Identification in *Saccharomyces cerevisiae* of a New Stable Variant of Alkyl Hydroperoxide Reductase 1 (Ahp1) Induced by Oxidative Stress*

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Yeasts lacking cytoplasmic superoxide dismutase (Cu,Zn-SOD) activity are permanently subjected to oxidative stress. We used two-dimensional PAGE to examine the proteome pattern of *Saccharomyces cerevisiae* strains lacking Cu,Zn-SOD. We found a new stable form of alkyl hydroperoxide reductase 1 (Ahp1) with a lower isoelectric point. This form was also present in wild type strains after treatment with tert-butyl hydroperoxide. *In vitro* enzyme assays showed that Ahp1p had lower specific activity in strains lacking Cu,Zn-SOD. We studied three mutants presenting a reduced production of the low pI variant under oxidative stress conditions. Two of the mutants (C62S and S59D) were totally inactive, thus suggesting that the acidic form of Ahp1p may only appear when the enzyme is functional. The other mutant (S59A) was active *in vitro* and was more resistant to inactivation by tert-butyl hydroperoxide than the wild type enzyme. Furthermore, the inactivation of Ahp1p in *vivo* is correlated with its conversion to the low pI form. These results suggest that in *vivo* during some particular oxidative stress (alkyl hydroperoxide treatment or lack of Cu,Zn-SOD activity but not hydrogen peroxide treatment), the catalytic cysteine of Ahp1p is more oxidized than cysteine-sulfenic acid (a natural occurring intermediate of the enzymatic reaction) and that cysteine-sulfenic acid or cysteine-sulfonic acid variant may be inactive.

Oxygen is an essential element of life, but it is also at the origin of the generation of damaging reactive oxygen species (ROS) that are produced by cellular metabolism and mitochondrial respiration (1, 2). These highly reactive O₂ derivatives act on all cellular macromolecules (DNA, proteins, and lipids) (3–5). The production of ROS is normally balanced by their consumption by a set of non-enzymatic and enzymatic defenses. When there is an imbalance in favor of the oxidant forces, the cell is in a state of oxidative stress. In the yeast *Saccharomyces cerevisiae*, the cytosolic Cu,Zn-SOD (gene name SOD1) is one of the major enzymes involved in the battle against oxidative stress. Cu,Zn-SOD catalyzes the disproportionation of superoxide anions, producing O₂ and H₂O₂. A deficiency of this enzyme has serious consequences for the yeast cell during aerobicosis; lysine and methionine auxotrophies appear, and the mutation rate is increased (see Refs. 6 and 7 for review). To be functional, the Cu,Zn-SOD must have a copper atom correctly inserted into its catalytic site. This is achieved by a protein, the copper chaperone for superoxide dismutase (Lys7p for the yeast *S. cerevisiae*), which carries the copper to the superoxide dismutase and enables the correct insertion of the metal ion into the catalytic site of the enzyme. Therefore, Lys7p deficiencies have the same consequences as Cu,Zn-SOD deficiencies, and the characteristic phenotype of sod1 mutants is nearly identical to that of lys7 mutants (8, 9).

One of the oxidative protective mechanisms present in the cell is the peroxiredoxin family (10, 11). This class of enzymes, present in prokaryotes and eukaryotes, promotes the elimination of H₂O₂ and alkyl hydrogen peroxides, and in bacteria the AhpC eliminates peroxynitrite (12). All the peroxiredoxins contain a catalytic cysteine that is oxidized during the reduction of peroxides and is itself reduced by a thiol reducing equivalent. *In vivo* the best known reducing system encountered in eukaryotes consists of NADPH, thioredoxin, and thioredoxin reductase (the thioredoxin system) (13).

Five peroxiredoxins have been discovered in *S. cerevisiae*, each with a special cellular localization and function (14). For example, all null mutants of two of the cytosolic peroxiredoxin proteins, Tsa1p and Ahp1p, are differentially sensitive to specific oxidants. The Tsa1p null mutant is very sensitive to H₂O₂, whereas the Ahp1p null mutant is very sensitive to tert-butyl hydroperoxide (t-BOOH), an alkyl hydroperoxide (15). This indicates that these proteins have separate specialized functions within the cell.

