A New Antioxidant with Alkyl Hydroperoxide Defense Properties in Yeast*

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To isolate new antioxidant genes, we have searched for activities that would rescue the tert-butyl hydroperoxide (t-BOOH)-hypersensitive phenotype of a Saccharomyces cerevisiae strain deleted for the gene encoding the oxidative stress response regulator Skn7. We report the characterization of AHP1, which encodes a 19-kDa protein similar to the AhpC/TA protein family within a small region encompassing Cys-62 of Ahp1p and the highly conserved N-terminal catalytic AhpC/TA cysteine. Ahp1p contains a peroxisomal sorting signal, suggesting a peroxisomal localization. AHP1 exerts strong antioxidant protective functions, as demonstrated both by gene overexpression and deletion analyses, and is inducible by peroxides in an Yap1- and Skn7-dependent manner. Similar to yeast Tsa1p, Ahp1p forms a disulfide-linked homodimer upon oxidation and in vivo requires the presence of the thioredoxin system but not of glutathione to perform its antioxidant protective function. Furthermore, in contrast to Tsa1p, which is specific for H2O2, Ahp1p is specific for organic peroxides. Therefore, with respect to substrate specificity, Ahp1p differs from Tsa1p and is similar to prokaryotic alkyl hydroperoxide reductase AhpC. These data suggest that Ahp1p is a yeast orthologue of prokaryotic AhpC and justifies its name of yeast alkyl hydroperoxide reductase.

The incomplete reduction of molecular oxygen during respiration and the lipid metabolism in peroxisomes leads to the formation of reactive oxygen species (ROS) (1). ROS are potent oxidants and can damage all cellular constituents (2). They cause DNA base modifications and strand breaks and are therefore mutagenic. They can damage proteins and inactivate enzymes. Oxidation of membrane lipids can initiate free radical chain reactions, which alter cellular membranes and give rise to other very toxic reactive species such as lipid hydroperoxides, alkoxyl and peroxyl radicals, lipid epoxides, and aldehydes. To protect against the toxicity of ROS, aerobic organisms use an array of defense mechanisms (1, 3, 4). Among these, cytosolic and mitochondrial superoxide dismutases eliminate the O2•− radical, cytosolic and peroxisomal catalases remove H2O2, and glutathione peroxidases reduce both H2O2 and alkyl hydroperoxides. The AhpC/TA family, also referred to as peroxiredoxin, is a large family of newly discovered peroxidases that are highly conserved from prokaryotes to eukaryotes (5).

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Yeast AhpC/TSA Family Member with Organic Peroxide Specificity

sln7::TRP1 construct used to create yMT2 was a gift from Dr. H. Bussey (McGill University, Montreal, Canada) (27). The yap1::LEU2 construct used to create yMT1 was prepared by removing the YAP1 coding sequence from the BamHI site (+186) to the KpnI site (+1650) relative to the ATG and replacing it with the LEU2 gene. The ahp1::TRP1 deletion construct used to generate yMT5 was prepared by replacing the full AHP1 open reading frame (ORF) with the TRP1 gene. Basically, oligonucleotide primers were used to PCR-amplify 5’ (−680 to +5) and 3’ (+532 to +1530) regions of the AHP1 gene relative to the ATG. These PCR-amplified fragments were then subcloned upstream and downstream of the TRP1 gene. The deletion construct used to create yMT4 (sln7::LEU2) was prepared by removing a 700-base pair DNA fragment from the HindIII site at −180 base pairs relative to the ATG up to the BamHI site at +511 and replacing it with the LEU2 gene. yMT6 and yMT7 were prepared by a one-step amplification protocol that replaced the entire TSA1 or TRR1 ORF with the TRP1 gene (28). The cDNA library plasmid pY1 contained the entire AHP1 ORF from −119 relative to the translation start to +614, 82 base pairs downstream of the stop codon. To generate pAHP1, the AHP1 gene was isolated by PCR amplification of total yeast DNA with oligonucleotides that primed from −984 to +614, and then cloned into the pUCL8 vector from the BamHI and NotI sites.

