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Glutathione peroxidase induction protects *Saccharomyces cerevisiae sod1Δsod2Δ* double mutants against oxidative damage

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Abstract

Saccharomyces cerevisiae mutants deficient in superoxide dismutase genes (*sod1Δ*, *sod2Δ* and the double mutant) were subjected to H_2O_2 stress in the stationary phase. The highest sensitivity was observed in the *sod2Δ* mutant, while the *sod1Δsod2Δ* double mutant was not sensitive. *sod* mutants had lower catalase activity (44%) than wild-type cells, independent of H_2O_2 stress. Untreated cells of *sod1Δsod2Δ* double mutants showed increased glutathione peroxidase activity (126%), while *sod1Δ* had lower activity (77%) than the wild type. Glutathione levels in *sod1Δ* were increased (200-260%) after exposure to various H_2O_2 concentrations. In addition, the highest malondialdehyde levels could be observed without H_2O_2 treatment in *sod1Δ* (167%) and *sod2Δ* (225%) mutants. In contrast, the level of malondialdehyde in the *sod1Δsod2Δ* double mutant was indistinguishable from that of the wild type. These results suggest that resistance to H_2O_2 by *sod1Δsod2Δ* cells depends on the induction of glutathione peroxidase and is independent of catalase, and that glutathione is a primary antioxidant in the defense against H_2O_2 in stationary phase *sod1Δ* mutants.

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- Superoxide dismutase
- Glutathione
- Hydrogen peroxide
- *Saccharomyces cerevisiae*
- Reactive oxygen species

Introduction

Oxygen metabolism may lead to the production of reactive oxygen species (ROS), i.e., superoxide and hydroxyl radicals and hydrogen peroxide (H_2O_2), by sequential one-electron reductions. ROS damages all cellular components, including protein, DNA and lipids. A primary source of superoxide is the electron transport chain in the inner membrane of mitochondria, where about 2% of the oxygen consumed during respiration is incompletely reduced to ROS. To counteract the oxidative stress resulting from ROS, cells possess a range of nonenzymatic and enzymatic defense systems, including glutathione (GSH), thioredoxin, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (1).

A first line of defense includes the SOD enzymes that catalyze the disproportionate cleavage of superoxide to H_2O_2 and water.

H_2O_2 is enzymatically catabolized in aerobic organisms by catalase and several peroxidases. *Saccharomyces cerevisiae*, like most other eukaryotes, contains CuZnSOD (the product of the *SOD1* gene) in the cytosol, nucleus, and lysosomes and MnSOD (the product of the *SOD2* gene) in the mitochondrial matrix.

MnSOD is believed to be the major means of protection against mitochondrial superoxide (2). The sources of ROS relevant to CuZnSOD are less clear because of the location of this enzyme within the cytosol. Neither MnSOD nor CuZnSOD is strictly essential; however, the loss of CuZnSOD has dramatic phenotypic consequences in yeast. Yeast strains lacking CuZnSOD (*sod1Δ*) show several defects during aerobic growth. These include reduced growth rates in glycerol and ethanol, auxotrophy for lysine and methionine or cysteine, a higher rate of spontaneous mutation, more rapid loss of viability in the stationary phase, increased iron uptake, elevated levels of "free iron", and sensitivity to redox-cycling drugs such as paraquat or menadione (3-8). *sod2Δ* mutants are oxygen-sensitive and, when required to utilize oxygen, grow poorly and are particularly sensitive to paraquat (6). s*od1Δsod2Δ* double mutants are more severely affected, exhibiting essentially all the characteristics of the single mutant phenotypes.

The full growth cycle of a yeast culture begins with the exponential phase of growth (log phase) and progresses through the diauxic shift to the true stationary phase. In the log phase, the cells use glucose to produce energy via glycolysis. The diauxic shift occurs when fermentable nutrients become limited and energy metabolism shifts to respiration, the growth rate slows, and cells utilize ethanol and other two- and three-carbon compounds for energy production. In the total absence of any nutrients, the cells enter the true stationary phase; no cell division occurs, the metabolic rate slows and cells can survive for weeks to months.

