCl, pH 8.0, 120 mM NaCl, and 20 mM GSH). Alternatively, we also released the S. pombe proteins without the GST tag from the Sepharose beads by incubating a 200-μl bed volume of beads with 10 μg of TEV protease (Invitrogen, Carlsbad, CA) overnight at 4°C. Size differences among GST-containing or untagged protein samples Tpx1, Trr1, and Trx1 were observed by electrophoretic separation on 15% denaturing polyacrylamide gels and Coomassie staining.

**Peroxidase Activity Assays**

NADPH oxidation was monitored as a decrease in optical density at 340 nm by using a Ultrospec 3100 pro UV/Visible spectrophotometer (GE Healthcare) in a 500-μl reaction mixture containing 50 mM HEPES-NaOH, pH 7.0, 0.25 mM NADPH, 6 μg of Tpx1, 20 μg of Tpx1, 1 μg of Tpx1, and H2O2. Reactions were started by the addition of the indicated concentrations of H2O2. As peroxidase activity of Tpx1 undergoes substrate-mediated inactivation, an initial linear portion of absorbance change (10 s) was used for the calculation of peroxidase activity, as described previously (Koo et al., 2002).

To determine the H2O2 detoxification driven by Tpx1, Tpx1 C48S, and Tpx1 C169S in the presence of small thiols, we incubated 5–20 μg of enzyme with the Trx system (consisting of the same reaction mixture as described in the previous paragraph), 100 or 100 mM diithothreitol (DTT) (in 50 mM HEPES-NaOH, pH 7.0), or 50 μM GSH (in 100 mM Tris-HCl, pH 8.0) to avoid acidification of the assay mixture. We started the reaction by the addition of 50 μM H2O2, and 150-μl aliquots were taken at different time points and stopped by the addition of 20 μl of 100% TCA. Samples were then centrifuged to eliminate proteins, and 100 μl of the supernatant were used to determine the remaining H2O2 concentration. This was achieved by addition of 27 μl of 10 mM ferrous ammonium sulfate and 13.5 μl of 2.5 M potassium thiocyanate. The red-colored ferrithiocyanate complex, which occurs as a result of the oxidation of Fe(II) by H2O2, was quantified by measuring the OD at 480 nm and was compared with H2O2 standards.

**Preparation of S. pombe TCA Extracts and Immunoblot Analysis**

For in vivo redox state analysis of Tpx1, S. pombe cultures (5 ml) at an OD600 of 0.5 were pelleted just after the addition of 100% TCA, to a final concentration of 10%, and washed in 20% TCA. The pellets were lysed by vortexing, after the addition of glass beads and 12.5% TCA. Cell lysates were pelleted, washed in acetone, and dried. Alkylation of free thiols was performed by resuspension of the pellets in 50 μl of a solution containing 75 mM iodoacetamide, 1% SDS, 100 mM Tris-HCl, pH 8.0, and 1 mM EDTA, and incubation at 25°C for 15 min. Alkylated samples were diluted fivefold and electrophoretically separated by reducing SDS-PAGE, as indicated, and proteins were immunodetected using polyclonal anti-Tpx1 antiserum raised against an E. coli fusion protein of GST-Tpx1, following standard rabbit immunization procedures. For the detection of sulfonlated Tpx1, the undiluted alkylated samples were separated by nonreducing SDS-PAGE and immunodetected with anti-peroxiredoxin-SO3 antibody (LabFrontier, Seoul, South Korea).

**RESULTS**

**Tpx1 Is Not Required for Growth under Anaerobic Conditions**

Using classic genetic techniques, we could not isolate haploid S. pombe cells carrying a deletion of the tpx1 gene, and we therefore generated a conditional knockout strain with Tpx1 expression under the control of a thiamine-repressible nmt promoter (Vivancos et al., 2005). To test whether the essential role of Tpx1 in fission yeast was due to its peroxidase activity, we attempted to isolate haploid cells carrying the tpx1 deletion from diploid cells, in which one of the tpx1 loci is substituted by the kanamycin cassette, by growing them under anaerobic conditions inside sealed Anaerocult sachets. The chemical mixture inside the sachets contains components that chemically bind oxygen rapidly and effectively, creating an oxygen-free atmosphere. Positive clones were isolated, grown anaerobically in liquid, and doubly tested for lack of Tpx1 and their inability to oxidize Pap1 in response to H2O2 (data not shown). Deletion of tpx1 increased the sensitivity of cells to aerobic growth, but cells grew as wild type in the absence of oxygen (Figure 1A). On the contrary, cells lacking Pap1, Sty1, or both grew as efficiently under aerobic or anaerobic conditions (data not shown).