Selection of Yeast cDNAs

A S. cerevisiae cDNA library under control of the repressible GAL1 promoter in the centromeric vector pRS316 (29) was transformed into strain yMT2 (sln7::LEU2). Transformed cells were plated onto SMG lacking uracil and containing 1 μM orotidine. URA3 transformants were pooled from each plate separately, diluted, and then plated onto SMGuracil lacking uracil and containing t-BOOH (0.5 mM). Plates were incubated at 30 °C for 5–12 days. Plasmids recovered from viable colonies were analyzed by restriction digest and sequencing. Sequencing was performed using an automated Perkin Elmer DNA sequencer according to the sequences of the proteins that are highly similar to Ahp1p.

Sensitivity Assays

Patch Assays—Aliquots (10 μl) containing approximately 106 or the indicated cell number of an overnight culture were spotted on YPD plates containing oxidants at the indicated concentration. Plates were monitored after 3–6 days of incubation at 30 °C.

Challenge Assay—Cells from an exponential growth culture were washed, resuspended in phosphate-buffered saline at a density of OD600 of 0.1, and not exposed or exposed to peroxides at different concentrations for 1 h. Cells were then diluted and plated for colony survival.

Northern Blot Analysis

Yeast cells from overnight culture were diluted to an OD600 of 0.01 in SC medium and incubated with shaking at 30 °C until they reached an OD of 0.3. The cells were then aliquoted and incubated in the absence or in the presence of H2O2 (0.2 mM) or t-BOOH (0.2 mM) for 20 min. Total RNA was prepared by the hot phenol method (30). For each condition tested, 20-μg RNA samples were loaded per lane on an agarose gel containing formaldehyde, separated by electrophoresis, transferred to a Bio-Rad nylon membrane, and hybridized with an an HAP1 32P-labeled DNA specific probe produced by random priming (Boehringer Mannheim). Hybridization of each blot with a small nuclear RNA 32P-labeled DNA probe served as a RNA loading control. Prehybridization, hybridization, and washes were carried out as described elsewhere (26). Hybridized membrane were exposed for autoradiography.

Two-dimensional Gel Electrophoresis and Protein Spot Identification

Two-dimensional gel electrophoresis and protein spot identification were performed as described elsewhere (13). Alkylation of protein free sulfhydryls before the second dimension was achieved as follows. The first dimensional gel rod was first reduced by a 10-min incubation in buffer A (30% glycerol, 6 M urea, 2.5% SDS, 0.01% bromphenol blue, 0.2 M Bis-Tris, 0.1 M HCl, and 67 mM dithiothreitol (DTT)). Alkylation was then performed by incubating the gel rod in buffer in which DTT was omitted, and iodoacetamide (222 mM) was added instead.

RESULTS

Selection of Genes Increasing the Tolerance to t-BOOH of a skn7 Null Strain—A S. cerevisiae strain carrying a skn7 null mutation fails to grow on medium containing 0.5 mM of t-BOOH (a synthetic alkyl hydroperoxide), whereas the isogenic wild type strain will tolerate up to 1.5 mM of this oxidant. We have searched for genes whose overexpression would rescue the t-BOOH hypersensitivity of the skn7 null strain using a galactose-inducible yeast cDNA library (29). This library was used to bypass the possible Skn7 requirement for the expression of candidate genes. We expected to identify genes controlled by Skn7 and/or encoding alkyl hydroperoxide antioxidant defense activities. Strain yMT2 (sln7::TRP1) was transformed with the GAL1-controlled cDNA library. Forty thousand transformants were obtained and incubated on plates containing 0.5 mM t-BOOH. Viable colonies were recovered from these plates after 7 days and the corresponding library plasmids isolated. Five plasmids, able to increase the t-BOOH tolerance of the yMT2 strain upon retransformation, were shown to carry inserts of different sizes, but containing the same ORF. One of them, pY1, was further analyzed. pY1 was able to increase the tolerance to t-BOOH not only of yMT2 but also of yMT1 (a yap1 null strain) (Fig. 1). Therefore, the gene carried on the pY1 plasmid has antioxidant properties. This gene was called AHP1 for alkyl hydroperoxide reductase (see below) and further analyzed. The AHP1 gene is encoded at the YLR109W locus. Interestingly, its gene product has been recently identified as a 19-kDa protein by comparative two-dimensional gel electrophoresis in the search of yeast proteins induced by H2O2 (13).