Stationary phase yeast resemble the majority of cells of multicellular organisms in two important aspects: 1) their main source of energy is mitochondrial respiration, and 2) the cells have exited the cell cycle and entered the G0 phase. With increasing time in the stationary phase, damage accumulates. This damage cannot be excluded by cell synthesis and division, which is not occurring, and thus must be prevented or repaired. For this reason the yeast *S. cerevisiae* has been extensively exploited as a model for advancing our understanding of cellular defenses against ROS.

In the present study we investigated the role of nonenzymatic and enzymatic defense systems after H_2O_2 treatment in *sod* mutants. We found that *sod1Δ* and *sod1Δsod2Δ* double mutants show very little or no sensitivity to H_2O_2 in the stationary phase. The double mutant displays increased GPx activity and reduced malondialdehyde (MDA) levels compared to wild-type cells, whereas CAT is decreased. The *sod1Δ* mutant has the highest total GSH content after H_2O_2. Thus, the resistance to H_2O_2 in *sod1Δsod2Δ* cells is dependent on the induction of GPx, whereas CAT does not appear to be required. We suggest here that GSH is a primary antioxidant in the defense against H_2O_2 in *sod1Δ* mutants.

Material and Methods

Strains of *Saccharomyces cerevisiae*

The wild-type *S. cerevisiae* strain EG103 and the isogenic mutant strains *sod1Δ*, *sod2Δ* and *sod1Δsod2Δ* were kindly provided by Dr. E. Gralla (University of California, Los Angeles, CA, USA). Disruption of the *SOD1* and *SOD2* genes was performed as described previously (4,9). MG5312 was a gift from Dr. M. Brendel (J.W. Goethe-University, Frankfurt, Germany). The relevant genotypes of the strains are listed in Table 1.

Media and growth conditions

Yeast strains were grown at 30ºC in YPD liquid medium containing 2% glucose, 1% yeast extract, 2% bacto-peptone, or selective medium supplemented with the appropriate nutrients (SD medium with 2% glucose, 0.67% yeast nitrogen base without amino acids, plus nutrients). The flask volume/medium ratio was 2:1 (microaerophilic conditions) and flasks were shaken at 200 rpm. For solid medium 2% agar was added (10). The cell lines lacking CuZnSOD were continually monitored for suppressor activity (4,6).

Hydrogen peroxide treatment

Yeast cells were grown to the stationary phase (1-2 x 10^8 cells/ml) in YPD medium at 30ºC. Cells were harvested and washed in sterile saline (0.9% NaCl) and the cell pellets were resuspended in saline and treated with increasing concentrations of H_2O_2 (0.5-10 mM) at 30ºC for 1 h.

For dose-response curves, aliquots of cells were diluted in saline and plated in triplicate onto YPD to obtain viable counts after 3-5 days of growth at 30ºC.

Enzyme activities

Crude extracts were prepared by glass bead lysis as follows: cells were suspended in lysis buffer (50 mM Tris, 150 mM NaCl, 50 mM EDTA, pH 7.2) with an equal volume of acid-washed 425-600 µm glass beads and phenylmethylsulfonyl fluoride, vortexed for 10-15 cycles (30 s each), followed by 30 s of cooling. The mixture was then microcentrifuged for 2 min to remove the cellular debris and glass beads (6).

CAT activity was determined spectrophotometrically by monitoring the disappearance of H_2O_2 at 240 nm (11). GPx activity was determined by monitoring the NADPH consumption rate at 340 nm (12). Protein concentration was determined by the Bradford assay (13).