**Tpx1 Is a Peroxidase with Very High Affinity for H2O2**

In most cell types, Prxs are not essential for aerobic growth. However, the lack of Tpx1 seems to drive cells grown in the presence of oxygen toward a basal oxidative stress, resulting in growth arrest. We decided to test whether Tpx1 scavenges the majority of endogenous H2O2 in S. pombe by assaying its capacity to decompose low concentrations of peroxides in vitro. The enzymatic activity of Prxs can be measured by coupling them to Trx1 and Trr1 in the presence of NADPH, following the reaction illustrated in Figure 2A. Traditionally, to assay the activity of eukaryotic Prxs, the three protein components of this reaction were purified from cell extracts by a series of multiple purification steps. We have developed an alternative strategy, in which Tpx1, Trx1, and Trr1 of S. pombe are purified from E. coli cells. We constructed expression vectors harboring full-length tpx1, trxl, and trrl genes.
fused to the GST-coding gene. The fusion proteins (GST-Tpx1, GST-Trx1, and GST-Trr1) were purified almost to homogeneity from *E. coli* extracts by affinity binding to GSH-beads. The GST tag interfered with the activity of some of the purified proteins, so we cleaved it with TEV protease, yielding highly purified recombinant Tpx1, Trx1, and Trr1. All the assays described hereafter were performed using these purified *E. coli*-expressed proteins, even though identical activities were detected in extracts of *S. pombe* cells overexpressing the wild-type or mutant Tpx1 (data not shown).

We measured Tpx1 activity by following NADPH oxidation at 340 nm in a reaction mixture containing NADPH, Trr1, Trx1, and Tpx1. The decrease of absorbance at 340 nm was monitored at room temperature in a 500-µl peroxidase reaction mixture containing 6 µg of Trr1, 20 µg of Trx1, and 1 µg of Tpx1 and low concentrations (≤50 µM; B), medium concentrations (50–200 µM; C), and higher doses of H2O2 (1–25 mM; D). The background of NADPH oxidation in the absence of peroxide is also present (control). (E–G) Effects of H2O2 concentration on the peroxidase activity of Tpx1 and Trx1. (E) Global Tpx1 peroxidase activity (micromoles of NADPH per minute per milligram) at different concentrations of H2O2. Reactions were performed as described for B to D. An initial linear portion of absorbance change (10 s) was used for the calculation of peroxidase activity. (F) Effects of H2O2 concentration on the peroxidase activity of Trr1 and Trx1 in the absence of Tpx1. Experiments were performed as described in E, but in the absence of Tpx1. (G) Net Tpx1 peroxidase activity micromoles of NADPH per minute per milligram) at different concentrations of H2O2. The results from F were subtracted from those of E, yielding the Tpx1-dependent peroxidase activity of the reaction mixture.

We measured Tpx1 activity by following NADPH oxidation at 340 nm in a reaction mixture containing NADPH, Trr1, Trx1, Tpx1, and varying concentrations of H2O2. At low peroxide concentrations (5–50 µM; Figure 2B), the rates of NADPH oxidation (the slope at the different time points) did not change with time and was proportional to H2O2 concentration, as expected for enzymes following Michaelis kinetics. As described for other eukaryotic Prxs, at intermediate H2O2 concentrations (50–200 µM), the rate of NADPH oxidation decreased with time, and the rate of this decrease was enhanced at higher H2O2 concentrations (Figure 2C). Surprisingly, high peroxidase activities were again achieved at peroxide concentrations above 1 mM, with the slopes at the different time points remaining constant at these H2O2 concentrations (Figure 2D). We then used the initial linear portion of absorbance change (10 s) to calculate the peroxidase activity at different concentrations of H2O2, and the results are shown in Figure 2E. An exact $K_m$ value could not be accurately established because the changes in $A_{340}$ were not large enough to allow determination of initial rates. Furthermore, at saturating concentrations of H2O2 (≥50 µM), the substrate-mediated inactivation of Tpx1 distorts the typical Michaelis kinetics. Nevertheless, applying the Lineweaver–Burk double-reciprocal plot to the Tpx1 activities at H2O2 concentrations from 5 to 50 µM, we could calculate that the apparent $K_m$ of Tpx1 for H2O2 is ~2–3 µM. The H2O2-dependent inactivation of Tpx1 occurred at peroxide concentrations >50 µM. Thioredoxin has been suggested to act as a H2O2 peroxidase by itself (Hirota *et al.*, 2002); so, to measure the H2O2-scavenging activity of Trx1 alone, we monitored NADPH oxidation by Trx1-Trr1 in the absence of Tpx1. As shown in Figure 2F, Trx1 was able to reduce H2O2, but with a $K_m$ value of at least 10 mM. This low affinity suggests that Trx1 is unlikely to contribute to H2O2 scavenging in vivo. Subtraction of the activity values for Trx1 (Figure 2F) from those of Tpx1 in the presence of the Trx1 system (Figure 2E) allowed us to calculate values for Tpx1 activity, which are shown in Figure 2G.
Overoxidation of Cys-48 of Tpx1 to a Cys-Sulfinic Acid Occurs at a Covalent Tpx1 Dimer Interface