Similarity of Ahp1p with Peroxisomal-like Proteins and with the AhpC/TSA Protein Family—Ahp1p is a protein predicted to be 176 amino acids long with a molecular mass of 19,108 Da. Ahp1p is a protein predicted to be one of the three other prokaryotic proteins. Ahp1p has also a weak, yet significant similarity to the peroxiredoxin AhpC/TSA family. The sequences of the proteins that are highly similar to Ahp1p are compared in Fig. 3. Of the three Ahp1p cysteines, two are conserved in all three other prokaryotic proteins. Ahp1p has also a weak, yet significant similarity to the peroxiredoxin AhpC/TSA family. The sequences of the proteins that are highly similar to Ahp1p are compared in Fig. 3. Of the three Ahp1p cysteines, Cys-62 is the only one conserved among these related proteins and occupies a region with a high density of amino acid identities. Cys-31 is conserved only in Lipomyces kononenkoae peroxisomal-like protein and in Aspergillus fumigatus Asp3 protein.

FIG. 1. The AHP1 gene partially rescues the t-BOOH-hypersensitive phenotype of skn7 and yap1 null strains. skn7 and yap1 null strains not carrying Δskn7, Δyap1) or carrying the pY1 plasmid (Δskn7 pGAL1AHP1, Δyap1pΔGALA1HAP1), which expresses the AHP1 gene under control of the GAL1 promoter and isogenic wild type strain, were tested by the patch assay for their ability to grow on SDGal solid medium containing t-BOOH at the indicated concentration. For each strain, 10 μl of an overnight culture (OD600 2–3) diluted to 20, 5, or 1 x 10^5 cells (left to right) were spotted on test plates.
Yeast AhpC/TSA Family Member with Organic Peroxide Specificity

**Ahp1p Is an Antioxidant Specific for Organic Peroxides**—A null mutation of the **AHP1** gene that removes all its coding sequence was created. The viability of the resulting **ahp1** null strain demonstrates that this gene is not essential. The **ahp1** null strain does not have any particular growth phenotype on glucose, galactose, acetate, pyruvate, or oleate media or under anaerobic conditions. The effect of the **ahp1** null mutation was then tested on the stress tolerance to t-BOOH by a patch assay. Compared with its isogenic wild type control, the **ahp1** null strain is hypersensitive to t-BOOH (Fig. 4A). Conversely, high expression of **AHP1** from a multicopy plasmid dramatically

**Fig. 2. Nucleotide sequence of the AHP1 gene and the predicted amino acid sequence of the Ahp1 protein.** The nucleotide sequence of the **AHP1** gene and its flanking regions is shown in the 5' to 3' direction. The predicted amino acid sequence of Ahp1p is indicated below the relevant nucleotides. The Yap1 response element (TTAGTAA) and the three putative stress response elements (CCCCT) are indicated in **boldface** and **underlined**. The two TATA boxes are also indicated. The three cysteines and the AHL peroxisomal-like sorting signal of Ahp1p are indicated in **boldface**.