We report the oxidative modification of Ahp1p (YLR109w, TPxII) in cells lacking Cu,Zn-SOD activity. This enzyme was discovered in 1999 (15–17) and is very abundant in the cytosol of the yeast. It seems to play an important role in the scavenging of alkyl hydroperoxide, especially during the stationary phase (14).
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**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions**—The *S. cerevisiae* strains used in this study are listed in Table I. The VP2101 and VP1909 strains were obtained by sporulation of strains (isogenic to S288C) FY-73A6 and FG-73A6, respectively. The *nat1,lys7* strain named W303-NL1 was constructed by the deletion/replacement of the *LYS7* gene (8) in the W303del Nat1 strain (20). Standard genetic methods and culture media were used (21, 22). Yeast cultures were maintained at 28 °C.

**Plasmids**—pAH1 was constructed by amplifying the ~681 bp 5′ flanking region of *AHP1* with an upstream primer that introduced an EcoRI site (5′-AAAAAATCTTCAACTAGGCAAGCAGACTT) and a downstream primer that introduced a BamHI site (5′-AAGAGATCTGAGCTCTTGCGCAGACTC). The amplified DNA fragment was inserted into the EcoRI-BamHI sites of pRS426 (23). The three mutations within Ahp1p, His6-Ahp1, were used (21, 22). Yeast cultures were maintained at 28 °C.

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**Two-dimensional Gel Electrophoresis and t-BOOH Treatment**—Two-dimensional analyses of the mutated sequences of Ahp1p were performed as described by Boucherie et al. (25). Yeast cultures were maintained at 28 °C.

**Modification of Ahp1p in Oxidatively Stressed Cells**—We carefully compared two-dimensional electrophoresis gels of total soluble proteins extracted from cells lacking superoxide dismutase activities (Δsod1 or Δlys7) with those for wild type cells. These analyses indicated that the amount of several proteins was modified (increased or decreased intensities) in cells lacking Cu,Zn-SOD. Most importantly, an intense novel protein spot, which was absent on two-dimensional maps of wild type cells, was detected in the cells lacking superoxide dismutase activities (Fig. 1A). This new spot was located close to a protein spot with a molecular mass of 19 kDa and an isoelectric point of 4.56 that had previously been identified as Ahp1p (24). Under normal growth conditions the basal levels of Ahp1p are high, but it was present at higher levels in both Δsod1 and Δlys7 strains. This overexpression is consistent with previous observations (28, 29) during H₂O₂ oxidative stress.

**RESULTS**

**Modification of Ahp1p by Oxidative Stress**—We looked for this form of Ahp1p when cells were subjected to 530 region of *AHP1* with a lower isoelectric point. The novel spot has the same apparent molecular weight as Ahp1p, but its isoelectric point is lower (4.44). To identify this protein, the spot was cut out of the gel and subjected to N-terminal microsequencing. No information could be obtained because the N terminus of the amino acid sequence is blocked, possibly by N-acetylation. Thus, a Δnat1,Δlys7 strain was constructed to bypass this problem. The novel spot was still present at higher levels in both Δsod1 and Δlys7 strains. This overexpression is consistent with previous observations (28, 29) during H₂O₂ oxidative stress.

**Purification of His6-Ahp1p**—VP2101 (wild type) or VP1909 (Δlys7) yeast cells carrying pV6H were grown for 36 h on selective SC-URA medium and then harvested. The cells were broken with glass beads in buffer A (0.05 m NaP₀, 0.3 m NaCl, pH 8.0). The cellular extract was loaded onto a nickel-nitri-trietionic acid column (Qiagen) equilibrated with the same buffer. The column was then washed with the same buffer and then washed twice with buffer A but with a pH of 6.3. The His₆-Ahp1 protein was eluted with buffer A supplemented with 500 mM imidazole, pH 7. Protein concentrations were determined by use of the Bio-Rad protein assay, using bovine serum albumin as the standard. The quantities of the monomer and the dimer were determined after gel electrophoresis and staining with Coomassie Blue by spectrophotometric scanning of the entire gel lane at 540 nm on a Beckman type DU-70 spectrophotometer followed by area integration of each peak.