Assay of total glutathione

Total GSH was monitored by a recently developed microbiological method that uses a GSH auxotrophic yeast strain as a sensor for the presence of GSH, GSSG and γ-Glu-Cys (14). The indicator strain, *S. cerevisiae* MG5312 (Table 1), homozygous for the mutant allele *gsh1ΔURA3*, was grown to a cell density of 2 x 10^8 cells/ml, washed twice with and then suspended in potassium phosphate buffer (25 mM KH_2PO_4, 50 mM Na_2HPO_4, pH 7.0) to a concentration of 2 x 10^7 cells/ml. One hundred microliters of this suspension was added to 3.5 ml top-agar (0.4% agar in potassium phosphate buffer) at a temperature of 48ºC and poured immediately onto synthetic medium lacking GSH. After the top-agar had solidified, 20 µl of the samples to be tested was applied to a sterile paper disc 10 mm in diameter on the agar surface. Plates were incubated at 30ºC for 3 days. Growth of MG5312 depends on the presence either GSH or GSSG. Growth zones on agar plates seeded with MG5312 permit the quantitative determination as little as 0.1 µg GSH (for details see Ref. 14).

Preparation of yeast samples. After H_2O_2 treatment yeast cells were harvested, washed twice and suspended in potassium phosphate buffer to a final concentration of 4%. Cell suspensions were heat-treated for 15 min at 85ºC and cell debris was removed by centrifugation at 11,700 *g*.

Table 1. Yeast strains used in this study.

Yeast strain	Genotype	Ref.
EG103	(DBY746) *MATa leu2-3, 112 his3Δ1 trp1-289 ura3-52 GAL+*	4
EG118	EG103 with *sod1Δ::URA3*	4
EG110	EG103 with *sod2Δ::TRP1*	9
EG133	EG103 with *sod1Δ::URA3 sod2Δ::TRP1*	9
MG5312	MATa/MATα *ade2-101/ade2-101 LEU2/leu2Δ1 LYS2/lys2-801 TRP1/trp1-Δ1 TRP5/trp5-a ura3-52/ura3-52 gsh1-Δ1::URA3/gsh1-Δ1 URA3 (rho-)*	14

Malondialdehyde determination

MDA was measured by HPLC by the method described by Esterbauer and Cheeseman (15). Briefly, an aqueous sample containing MDA, pH 6.5-8.0, was separated by HPLC using an amino-phase column with acetonitrile, 30 mM Tris buffer, pH 7.4 (1:9 v/v). The effluent was monitored at 267 nm (15).

Statistical analysis

Results are reported as means ± SD and were analyzed by the Student *t*-test. Values of $P < 0.05$ were considered to be statistically significant.

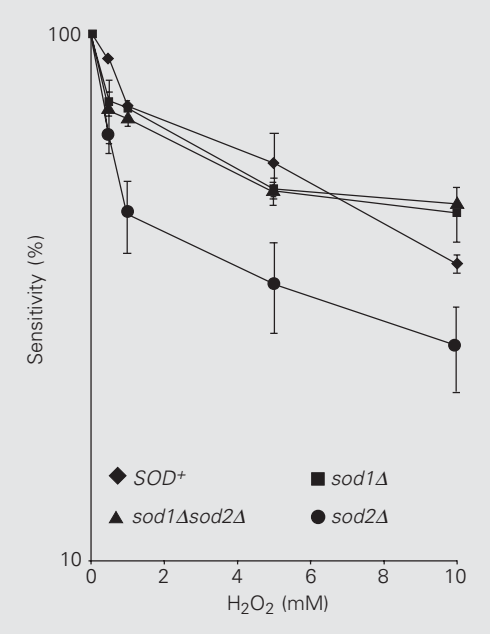

Figure 1. Sensitivity of *sod* null mutants exposed to H_2O_2. The strains used were: *SOD+*, *sod1Δ*, *sod2Δ* and *sod1Δsod2Δ*. Data are reported as the mean ± SD of three independent experiments.

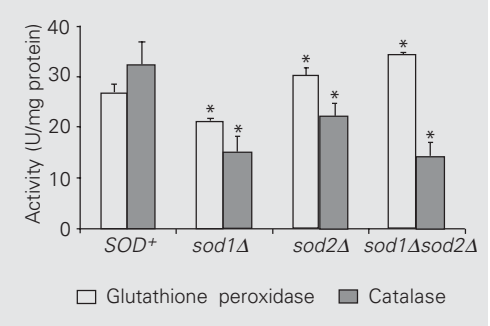

Figure 2. Activity of catalase and glutathione peroxidase in untreated wild-type and *sod* mutant cells. Data are reported as the mean ± SD of three independent experiments. *P < 0.05 compared to *SOD+* (Student *t*-test).