mophilus influenzae Y572 protein has an additional C-terminal domain with a very strong similarity to the redox-active center of glutaredoxins (Fig. 3). *C. bondinii* PMP20 A and B are two isoforms of a protein that was identified as the most abundant peroxisomal proteins of methanol-exposed cells but was not assigned any specific function (32, 33). None of the other proteins shown in Fig. 3 have an identified function. Nevertheless, the presence of a glutaredoxin-like domain in Y572 suggests that this protein might be involved in redox reactions.
increases the resistance of the wild type strain toward t-BOOH (Fig. 4C). Note that the difference in t-BOOH sensitivity of wild type cells in panels A and C is related to the different strain backgrounds used in these experiments (see figure legend and "Experimental Procedures"). The role of AHP1 in the stress tolerance to \( H_2O_2 \) was then examined. In sharp contrast to its null mutant is hypersensitive to t-BOOH, whereas the \( trx_1 \) null strain has a normal tolerance toward t-BOOH, similar to that seen in the wild type strain (compare Fig. 5A with Fig. 4C). However, the \( trx_1trx_2 \) null strain is hypersensitive to t-BOOH. In the \( trx_1 null \) strain, no effect can be seen upon overexpression of AHP1. A thioredoxin reductase (TRR1) deletion mutant also displayed a t-BOOH-hypersensitive phenotype that was not improved by overexpression of AHP1 (data not shown).

The antioxidant properties of AHP1 were also tested toward several other oxidants and a metal (Fig. 4D). The \( ahp_1 \) null mutation results in a slight decrease in the tolerance to the thioloxidant diamide and to cadmium and surprisingly to an increase in the resistance to the superoxide generating redox cycling drug menadione. However, high copy expression of AHP1 has no effect on the wild type strain tolerance to any of these compounds.

The Antioxidant Function of Ahp1p Is Independent of GSH and Dependent upon the Thioredoxin System—Tsa1p reduces peroxides with electrons donated by thioredoxin, thioredoxin reductase, and NADPH (7, 34), whereas AhpC reduces organic peroxides with electrons donated by the FAD-bound NADPH-dependent AhpF reductase (6). We thus sought to determine the potential requirement of an electron donor system for the Ahp1p antioxidant function. The effect of overexpressing AHP1 on t-BOOH resistance was tested in strains deleted of thioredoxin 1 (TRX1), thioredoxin 2 (TRX2), or both TRX1 and TRX2 (Fig. 5A). The \( trx_1 \) null strain has a normal tolerance toward t-BOOH, whereas the \( trx_2 \) null mutant is slightly more sensitive than the wild type strain to this oxidant (15) (compare Fig. 5A with Fig. 4C). However, the \( trx_1trx_2 \) double null strain is hypersensitive to t-BOOH. In the \( trx_1 \) null strain, overexpression of AHP1 results in a significant increase in the resistance to t-BOOH, similar to that seen in the wild type strain (compare Fig. 5A and 4C). In the \( trx_2 \) null strain, the effect of overexpressing AHP1 can be seen only at the lower t-BOOH concentrations. In the \( trx_1trx_2 \) null strain, no effect can be seen upon overexpression of AHP1. A thioredoxin reductase (TRR1) deletion mutant also displayed a t-BOOH-hypersensitive phenotype that was not improved by overexpression of AHP1 (data not shown).

The effect of overexpressing AHP1 on t-BOOH resistance was also tested in strains deleted for \( \gamma \)-glutamylcysteine synthase (GSH1), the rate-limiting enzyme in glutathione (GSH) biosynthesis (Fig. 5B). The \( gsh_1 \) null strain appears slightly more resistant to t-BOOH than the wild type strain, suggesting that high levels of GSH are not required for the resistance to this oxidant (the \( gsh_1 \) null strain is auxotrophic for GSH and can only grow in the presence of at least minimal amounts of exogenous GSH supplement such as those present in YPD-rich medium; Ref. 35). In addition, the \( gsh_1 \) null mutation does not affect the gain in t-BOOH resistance resulting from AHP1
overexpression. Taken together, these data show that thioredoxins but not GSH are important in the protection against t-BOOH and provide indirect evidence for the requirement of the thioredoxin system, but not of GSH, in the antioxidant protective function of Ahp1p.