**Measurement of the Enzymatic Activity of His₆-Ahp1p**—The enzyme assay was adapted from the method described by Chao et al. (27). The reaction mixture (500 μl) was buffered at pH 7 by 50 mM Hepes. It contained 150 μM NADPH, 50 μM Escherichia coli thioredoxin reductase (Sigma, catalog number T7051), 5 μM *E. coli* thioredoxin (Promega, catalog number T7915), and t-BOOH, cumene hydroperoxide (Cum-OOH) or H₂O₂, as the substrates. After 3 min, ~100 nM of His₆-Ahp1p was added, and the decrease of absorbance at 340 nm was monitored.

| Strain  | Genotype           | Laboratory collection | Source                  |
|---------|--------------------|-----------------------|-------------------------|
| FY-A6   | MATα ura3–52 trp1Δ63 his3Δ200 |                       | Laboratory collection |
| FY-3A6  | MATα/MATα ura3–52/ura3–52 TRP1/trp1Δ63 his3Δ200/his3Δ200 |                       | Laboratory collection |
| FY-A61  | MATα ura3–52 trp1Δ63 his3Δ200 lys7::HIS3 | 8                      | This study              |
| FG-73A6 | MATα/MATα ura3–52/ura3–52 TRP1/trp1Δ63 his3Δ200/his3Δ200 LYS7::HIS3 | 8                      | This study              |
| FY-A6asod1 | MATα ura3–52 trp1Δ63 his3Δ200 sod1::URA3 | 8                      | This study              |
| VP2101  | MATα trp1 – 52 trp1Δ63 his3Δ200 | 8                      | This study              |
| VP1909  | MATα ura3–52 trp1Δ63 his3Δ200 lys7::HIS3 | 8                      | This study              |
| IYP198  | MATα his6–2 lys2–80gamber ade2–101chrr leu2–Δ1 | 18                     | This study              |
| IYP198Ahp1 | MATα ura3–52 lys2–80gamber ade2–101chrr leu2–Δ1 | 19                     | This study              |
| W303del Nat1 | MATα ade2–1, ura3–1, his3–11, trp1–1, leu2–3, can1–100, nat1::LEU2 | 20                     | This study              |
| W303-NL1 | MATα ade2–1, ura3–1, his3–11, trp1–1, leu2–3, can1–100, nat1::LEU2, lys7::HIS3 | This study              | This study              |

**TABLE I**

**Yeast strains used in this study**
Abnormal Dimerization of Ahp1p Isolated from a Strain Lacking Cytosolic Superoxide Dismutase Activity—The mechanistic scheme proposed for the antioxidan role of the peroxide oxidase family and Ahp1p involves the oxidation step of the catalytic Cys62, which generates a sulfenic acid (SOH). This reaction further takes place with the SH of the Cys120 of another molecule of Ahp1p creating a disulfide bond (16, 30). This bond forms between the Cys62 (the catalytic cysteine) of one monomer and the Cys120 of the other monomer, forming a covalent dimer of the enzyme. This bond is then reduced by the thioredoxin (Trx)/thioredoxin reductase (Trr) system which derives its reducing power from the electrons of NADPH. In this way, the ability of the enzyme to make a disulfide bridge between two subunits and to become covalently homodimerized is essential for its activity.

We investigated the ability of the forms of Ahp1p found during oxidative stress to form this disulfide bridge. A His6-tagged Ahp1p was expressed from a multicopy plasmid (pV6H) in which the coding sequence was under the control of a strong constitutive promoter (phosphoglycerate kinase promoter) in a wild type strain of S. cerevisiae and in its isogenic Δlys7 strain. We used two-dimensional electrophoresis to check for the presence of the modified form of Ahp1p, in these conditions in a Δlys7 strain carrying the overexpression plasmid (Fig. 1C). The His6-tagged Ahp1p is about 1 kDa heavier, and its pI is ~1 unit higher because of the addition of the histidines. Nevertheless, two forms of this tagged Ahp1p were still present in these conditions, although the acidic form was slightly less abundant in the tagged Ahp1p than in the native protein. The proportion of the tagged form with the lowest pI was about 25% of the total pool of tagged protein. This might be due to the high level of overproduction of this protein in the cell, as a multicopy vector and a strong promoter were used, or alternatively it may be due to a slight influence of the six-histidine tag. The second possibility seems unlikely because in cells expressing both the normal and the tagged Ahp1p, the ratios between modified and unmodified proteins are similar (data not shown).

The tagged enzyme was prepared from the wild type and the Δlys7 strains and purified on a nickel column; therefore it was highly pure (Fig. 2A). Nevertheless, a faint fast migrating band was still present below His6-Ahp1p at the end of the purification procedure. This band was not recognized by anti-penta-His antibodies. This band never disappeared regardless of the ionic strength of the washing solutions (up to 2 M NaCl); thus it can be assumed that it is native Ahp1p, which was copurified with the tagged protein because of the presence of covalent disulfide bonds between the two proteins. Furthermore, when the tagged protein was purified from a ΔΔlys1 strain carrying pV6H, this band was almost totally absent. This residual portion is probably the part of protein translated from the second AUG located just after the sequence coding for the six histidines in the N terminus.

