Mitochondrial Protein Oxidation in Yeast Mutants Lacking Manganese- (MnSOD) or Copper- and Zinc-containing Superoxide Dismutase (CuZnSOD)

EVIDENCE THAT MnSOD AND CuZnSOD HAVE BOTH UNIQUE AND OVERLAPPING FUNCTIONS IN PROTECTING MITOCHONDRIAL PROTEINS FROM OXIDATIVE DAMAGE*

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Kristin M. O’Brien‡, Reinhard Dirmeier, Marcella Engle, and Robert O. Poyton§

From the Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, Colorado 80309-0347

Saccharomyces cerevisiae expresses two forms of superoxide dismutase (SOD): MnSOD, encoded by SOD2, which is located within the mitochondrial matrix, and CuZnSOD, encoded by SOD1, which is located in both the cytosol and the mitochondrial intermembrane space. Because two different SOD enzymes are located in the mitochondrion, we examined the relative roles of each in protecting mitochondria against oxidative stress. Using protein carbonylation as a measure of oxidative stress, we have found no correlation between overall levels of respiration and the level of oxidative mitochondrial protein damage in either wild type or sod mutant strains. Moreover, mitochondrial protein carbonylation levels in sod1, sod2, and sod1sod2 mutants are not elevated in cells harvested from mid-logarithmic and early stationary phases, suggesting that neither MnSOD nor CuZnSOD is required for protecting the majority of mitochondrial proteins from oxidative damage during these early phases of growth. During late stationary phase, mitochondrial protein carbonylation increases in all strains, particularly in sod1 and sod1sod2 mutants. By using matrix-assisted laser desorption ionization time-of-flight mass spectrometry, we have found that specific proteins become carbonylated in sod1 and sod2 mutants. We identified six mitochondrial protein spots representing five unique proteins that become carbonylated in a sod1 mutant and 19 mitochondrial protein spots representing 11 unique proteins that become carbonylated in a sod2 mutant. Although some of the same proteins are carbonylated in both mutants, other proteins are not. These findings indicate that MnSOD and CuZnSOD have both unique and overlapping functions in the mitochondrion.

Some of the oxygen that is consumed by the mitochondrial respiratory chain is not completely reduced to H₂O (1, 2) but instead is partially reduced to produce superoxide, which can be dismutated, by superoxide dismutase (3), to oxygen and hydroperoxide. In the presence of certain transition metals (e.g. iron or copper), hydrogen peroxide can be converted to the hydroxyl radical. These one-, two-, and three-electron-reduced forms of oxygen, respectively, are the reactive oxygen species (ROS) that are primarily responsible for cellular oxidative stress (4). Although complex III of the mitochondrial respiratory chain is thought to produce the majority of superoxide, particularly under conditions that increase the reduction state of the electron transport chain, electron leakage at NADH dehydrogenase (complex I) may also be responsible for some superoxide production (5). In addition, cytochrome c oxidase, which generates one-, two- and three-electron-reduced forms of oxygen as part of its catalytic redox chemistry, may be responsible for the generation of ROS, under some conditions (6).

Superoxide dismutase (SOD) constitutes a primary cellular defense against oxidative stress in most organisms. There are two forms of SOD in eukaryotic cells. A manganese-containing enzyme, MnSOD, is located in the mitochondrial matrix. A copper- and zinc-containing enzyme, CuZnSOD, is more widely distributed; it has been localized in different species to the cytosol, peroxisomes, lysosomes, the nucleus, and the mitochondrial intermembrane space (7–13). Several diseases are associated with defects in CuZnSOD (14, 15). The best understood of these is amyotrophic lateral sclerosis, which is characterized by the degeneration of motor neurons and death as a result of respiratory failure. The damaging effects of CuZnSOD on motor neurons may be caused either by the production of peroxide by a toxic gain of function mutant enzyme (16, 17) or by the ability of CuZnSOD to catalyze the nitration of tyrosines on neurofilament proteins (18). Patients with amyotrophic lateral sclerosis have abnormally shaped mitochondria and a reduced rate of respiration because of damage of electron transport chain proteins (19–21). To better understand the relationship(s) between amyotrophic lateral sclerosis and superoxide dismutase, it would be helpful to know exactly which mitochondrial proteins are targets of oxidative damage in patients with defects in MnSOD or CuZnSOD.