Ahp1p Can Form a Disulfide-linked Homodimer in Vitro—Ahp1p was identified on two-dimensional gel electrophoresis of total yeast proteins as a H2O2-inducible spot migrating at a pI of 4.5 and a molecular weight of 19,000. Interestingly, an unidentified H2O2-induced spot with a pI identical to that of Ahp1p and a molecular weight of 40,000 was observed (Fig. 6). We suspected that this unknown spot could represent a dimeric form or other covalent modifications of Ahp1p. This spot was indeed identified as Ahp1p by peptide mapping using matrix-assisted laser desorption ionization-time of flight/mass spectrometry (36). We next asked whether the 40-kDa spot could represent a disulfide-linked dimer of Ahp1p. Cellular extracts subjected to two-dimensional gel electrophoresis are initially reduced in a buffer containing 160 mM β-mercaptoethanol. However, some substrates could be potentially reoxidized dur-
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Fig. 6. Ahp1p can form a disulfide-linked homodimer in vitro. Autoradiogram of two-dimensional gel electrophoresis performed with total cellular extracts from F5-methionine-labeled exponentially growing wild type YPH88 cells. The 19-kDa Ahp1p protein and the 40-kDa Ahp1p dimer identified by mass spectrometry are indicated by an arrow. A, extracts prepared from untreated cells. B, extracts prepared from cells exposed to H2O2 (0.4 mM) for 15 min. C, alkylated extracts prepared from untreated cells. In this case, the two-dimensional gel electrophoresis procedure was modified by the following change; after the first-dimensional electrophoresis, the resulting polyacrylamide rod was incubated in a buffer containing iodoacetamide before subjecting it to the second electrophoretic dimension.

**DISCUSSION**

Oxidative damage to lipids can lead to a variety of alkyl hydroperoxides, which are particularly detrimental to their ability to initiate and propagate free radical chain reactions. Enzymes involved in the breakdown of alkyl hydroperoxides are thus important for protection against oxidative stress. We report here the characterization of AHP1, which encodes a yeast antioxidant important for the protection against alkyl hydroperoxides, and related to the AhpC/TSA peroxiredoxin family.

Gene deletion and overexpression analyses have demonstrated that AHP1 exerts strong antioxidant protective functions. Furthermore, the observation that AHP1 mRNA and protein levels are induced by peroxides in a Yap1- and Skn7-dependent manner also supports the notion that Ahp1p is involved in protecting yeast cells from oxidative stress. Therefore, AHP1 can be added to the growing list of yeast antioxidant genes of the Yap1 and Skn7 regulons, which includes TRX2-encoded thioredoxin (15), GSH1-encoded \( \gamma \)-glutamylcysteine synthetase (19), GLR1-encoded glutathione reductase (20), and TRR1-encoded thioredoxin reductase (17).

Sequence analysis relates this antioxidant to the AhpC/TSA protein family. Although mostly confined to a small region surrounding Cys62 of Ahp1p and the highly conserved N-terminal cysteine of the AhpC/TSA proteins family (5), this homology is functionally important because the N-terminal cysteine of yeast Tsa1p is the catalytic center of its peroxidase function (7, 34). The proposed catalytic mechanism for Tsa1p involves substrate peroxide reduction by Cys N terminus, which upon oxidation reacts with the Cys C terminus of another subunit to form an intermolecular disulfide. This disulfide is subsequently reduced with electrons donated by thioredoxin, thioredoxin reductase, and NADPH (7, 34). It was observed that Ahp1p forms a disulfide-linked homodimer upon oxidation. Furthermore, genetic data suggested that the antioxidant protective function of AHP1 is independent of GSH but dependent upon both thioredoxin and thioredoxin reductase. Therefore, the structural similarity between Ahp1p and Tsa1p may be extended to a common mode of action, which suggests that a peroxidase function underlies the antioxidant properties of Ahp1p. Of the three Ahp1p cysteines, Cys-62 would be most likely to be involved in direct substrate peroxide reduction. However, it is not clear whether another cysteine would play a role in the putative Ahp1p catalytic function, nor which cysteine has been lost during electrophoresis. To test this hypothesis, the first dimension polyacrylamide rod was incubated in a buffer containing DTT to reduce any potentially reoxidized cysteine residues, and then treated in a buffer containing the cysteine alkylating agent iodoacetamide, which irreversibly blocks free sulfhydryls. As shown in Fig. 6, this procedure led to the complete disappearance of the 40-kDa spot. These data strongly suggest that the 40-kDa Ahp1p spot represents a disulfide-linked Ahp1p homodimer.