Once established that Tpx1 suffers H$_2$O$_2$-mediated inactivation in vitro (Figure 2G), we decided to study whether such inactivation was concomitant with the oxidation of Cys-48 to the sulfinic acid state. To detect hyperoxidized Tpx1, we used commercial polyclonal antibodies against a sulfonlated peptide encompassing the active site of mammalian Prx, which is identical in sequence to that of S. pombe Tpx1. These antibodies recognize sulfinic (and sulfonic) forms of Prxs and permit the detection of sulfonlated Prx enzymes in extracts from H$_2$O$_2$-treated cells with high sensitivity and specificity (Woo et al., 2003b). The presence of oxygen during the protein purification procedure was able to induce the formation of a disulfide bond in Tpx1 (data not shown). Nevertheless, as described for other Prxs, H$_2$O$_2$ is not sufficient to cause hyperoxidation of the peroxidatic Cys-SH of Tpx1 to Cys-SO$_2$H (Figure 3A). Instead, all catalytic components (Trx1, Trr1, and NADPH) are required. To our surprise, we detected two bands on immunoblots of in vitro hyperoxidized Tpx1 separated by nonreducing SDS-PAGE: a faster migrating band corresponding in size to a Tpx1–SO$_2$H monomer, and another band migrating as a disulfide-linked Tpx1–SO$_2$H dimer (Figure 3A). This dimeric form is DTT sensitive (data not shown), and it is not an artifact of purification procedures: immunoblot analysis of S. pombe extracts showed that the relative proportion of the monomeric and dimeric sulfinic acid forms depends on the H$_2$O$_2$ concentration (Figure 3B); in these blots, a nonspecific, H$_2$O$_2$-independent band was only present occasionally, and it is indicated with an asterisk (*). (C) Kinetics of Tpx1–SO$_2$H monomer and dimer formation in response to 1 mM H$_2$O$_2$ in vivo. Same as described in B, after treatment of wild-type strain 972 with 1 mM H$_2$O$_2$ for the times indicated in the figure.

Cys-169 of Tpx1 Is Not Required for Its Peroxidatic Activity In Vivo

Tpx1 contains two Cys residues, Cys-48 and Cys-169, both of which are essential for its role in redox signal transduction to Pap1 (Bozonet et al., 2005; Vivancos et al., 2005). Tpx1 belongs to the typical 2-Cys Prx family. Members of this family are H$_2$O$_2$ scavengers, and the peroxidase reaction of these enzymes also requires both conserved Cys residues, because the oxidized enzyme intermediate generated during the catalytic cycle is a dimer in which the subunits are linked by one or two intermolecular disulfide bonds between the peroxidatic and the resolving Cys of the other subunit (for review, see Wood et al., 2003b). We analyzed what effect mutations of each one of the two Cys residues would have on cell viability. Surprisingly, a protein lacking the resolving Cys, Tpx1.C169S, was able to support aerobic growth of S. pombe cells (Figure 4A, middle), whereas the absence of the peroxidatic residue yielded a protein unable to sustain growth in the presence of oxygen. This result indicates that the Cys169 residue is not essential for the peroxidatic activity of Tpx1. Nevertheless, the ability of Tpx1.C169S to decompose high concentrations of peroxides is limited, because expression of Tpx1.C169S decreased cell tolerance to extracellular H$_2$O$_2$ to a greater extent than that of Δpap1 or Δsrx1 cells (Figure 4A, right).