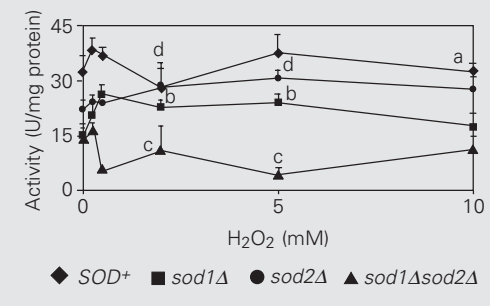

Figure 3. Activity of catalase in stationary wild-type and *sod* mutant cells exposed to H_2O_2. The strains used were: *SOD+*, *sod1Δ*, *sod2Δ* and *sod1Δsod2Δ*. Data are reported as the mean ± SD of three independent experiments. [a]P < 0.05 compared to *SOD+*-untreated cells; [b]P < 0.05 compared to *sod1Δ*-untreated cells; [c]P < 0.05 compared to *sod2Δ*-untreated cells; [d]P < 0.05 compared to *sod1Δsod2Δ*-untreated cells (Student *t*-test).

Results

Wild-type and *sod* null mutant cultures in the stationary phase of growth under microaerophilic conditions were treated with increasing levels of H_2O_2 for 1 h at 30ºC under non-growth conditions. The results (Figure 1) showed that the *sod2Δ* mutant was more sensitive to H_2O_2 than its isogenic parent strain, while the sensitivity of *sod1Δ* was similar to that of the wild type. Surprisingly, the sensitivity of the *sod1Δsod2Δ* double mutant was indistinguishable from that of the wild type.

In order to determine the possible mechanisms that may contribute to H_2O_2 resistance in the *sod1Δsod2Δ* and *sod1Δ* mutants, antioxidant enzyme activities were assayed (Figures 2-4). Decreased CAT activity was observed either with or without H_2O_2 treatment in simple and double *sod* mutants (Figures 2 and 3). On the other hand, GPx activities were significantly increased in *sod2Δ* and *sod1Δsod2Δ* mutants without treatment, while *sod1Δ* showed lower GPx activity than the wild type (Figure 2). After H_2O_2 treatment, GPx levels were significantly increased when exposed to 0.25-0.5 mM H_2O_2 and strongly reduced at 2.5 mM H_2O_2 in the wild-type and mutant strains (Figure 4). At the highest doses of H_2O_2 (5-10 mM), GPx levels were the same as in untreated cells for *sod2Δ*, while wild-type and *sod1Δsod2Δ* levels increased over control cell levels and *sod1Δ* maintained lower GPx activities.

The total GSH levels in these mutants were increased at 0.5 mM H_2O_2 and were reduced 6- to 7-fold at 1 mM H_2O_2 in the wild type, while the strain lacking both SODs showed a reduction in GSH levels both with and without treatment (Figure 5). The single *sod1Δ* mutant showed increased GSH at 0.5 to 5 mM H_2O_2. Little increase in the GSH level was caused by H_2O_2 treatment in the single *sod2Δ* mutant.

The lipid peroxidation index was determined on the basis of MDA levels (Figure 6). The highest MDA levels were observed without H_2O_2 treatment in simple *sod* mutants. On the other hand, the MDA level was indistinguishable from that of the wild type in the *sod1Δsod2Δ* double mutant.

Discussion

Metabolically active cells consume oxygen intensively; under limited aeration, this leads to low oxygen concentration within the cells. In stationary cultures, metabolism is considerably reduced and the partial pressure of oxygen can increase as a result of decreased metabolism. Under these conditions of high oxidative stress, one-electron reactions of autoxidation-prone cellular components may produce superoxide and other ROS. Mitochondrial respiration has been suggested to be the major source of ROS under these conditions, even or especially under conditions of low aeration.