**AHP1 Is Regulated by Yap1 and Skn7—** Ahp1p is an abundant protein under non induced conditions, and its synthesis rate is increased by about 3-fold upon exposure to H2O2 (13). We therefore analyzed whether the peroxide stimulation of Ahp1p was dependent upon Yap1 and Skn7. Northern blots were performed using an AHP1 specific probe and total RNA from exponentially growing wild type, yap1, and skn7 null cells that had not been treated or were treated with 0.2 mM amounts of either t-BOOH or H2O2. In wild type cells, AHP1 mRNA levels were significantly increased upon H2O2 (Fig. 7A) or t-BOOH (Fig. 7B) exposure, confirming the results of the two-dimensional gel analysis. In contrast, in yap1 or in skn7 null strains, both uninduced and t-BOOH-induced AHP1 mRNA levels were significantly decreased, although they could still be moderately induced by peroxide. However, this residual induction was not seen in the yap1skn7 double null strain, although some uninduced levels persisted (Fig. 7C). Consistent with a transcriptional control by Yap1, the 5’ AHP1 flank contains the sequence TTAGTAA at position (~484) from the start codon, which perfectly matches a Yap1 response element (Fig. 1) (37).
The slight increase in $H_2O_2$ sensitivity, $(0), trol; relative values are given for each lane:

$\text{quantification of the autoradiogram normalized to the } U_3$

$\text{type cells (Yap1 and Skn7).}$

$\text{Northern blot analysis of SKN7 (AHP1)}$

$\text{lanes 2 and 3) or were treated for 20 min by } H_2O_2 (0.2 \text{ mM}) (1.77).$

The antioxidant protective function of $AHP1$ is an oxidative stress-inducible gene regulated

by Yap1 and Skn7. Northern blot analysis of $AHP1$ transcription, A, total RNA was isolated from exponentially growing (OD$_{600}$ 0.3) wild type cells (WT), and isogenic strains with null deletion of YAP1 ($\Delta$yap1), SKN7 ($\Delta$skn7), which had not been treated or were treated for 20 min with $H_2O_2 (0.2 \text{ mM})$ as indicated. The resulting RNA were subjected to Northern blot which was probed for $AHP1$ and $U_3$ as a loading control, as described under “Experimental Procedures.” B, same as in A, but cells were induced by $t$-BOOH (0.2 mM) instead of $H_2O_2$. C, total RNA was isolated from wild type (lane 1 to 3) and from an isogenic strain with a null deletion of Yap1 and Skn7 ($\Delta$yap1$\Delta$skn7). Cells were not treated (lanes 1 and 4) or were treated for 20 min by $H_2O_2 (0.2 \text{ mM}) (lunes 2 and 5) or by $t$-BOOH (0.2 mM) (lanes 3 and 6). Densitometric quantification of the autoradiogram normalized to the $U_3$ loading control; relative values are given for each lane: 1 (2.83), 2 (8.16), 3 (6.37), 4 (0), 5 (1.68), and 6 (1.77).

teine(s) is (are) involved in homodimer disulfide bond formation. Site-directed mutagenesis of individual cysteines of Ahp1p will answer these questions.

The antioxidant protective function of $AHP1$ appears specific for organic peroxides. The slight increase in $H_2O_2$ sensitivity, and perhaps the altered diamide tolerance phenotype seen in the $\text{aqhp1}$ null mutant, could be related to the lipid hydroperoxides which might be generated upon exposure to these oxidants. Although not an oxidant, cadmium may also lead to the production of ROS through metal-catalyzed electron transfer $(38, 39)$, and its toxicity might therefore be exacerbated by the lack of $AHP1$. It is unlikely that cadmium is a direct substrate for Ahp1p. The menadione hyperresistance phenotype of the $\text{aqhp1}$ null strain is not understood but could be related to a deregulated expression of superoxide defense genes. Interestingly, an in vivo comparison of the substrate specificities of Ahp1p and Tsa1p showed that, whereas Ahp1p activity is specific for organic peroxides, Tsa1p activity is specific for $H_2O_2$. This observed in vitro substrate specificity of Tsa1p is fully consistent with in vivo measurements of its catalytic activity toward $t$-BOOH and $H_2O_2 (7)$. Therefore, with respect to its substrate specificity, Ahp1p differs from Tsa1p and appears related to the Escherichia coli and Salmonella typhimurium alkyl hydroperoxide reductase AhpC, which is specifically involved in the breakdown of lipid and other alkyl hydroperoxides, but not of $H_2O_2 (6)$.