As stated above, inactivation by sulfinic acid formation can only occur in Prxs when the protein is engaged in the catalytic cycle. We were able to detect Tpx1.C169S–SO$_2$H in extracts from cells treated with H$_2$O$_2$ (Figure 5A), which demonstrates that Tpx1.C169S is catalytically active in vivo. As determined experimentally, disulfides in typical 2-Cys Prxs are specifically reduced by thioredoxin, but they can also be reduced in vitro by a small molecular thiol, such as DTT (Chae et al., 1994a). Indeed, it has been demonstrated that DTT can support full activity in vitro of a Prx lacking its resolving Cys, whereas the Trx system cannot (Chae et al., 1994a). According to the model proposed by Rhee and colleagues, a molecule of DTT could replace the resolving Cys in the formation of a disulfide with the peroxidatic Cys-SOH, and subsequently another molecule of DTT could reduce the mixed disulfide. We assayed the activity of wild-type and mutated Tpx1 by measuring the removal of H$_2$O$_2$ from the reaction mixture by using the ferrithiocyanate method (see Materials and Methods). Wild-type Tpx1 was able to scavenge peroxides in the presence either of the Trx1 system or, weakly, of DTT (Figure 5B). The Tpx1–C48S mutant enzyme was completely inactive under all condit-
Figure 4. The resolving Cys of Tpx1 is not essential for aerobic growth. (A) Survival of different strains in aerobiosis. Strains 972 (WT), AV25 (Δtpx1), AV18 (Δsrx1), EA38 (Δsrx1), AV49 (Δtpx1 tpx1), AV49.C48S (Δtpx1 tpx1.C48S), and AV49.C169S (Δtpx1 tpx1.C169S) were grown anaerobically in rich media (YES5) to a final OD600 of 0.5, and the number of cells indicated at the top of the panels was spotted onto YES5 plates containing or not containing 0.8 mM H2O2. Plates were incubated at 30°C for 3–4 d in aerobic or anaerobic conditions, as indicated. (B) Protein carbonylation generated for 3–4 d in aerobic or anaerobic conditions, as indicated. (A) Survival of different strains in aerobiosis. Strains 972 (WT), AV25 (Δtpx1), AV49.C48S (tpx1.C48S), and AV49.C169S (tpx1.C169S) were grown aerobically for 12 h to a final OD600 of 0.5, and protein carbonylation was detected by reaction of carbonyl groups with DNP, followed by SDS-PAGE and Western blot analysis by using anti-DNP antibodies (α-DNP; top) or anti-Sty1 antibodies as a loading control (α-Sty1; bottom). Quantification data (fold induction) was obtained as described in Figure 1B.

DISCUSSION

Several roles have been ascribed to peroxiredoxins in vivo: H2O2 scavengers (Rhee et al., 1994), components of signal transduction pathways (Veal et al., 2004; Bozonet et al., 2005; Vivancos et al., 2005) and chaperones after heat stress (Jang et al., 2004; Chuang et al., 2006). The only 2-Cys Prx of S. pombe, Tpx1, has been previously reported to participate in signal transduction in response to mild extracellular oxidative stress, but we have demonstrated in the present study that it also plays an essential role as a scavenger of H2O2 generated during oxidative metabolism. Δtpx1 cells shifted from anaerobic to aerobic conditions suffered growth arrest; this was probably a consequence of intracellular oxidative stress, as indicated by their elevated levels of protein carbonylation. Steady-state levels of H2O2 in aerobically growing cells are reported to be ~10–7 M (Seaver and Imlay, 2001). As shown for other Prxs, the Vmax of Tpx1 indicates that this is a slow detoxifying enzyme compared with catalases or GSH peroxidases; but its high-affinity for H2O2 (with a Km ~2 μM) and its abundance (0.1–1% of total soluble proteins in S. pombe; data not shown) indicate that Tpx1 is very efficient at removing low concentrations of H2O2.

Other model organisms also have been shown to depend on Prxs for survival under aerobic conditions, such as the oxygen-sensitive microaerophile Helicobacter pylori (Baker et al., 2001). In the yeast S. cerevisiae, although cells lacking all five Prxs are still able to grow aerobically, they are hypersensitive to oxidative stress and are genomically unstable.
zymes contains two disulfide bonds, but that several minor dimeric forms carrying only one disulfide are also likely to be formed, resulting in a variable compactness and different mobilities on nonreducing SDS-PAGE (Chae et al., 1994c). The sulfonlated dimer in our experiments migrates slower than the major, two disulfide-containing dimer, and it has the same electrophoretic mobility as the minor species carrying only one disulfide (data not shown). Therefore, only the minor band corresponding to the one disulfide-containing Tpx1 dimer undergoes sulfonlation. According to structural studies on Prxs, the peroxidatic and resolving Cys residues are situated far apart in the corresponding Prx dimers (Wood et al., 2003b), and formation of a disulfide probably requires and induces significant conformational changes. Perhaps formation of the first disulfide in a Tpx1 dimer temporarily prevents the second Cys-48-SOH from stabilizing within its structural pocket in the active site, increasing its susceptibility to hyperoxidation to Cys-SO₂H.

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