The oligomerization state of the purified protein was examined on SDS-PAGE under non-reducing conditions to check for the presence of disulfide covalent bridges (Fig. 2B). All of the enzyme purified from the wild type strain was completely in the disulfide-linked dimeric forms (molecular mass ~40 kDa) when the sample was not artificially reduced (with dithiothreitol or 2-mercaptoethanol). Two major dimeric forms were seen. These are probably dimers with one and two disulfide linkages between the opposite Cys62 and Cys120 as hypothesized by Jeong et al. (16). The protein purified from the Δlys7 cells presented a different pattern of disulfide-linked dimerization; at least four bands with a similar molecular weight to the dimer were revealed by Coomassie Blue staining or Western blot analysis with a shorter exposure time than that in Fig. 2B,
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Fig. 2. Electrophoretic analyses of purified His6-Ahp1p. A, the tagged protein (2 μg) purified from a wild type strain (W) and from a Δlys7 strain was analyzed on a 12.5% reducing SDS-PAGE. Left panel, Coomassie Blue staining; right panel, Western blot analysis of the same gel with anti-penta-His antibody. B, left panel, His6-Ahp1p (same amount as in A) was examined on a 10% non-reducing SDS gel. Left panel, Coomassie Blue staining; right panel, Western blot analysis of this gel with anti-penta-His antibody.

right panel. The two major forms found in His6-Ahp1p extracted from a wild type strain were still present, but two additional forms were also present in equivalent amounts. Furthermore, a part of the tagged protein was still in the monomeric form under non-reducing conditions. Under these conditions between 7 and 25% of the total protein was in the monomeric form, depending on the preparation, as calculated by spectrophotometric scanning of the stained SDS-PAGE and area integration of the peaks.

In this oxidative stress context, the pattern of disulfide-linked dimerization of the protein is abnormal, with new, unnatural dimeric forms, which might involve the linkage of the other cysteine of the enzyme, Cys31. Similarly, part of the protein had undergone alterations which affect its ability to form a dimer by disulfide bridges.

His6-Ahp1p Extracted from a Strain Lacking Cytosolic Superoxide Dismutase Has a Diminished Specific Activity—To assess the peroxide reductase activity of Ahp1p purified from cells under normal conditions or subjected to superoxide anion oxidative stress, an in vitro assay was set up (see "Experimental Procedures") combining a thioredoxin/thioredoxin reductase system from E. coli and the alkyl hydroperoxide reductase to be tested from S. cerevisiae. The Trx/Trr system from E. coli can functionally substitute for the yeast system as both proteins can functionally substitute for the yeast system as both proteins and the alkyl hydroperoxide reductase to be tested from S. cerevisiae. The Trx/Trr system from E. coli can functionally substitute for the yeast system as both proteins were required for the oxidation of NADPH in the presence of substrates of the enzyme (Fig. 3). This thioredoxin reduction system is well conserved in bacteria and yeast, as the percentage of similarity between the Trx1p of both organisms is 65% and that of the cytosolic Trr is 66%. Three substrates were tested as follows: H2O2, t-BOOH, and Cum-OOH. For each of these compounds, the catalytic constants of the enzyme (Vmax/Km) is 0.11 liters·min⁻¹·mg⁻¹ for H2O2, 0.2 liters·min⁻¹·mg⁻¹ for t-BOOH, and 1.9 liters·min⁻¹·mg⁻¹ for Cum-OOH were very close to those measured by Jeong and co-workers (16), confirming the relevance of using the bacterial reduction system with the yeast Ahp1p.

This assay was carried out with t-BOOH, and the specific activity of the purified His6-Ahp1p extracted from a wild type strain was compared with that of a Δlys7 strain. The data showed that the activity differs according to the origin of the enzyme. When His6-Ahp1p was extracted from a Δlys7 strain, its specific activity was consistently decreased by 10–20% compared with the enzymatic activity of the protein purified from the wild type strain.

The Shift of pI of Ahp1p Under Oxidative Conditions Is Dependent on a Fully Active Enzyme—We used three distinct mutants of Ahp1p, one mutation is the replacement of Cys46 (the catalytic cysteine), and the two others are replacements of Ser29. This amino acid was mutated because it represented a possible target for phosphorylation that could have explained the modification of pI. The S59D mutation could have mimicked a constitutive phosphorylation of Ahp1p, but as discussed later a phosphorylation is not implicated here. Nevertheless, those mutants gave us interesting information.