Eukaryotic cells have several enzymes involved in the breakdown of lipid hydroperoxides. The structurally related selenoproteins glutathione peroxidase $(40)$ and phospholipid hydroperoxide glutathione peroxidase $(41, 42)$ can reduce both alkyl hydroperoxides and $H_2O_2$. However, glutathione peroxidase is more active toward $H_2O_2$, whereas phospholipid hydroperoxide glutathione peroxidase is more specific for lipid hydroperoxides $(41, 43)$. Furthermore, only phospholipid hydroperoxide glutathione peroxidase can catalyze the reduction of lipid hydroperoxide derivatives of intact phospholipids. Glutathione S-transferase isozymes are also able to reduce lipid hydroperoxides with GSH $(44, 45)$. Another pathway for the reduction of lipid hydroperoxides is constituted by NADPH-dependent thioredoxin reductase $(46)$. However, an eukaryotic orthologue of prokaryotic AhpC has not been described. We suggest, based on substrate specificity and structural homology, that Ahp1p is a peroxidase and the yeast orthologue of prokaryotic AhpC.

Ahp1p is highly similar to C. bondinii PMP20 A and B and to several other fungi and prokaryotic proteins (see Fig. 1B). C. bondinii PMP20 A and B are two isoforms of a protein identified as the most abundant peroxisomal protein of methanol-exposed cells and suspected to play a role in methanol metabolism $(32, 33)$. However, these two proteins and their fungi and prokaryotic homologues (see Fig. 3A), which share with Ahp1p the same similarity to the AhpC/TSA family, may have also a peroxidase function. Consistent with this idea is the presence of a C-terminal glutaredoxin domain in the H. influenzae Y572, which may serve as an electron donor for the catalytic cysteine of this putative peroxidase. Based on immunostaining and cellular subfractionation experiments, PMP20 is a peroxisomal protein tightly associated with membranes $(33)$. Consistent with this localization, PMP20 A and B contain a C-terminal peroxisomal sorting signal sequence $(32)$. A similar peroxisomal sorting signal is present in Ahp1p and in the other PMP20-related fungi proteins, suggesting that they may all be similarly localized in peroxisomes. However, the abundance of Ahp1p observed on two-dimensional gels, which only analyze soluble proteins, and its presence in high quantity in the supernatant of a stationary cell culture (data not shown), may indicate that this polypeptide is also present in the cytosol and is shed out of the cell in the medium. A detailed analysis of Ahp1p subcellular location will provide an answer to this question.

In summary, $AHP1$ encodes an AhpC/TSA-related antioxidant with apparent substrate specificity for alkyl hydroperoxides. These data suggest that this antioxidant is a yeast orthologue of prokaryotic AhpC and justifies its name of Ahp1p for yeast alkyl hydroperoxide reductase. $AHP1$ is coordinately controlled with $TRX2$ and $TRRI$ by Yap1 and Skn7 and requires the presence of these two enzymes to exert in vivo its antioxidant protective function. In vitro measurements of the antioxidant activity of wild type and Ahp1p cysteine substitution mutants will provide a detailed analysis of its peroxidase function and will confirm the role of the thioredoxin system in supporting the antioxidant activity of Ahp1p. Further studies aimed at analyzing the redox characteristics of Ahp1p and
understanding the molecular basis of its substrate specificity will be of interest. In that respect, the propensity of Ahp1p to oxidize to a disulfide-linked homodimer during electrophoresis is quite striking and suggests a high reactivity to oxidation in vitro and probably also in vivo of one or more of its cysteines.

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