Oxidative stress may be a factor limiting the survival of microorganisms in long-term stationary culture. The increases in antioxidant content and in the levels of antioxidant enzymes, including SOD, in yeast entering the stationary phase may, therefore, constitute an adaptive response to the enhanced oxidative damage (16). The resistance to H_2O_2 in *sod1Δsod2Δ* cells is dependent on the induction of GPx rather than CAT, the latter apparently being unnecessary. In the absence only of MnSOD (*sod2Δ* mutant), GPx was induced, but this did not prevent

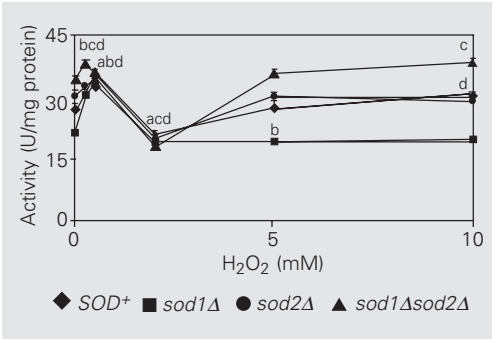

Figure 4. Activity of glutathione peroxidase in wild-type and *sod* mutant cells exposed to H_2O_2. The strains used were: *SOD+*, *sod1Δ*, *sod2Δ* and *sod1Δsod2Δ*. Data are reported as the mean ± SD of three independent experiments. [a]P < 0.05 compared to *SOD+*-untreated cells; [b]P < 0.05 compared to *sod1Δ*-untreated cells; [c]P < 0.05 compared to *sod2Δ*-untreated cells; [d]P < 0.05 compared to *sod1Δsod2Δ*-untreated cells (Student *t*-test).

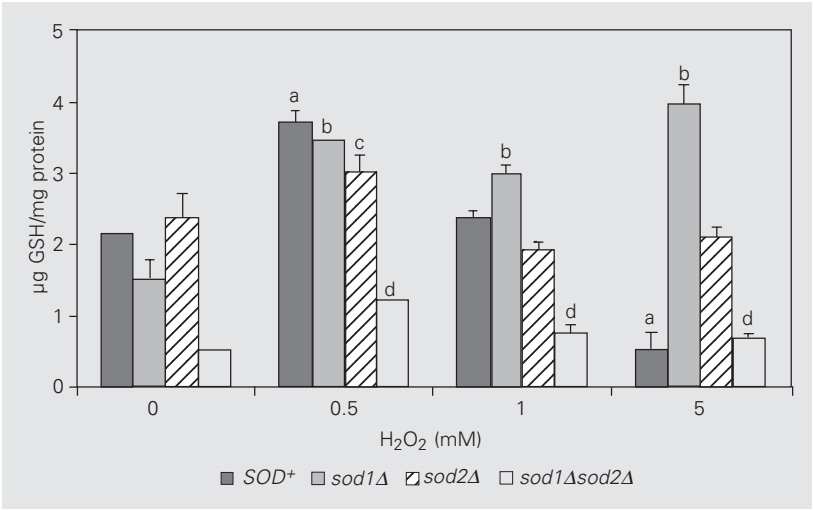

Figure 5. Total glutathione (GSH) content of wild-type and *sod* mutant cells exposed to H_2O_2. The total GSH was monitored by a microbiological method that uses a GSH auxotrophic yeast strain as a sensor for the presence of GSH, GSSG and γ-Glu-Cys. Data are reported as the mean ± SD of two independent experiments. [a]P < 0.05 compared to *SOD+*-untreated cells; [b]P < 0.05 compared to *sod1Δ*-untreated cells; [c]P < 0.05 compared to *sod2Δ*-untreated cells; [d]P < 0.05 compared to *sod1Δsod2Δ*-untreated cells (Student *t*-test).