The two first mutants of the enzyme were constructed on multicopy plasmids carrying the mutated sequences of AHP1 under their own promoter. The mutations were named S59D and C62S. The two plasmids carrying the altered sequences were unable to complement the growth defect of a Δahp1 strain when grown onto a solid medium in the presence of t-BOOH or Cum-OOH, indicating that these Ahp1p mutants were probably non-functional.

The proteome of Δahp1 cells expressing the mutated forms of Ahp1p and treated with t-BOOH were analyzed by two-dimensional polyacrylamide gel electrophoresis. Unlike wild type Ahp1p, the mutated Ahp1-C62S did not exhibit the shift of the isoelectric point that was usually seen during oxidative treatment (Fig. 4). For the Ahp1-S59D mutant, the isoelectric point was altered due to the addition of a charged amino acid (an aspartate residue). This caused the protein to migrate on the two-dimensional map to the same position as the previously identified acidic spot. However, the treatment with t-BOOH did not induce a pI shift in this mutant.
The two mutated sequences were subcloned into the pV6H plasmid, so that they were upstream of an N-terminal tag of six histidines. The two mutated proteins were purified, and their enzymatic activities were assessed by the \textit{in vitro} assay described above. As expected the C62S mutant did not exhibit any enzymatic activity when t-BOOH was used as a substrate, but surprisingly the S59D mutant was also inactive. It is logical that the enzymatic activity of Ahp1p is totally abolished when the catalytic cysteine, Cys\textsuperscript{452}, is mutated. In addition a non-reducing SDS-PAGE showed that Ahp1p-C62S could not dimerize via a disulfide bridge (data not shown). For the mutated form Ahp1p-S59D, the non-functionality of the enzyme is probably due to its inability to make a dimer by a disulfide bond. Indeed, unlike the wild type, this mutant only displayed the monomeric form on a non-reducing SDS-PAGE (not shown). This may be due to a steric effect caused by the side chain of the aspartate residue.

Thus, the two different mutations that inactivate Ahp1p prevent the appearance of the modified form of Ahp1p on twodimensional polyacrylamide gels during oxidative stress, suggesting that the functionality of the enzyme may be necessary for its formation.

**Normal Sensitivity to Inactivation Is Required to Observe a Modification of the pI during Oxidative Stress**—It is well known that peroxiredoxins are progressively inactivated by the substrate (17, 30). This inactivation specifically affects the peroxiredoxin system and not the thioredoxin system (30). We used His\textsubscript{6}-Ahp1p and an \textit{in vitro} assay to show that the inactivation was very rapid when the concentration of t-BOOH reached 10 mM (Fig. 5, \textit{left panel}). We have also studied another mutated form of Ahp1p (S59A), which has a smaller modification of its isoelectric point than the wild type protein during oxidative stress (Fig. 4). Nevertheless, this mutant was functional. Indeed, the strains expressing Ahp1p-S59A could grow on a medium containing 1 mM t-BOOH but less well than the strains expressing wild type Ahp1p. It is noteworthy that the strains carrying the mutated forms C62S and S59D could not grow at all with this concentration of t-BOOH. Furthermore, the \textit{in vitro} specific activity of His\textsubscript{6}-Ahp1p-S59A was identical to that of the wild type protein. However, one major difference was revealed (Fig. 5, \textit{right panel}); the S59A mutant of Ahp1p was much more resistant to inactivation by high concentrations of substrate. We investigated the ability of His\textsubscript{6}-Ahp1p and of His\textsubscript{6}-Ahp1p-S59A to form disulfide linkages after 1 h of incubation with increasing concentrations of t-BOOH and with the Trx/Trr reduction system permitting the enzyme to be fully operational (Fig. 6) and to reconstitute the intracellular conditions of an increased amount of oxidant substrates \textit{in vitro}. In Fig. 6, \textit{lane 1}, where there is nearly 100 times more t-BOOH than His\textsubscript{6}-Ahp1p, all of the enzyme was in the two dimeric disulfide linked forms previously observed in Fig. 2B after 1 h. When the substrate is so low, the reducing system that disrupts the disulfide bridges proceeds at a slower rate than the rate of association by disulfide linkage of the two subunits of His\textsubscript{6}-Ahp1p. When we increased the ratio of t-BOOH/His\textsubscript{6}-Ahp1p to 500 or 2000 the disulfide-linked dimeric form of His\textsubscript{6}-Ahp1p disappeared. This suggests that the cysteines of the enzyme are attacked. Here we can see clearly that when the quantity of alkyl hydroperoxide is high, and when the enzyme is incubated with all the components required to sustain its catalytic cycle, the formation of the disulfide bridges is progressively prevented. The same experiment, in the absence of the thioredoxin-reducing system showed that His\textsubscript{6}-Ahp1p was completely in the disulfide-linked dimeric forms (data not shown). Conversely, the mutated enzyme His\textsubscript{6}-Ahp1p-S59A was still in the dimeric form when the higher concentrations of t-BOOH were tested in presence of the thioredoxin reduction system. This confirms that this mutant form is not inactivated as was seen by monitoring the consumption of NADPH in the activity test.

In contrast to what was seen for the C62S and S59D mutant forms of His\textsubscript{6}-Ahp1p, the mutant form S59A remained active and was surprisingly more resistant to inactivation by t-BOOH. Nevertheless, all of the mutants exhibited little or no pI shift during oxidative stress.

**The in Vitro Inactivated Form of Ahp1p Has a More Acidic Isoelectric Point**—We investigated the isoelectric point of the enzyme at the end of the \textit{in vitro} test when the enzyme was no longer a dimer with a disulfide bond and when the enzymatic assay showed that it was inactive. As described above, His\textsubscript{6}-Ahp1p was allowed to react with a large concentration of t-BOOH in the presence of the thioredoxin reducing system, and the reaction mixture was analyzed by two-dimensional gel electrophoresis (Fig. 7A). We obtained one spot at the expected location on the two-dimensional gel compared with the two-dimensional map of proteins of a strain expressing His\textsubscript{6}-Ahp1p. To determine the exact form observed (normal or lower pI), we ran a second gel with the same sample added to an identical quantity of fresh His\textsubscript{6}-Ahp1p at the moment of loading on the gel (Fig. 7B). This fresh protein was considered as the reference position for a His\textsubscript{6}-Ahp1p with a normal isoelectric point. Other reference proteins were the Trr and Trx that were present in the reaction mixture (not shown). The map location of His\textsubscript{6}-Ahp1p after incubation with t-BOOH as a substrate corre-
sponded exactly to the position of the low pI form of His₆-Ahp1p and all the protein molecules displayed this pI.

**DISCUSSION**

During definite oxidative stress (deficiency of intracellular scavengers for superoxide anions or growth in the presence of the alkyl hydroperoxide t-BOOH), the Ahp1 is modified. This modification changes its global net electric charge but not the molecular weight.

To determine the nature of this protein modification, we first looked to see whether Ahp1p was phosphorylated, because many indications supported this possibility. First, the two-dimensional map of oxidatively stressed cells revealed a phosphorylated spot at the same place as the modified spot of Ahp1p (data not shown). Second, Ahp1p with a mutation of Ser59, which is a putative phosphorylation site, was not modified by oxidative stress. However, this phosphorylation hypothesis is not correct as shown by labeling the phosphorylated proteins of the /H9004 lys7 strain grown in a medium containing radioactive phosphorus. The purified His₆-Ahp1p extracted from the /H9004 lys7 strain was not radioactive.

We showed that the modification process can only occur if the enzyme is functional and can therefore achieve its catalytic cycle and when the enzyme is sensitive to inactivation by alkyl hydroperoxide substrates. During the catalysis, the thiol (—SH) of the active Cys⁶² becomes an unstable sulfenic acid (—SOH) that immediately reacts with another thiol of Cys¹²⁰ to form a disulfide bond (—S—S—) before being reduced by another enzyme (the thioredoxin system). We believe that when the catalytic cysteine is in the form of a —SOH, a second attack by alkyl hydroperoxides (or lipid hydroperoxides) could lead to the formation of a sulfenic acid (—SO₂H) or a sulfonic acid (—SO₃H), both of which are very stable (and cannot be reduced by dithiothreitol or 2-mercaptoethanol) and can irreversibly inactivate the enzyme. This would explain the change of pI of the protein, the lessening of the *in vitro* activity of the tagged His₆-Ahp1p extracted from cells submitted to oxidative stress, and the inability to find disulfide-linked dimer when the quantity of substrate *in vitro* is too high.

The *in vitro* activity decreased by ~25% of the activity of the protein extracted from a wild type strain during oxidative stress. We hypothesize that this is linked to the portion of the more acidic form observed on the two-dimensional gel for the tagged enzyme, as this portion never exceeded 25% for the tagged enzyme (Fig. 1C). This might be related to the high level of overproduction of the protein.