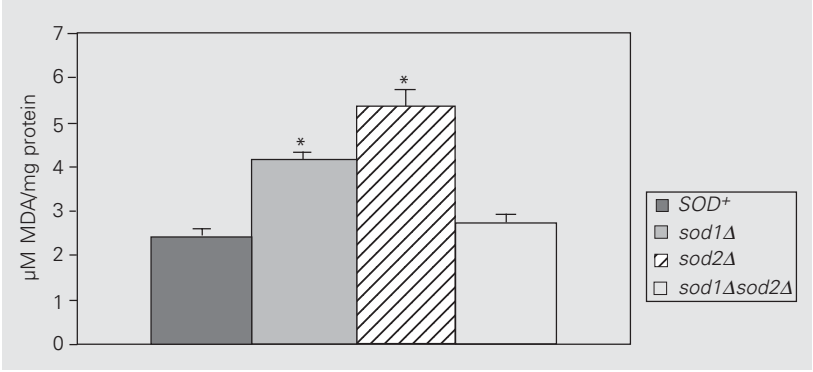

Figure 6. Malondialdehyde (MDA) determination in untreated wild-type and *sod* mutant cells. Data are reported as the mean ± SD of four independent experiments. *P < 0.05 compared to *SOD+* (Student *t*-test).

cell mortality (70% at 10 mM H_2O_2; Figure 1). However, the cells lacking CuZnSOD (*sod1Δ* mutant) were resistant to H_2O_2 (Figure 1). These results cannot be explained by GPx or CAT induction, because both GPx and CAT activities were lower in the *sod1Δ* mutant (Figures 2-4).

We also investigated the levels of total GSH in these mutants and in the wild type (Figure 5). The *sod1Δ* mutant showed increased GSH after H_2O_2 treatment. These results indicate that GSH is a primary antioxidant in the defense against H_2O_2 in *sod1Δ* cells. Mixed disulfides of GSSG with proteins accumulate in tissues subjected to oxidative stress, in both the mitochondria and the cytosol (17). GSSG can inhibit protein synthesis in animal and plant cells (18,19). These actions of GSSG may explain why cells keep intracellular GSSG levels very low under normal conditions, and why cells export GSSG when they are under oxidative stress (20-23). The *sod1Δsod2Δ* double mutant showed reduced total GSH levels with or without H_2O_2 treatment (Figure 5), while a slight increase in GSH levels was observed in the *sod2Δ* mutant after H_2O_2 treatment. We therefore suggest that GSSG and/or mixed disulfides are exported by these cells. It is known that GSSG levels are similar in exponential phase cells and their media during growth conditions and that the extracellular GSH level is elevated following treatment with H_2O_2 concentrations greater than 0.5 mM (24). We obtained similar results in the stationary phase (Figure 5).

The highest levels of antioxidant defenses, including GPx or total GSH, were observed in *sod* mutants. CAT levels, however, were lower and the enzyme was not induced after H_2O_2 treatment. SOD enzymes catalyze the disproportionate cleavage of superoxide, producing oxygen and H_2O_2; so *sod* mutants should contain less H_2O_2. H_2O_2 is enzymatically detoxified within the cells by CAT and GPx; however, GPx also detoxifies other peroxides. The acatalasemic mutant cells in the stationary phase were much more sensitive to H_2O_2 stress than wild-type cells. In addition, the ability of acatalasemic cells to show adaptation to H_2O_2 treatment has been shown to be distinctly inferior to that of the wild type (25). These results suggest that CAT is not essential for yeast cells under normal conditions, but plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response. The *sod* mutants did not induce CAT in spite of stress in their intracellular environment (Figure 3). If superoxide is not adequately removed it could inhibit CAT (26). However, *sod* mutants induced GPx (Figure 4).

These results suggest that *sod* mutants produce other type(s) of intracellular peroxides. It has been shown recently that the GPx genes of *S. cerevisiae* encode phospholipid hydroperoxide GPx and that these enzymes protect yeast against phospholipid hydroperoxides as well as nonphospholipid peroxides during oxidative stress (27,28). Therefore, we can suggest that single *sod* mutants have increased lipid peroxidation as well as protein damage (7,8). Our results showed that lack of CuZnSOD or MnSOD causes a significant increase, 71 and 124%, respectively, in the levels of MDA, an indicator of lipid peroxidation (Figure 6). However, cells lacking both CuZnSOD and MnSOD enzymes did not show elevated MDA levels. These findings are in agreement with the GPx induction and resistance to H_2O_2 observed in *sod1Δsod2Δ*.

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