The substrates of Ahp1p also have a deleterious effect on the activity of the enzyme. Chae et al. (30) used an *in vitro* assay to show that one of the four other members of the peroxiredoxin family in *S. cerevisiae*, the Tsa1p enzyme, is inactivated by H₂O₂ in a concentration- and a time-dependent manner. We found a similar decrease in the activity of His₆-Ahp1p when t-BOOH, cumene-OOH, or H₂O₂ were used as substrates. The non-reducing SDS-PAGE analysis showed that a disulfide bridge-mediated dimerization was impossible in the inactivated enzyme, and the two-dimensional analysis of this inactivated protein clearly showed that it corresponds to the low pI form of Ahp1p. These results are consistent with the fact that the decrease in activity of the enzyme is linked to an irrevers-
is upset in cells lacking cytoplasmic Cu,Zn-SOD activity, leading to the inefficient scavenging of the numerous oxidizing molecules; the intracellular reducing power is then decreased, as indicated by the decreased level of available NADPH (31).

The level of $O_2^-$ increases in cells lacking Cu,Zn-SOD activity and the superoxide-driven Fenton chemistry lead to an increase in the more powerful oxidant OH$^-$ (6). We assume that this molecule acts either directly on the cysteine-sulfenic acid of Ahp1p to increase its oxidation state or, alternatively, that it acts indirectly by considerably increasing the level of lipid hydroperoxides, which are probably the natural substrates of Ahp1p, increasing the amount of oxidized lipids beyond the scavenging capacity of the cell. This in turn enables these lipid hydroperoxides to react with the cysteine-sulfenic acid of Cys$^{82}$ before this catalytic intermediate can condense with the thiol of Cys$^{120}$ of the other subunit of Ahp1p.

In the strain lacking Cu,Zn-SOD, the quantity of normal Ahp1p is slightly increased, whereas the low pI variant is induced in nearly equal amount. It seems that the cells adapt the rate of synthesis of Ahp1p to match its rate of inactivation. This is true of exponentially growing cells and may not be the case for the stationary phase cells, as Cu,Zn-Sod plays a more important role. The absence of this key cytoplasmic enzyme might accelerate the inactivation of Ahp1p, and this would participate in the decrease of survival rate of these cells. It is noteworthy that the global electric charge of Ahp1p was not altered when cells were treated with $H_2O_2$. This might be because $H_2O_2$ is not the substrate of choice of Ahp1p inside the cell and that this oxidant is preferentially dealt with by other reducing systems like the catalases, glutathione peroxidases, or Tsa1p.

Mitsumoto et al. (32) showed that the isoelectric point of peroxiredoxins in human endothelial cells alters in response to oxidative stress. At least four out of the six known human members of the peroxiredoxin family can be modified by chemical oxidative stress (alkyl or hydrogen peroxide), but this is not the case for S. cerevisiae. For example, the pI of Tsa1p, a well characterized and abundant cytosolic peroxiredoxin of the yeast, was not modified when the cells were treated with both hydrogen or organic peroxides. This indicates that Ahp1p is particularly sensitive.

It has been shown recently that some Cys-SOH are present in native proteins of various organisms, either as stabilized structural residues, catalytic intermediates, or as a mechanism of redox sensing (see Refs. 33 and 34 for review). Little is known about the fate of those Cys-SOH during oxidative stress. It is not known whether the cysteine-sulfenic acid becomes more oxidized resulting in a sulfinic or sulfonic derivative when subjected to severe oxidative stress, as is possibly the case for Ahp1p. This would lead to the irreversible inactivation of many cellular mechanisms.

The inactivation of Ahp1p during oxidative stress may have important biological implications because it could be considered as a good model for the cellular dysfunctional mechanisms resulting from the oxidative stress involved in aging and in some neurodegenerative disorders (35, 36).

REFERENCES
1. Longo, V. D., Gralla, E. B., and Valentine, J. S. (1996) J. Biol. Chem. 271, 12275–12280
2. Cadenas, E. (1989) Annu. Rev. Biochem. 58, 79–110
3. Berlett, B. S., and Stadtman, E. R. (1997) J. Biol. Chem. 272, 20313–20316
4. Dean, R. T., Fu, S., Stocker, R., and Davies, M. J. (1997) Biochem. J. 324, 1–18
5. Moradas-Ferreira, P., Costa, V., Piper, F., and Mager, W. (1996) Mol. Microbiol. 19, 651–658
6. Gralla, E. B., and Kosman, D. J. (1992) Adv. Genet. 30, 251–319
7. Fridovich, I. (1995) Annu. Rev. Biochem. 64, 97–112
8. Gamonet, F., and Lauquin, G. J.-M. (1998) Eur. J. Biochem. 251, 716–723
9. Culotta, V. C., Klomp, L. W. J., Strain, J., Casarosa, R. L. B., Krens, B., and Gitlin, J. D. (1997) J. Biol. Chem. 272, 23469–23472
10. Jacobson, P. S., Morgan, R. W., Christman, M. F., and Ames, B. N. (1988)
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11. Chae, H. Z., Robinson, K., Poole, L. B., Church, G., Storz, G., and Rhee, S. G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7017–7021
12. Bryk, R., Griffin, P., and Nathan, C. (2000) Nature 407, 211–215
13. Carmel-Harel, O., and Storz, G. (2000) Annu. Rev. Microbiol. 54, 439–461
14. Park, S. G., Cha, M.-K., Jeong, W., and Rhee, S. G. (2000) J. Biol. Chem. 275, 5723–5732
15. Lee, J., Spector, D., Godon, C., Labarre, J., and Toledano, M. B. (1999) J. Biol. Chem. 274, 4537–4544
16. Jeong, J. S., Kang, S. W., Rhee, S. G., and Kim, K. (1999) Biochemistry 38, 776–783
17. Verdoucq, L., Vignols, F., Jacquot, J.-P., Chartier, Y., and Meyer, Y. (1999) J. Biol. Chem. 274, 19714–19722
18. Sikorski, S., and Hieter, P. (1989) Genetics 122, 19–27
19. Vida, K., Spector, D., Lagniel, G., Lopez, S., Toledano, M. B., and Labarre, J. (2001) J. Biol. Chem. 276, 8469–8474
20. Mullen, J. R., Kayne, P. R., Moerschell, R. P., Tsunasawa, S., Grishok, M., Calovito-Shepanski, M., Grunstein, M., Sherman, F., and Sternfels, R. (1989) EMBO J. 8, 2067–2075
21. Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J., and Struhl, K. (1992) Current Protocols in Molecular Biology, John Wiley & Sons, Inc., New York
22. Sherman, F., Fink, G., and Lawrence, C. (1974) Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
23. Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H., and Hieter, P. (1992) Gene (Amst.) 110, 119–122
24. Minet, M., and Lacroute, F. (1990) Curr. Genet. 18, 287–291
25. Bouchard, H., Dujardin, G., Monribot, C., Slonimski, P., and Perrot, M. (1995) Yeast 11, 601–613
26. Maillet, I., Lagniel, G., Perret, M., Bouchard, H., and Labarre, J. (1996) J. Biol. Chem. 271, 10263–10270
27. Chae, H. Z., Kang, S. W., and Rhee, S. G. (1999) Methods Enzymol. 300, 748–754
28. Lee, J., Godon, C., Lagniel, G., Perret, M., Garin, J., Labarre, J., and Toledano, M. B. (1999) J. Biol. Chem. 274, 16040–16046
29. Godon, C., Lagniel, G., Lee, J., Buhler, J. M., Kieffer, S., Perrot, M., Bouchard, H., Toledano, M. B., and Labarre, J. (1998) J. Biol. Chem. 273, 22480–22489
30. Chae, H. Z., Chung, S. J., and Rhee, S. G. (1994) J. Biol. Chem. 269, 27670–27678
31. Sieker, K. H., Kosman, D. J., and Culeotta, V. C. (1996) J. Biol. Chem. 271, 28831–28836
32. Mitsumoto, A., Takanezawa, Y., Okawa, K., Iwamatsu, A., and Nakagawa, Y. (2001) Free Radic. Biol. Med. 30, 625–635
33. Claiborne, A., Miller, H., Parsonsage, D., and Ross, R. P. (1993) FASEB J. 7, 1483–1490
34. Claiborne, A., Yeh, J. I., Mallet, T. C., Luba, J., Crane, E. J., Charrier, V., and Parsonsage, D. (1999) Biochemistry 38, 15407–15416
35. Ames, B. N., Shigenaga, M. K., and Hagen, T. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7915–7922
36. Mates, J. M., and Sanchez-Jimenez, F. (1999) Front. Biosci. 4, D339–D345