In Saccharomyces cerevisiae, CuZnSOD and MnSOD are encoded by the nuclear genes SOD1 and SOD2, respectively. As in other eukaryotes, yeast MnSOD is located in the mitochondrial matrix. Yeast CuZnSOD was initially thought to reside

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‡ Present address: Inst. of Arctic Biology, University of Alaska, Fairbanks, AK 99775-7000.

§ To whom correspondence should be addressed. Tel.: 303-493-3823; Fax: 303-492-3883; E-mail: Poyton@spot.colorado.edu.

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1 The abbreviations used are: ROS, reactive oxygen species; SOD, superoxide dismutase; MnSOD, manganese-containing SOD; CuZnSOD, copper- and zinc-containing SOD; SC, synthetic complete; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; HPLC, high pressure liquid chromatography; DNP, 2,4-dinitrophenol; DNPH, 2,4-dinitrophenyl hydrazine.
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soley in the cytosol (10) but more recently has also been found to reside in the mitochondrial intermembrane space as well (11). CuZnSOD accounts for 90–95% of the total superoxide dismutase activity in S. cerevisiae. As a result, yeast strains carrying deletion mutations in SOD1 are more highly compromised than yeast strains carrying deletion mutations in SOD2. For example, sod1 mutants are more sensitive to redox-cycling drugs than sod2 mutants, have auxotrophies for lysine and methionine or cysteine, grow poorly in air and not at all under hyperoxic conditions, and have higher mutation rates compared with wild type cells (10). The viability of a sod1 or sod2 mutant is enhanced by reducing oxygen tension or by abolishing respiration in these strains (22, 23), suggesting that CuZnSOD and/or MnSOD function to protect yeast cells from ROS generated by mitochondrial respiration. This role for these enzymes is also suggested by the finding that superoxide dismutase activity is necessary for the survival of S. cerevisiae during the stationary phase, because cells switch from fermentation-based growth to growth fueled by respiration (22, 24).

Although previous studies have examined the role of yeast MnSOD or CuZnSOD in protecting stationary phase yeast cells from oxidative stress, little is known about the roles of these two enzymes in protecting cells from oxidative damage during earlier times in their growth cycle (e.g. mid-logarithmic and early stationary phase), and it is not known whether CuZnSOD and MnSOD have different or overlapping roles in protecting specific cellular components from oxidative stress. Also unclear are the relationships between the level of respiration and oxidative stress in cells in any phase of the growth cycle.

In view of the importance of MnSOD and CuZnSOD in cellular defense against oxidative stress, their location within mitochondria, and the involvement of a CuZnSOD defect in amyotrophic lateral sclerosis, we sought to characterize the relative roles of both MnSOD and CuZnSOD in protecting mitochondria from protein oxidative damage. First, we assessed whether each superoxide dismutase is critical for protecting mitochondrial proteins from oxidative damage during mid-logarithmic, early, and late stationary growth and examined the relationship(s) between cellular respiration and oxidative stress during these phases of the growth cycle. Second, we identified specific mitochondrial proteins that are protected from oxidative damage during these phases of the growth cycle. For example, the relative roles of both MnSOD and CuZnSOD in protecting mitochondria from protein oxidative damage. First, we assessed whether each superoxide dismutase is critical for protecting mitochondrial proteins from oxidative damage during mid-logarithmic, early, and late stationary growth and examined the relationships between cellular respiration and oxidative stress during these phases of the growth cycle. Second, we identified specific mitochondrial proteins that are protected by each superoxide dismutase.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—The yeast strains used were: EG103 (MATα, leu2-3, his3Δ1, trpl-289, ura3-52), EG110 (EG103 with sod2Δ::TRP1), EG118 (EG103 with sod1::URA3), and EG133 (EG103 with sod1::URA3, sod2Δ::TRP1) (22). The cells were grown to the mid-logarithmic phase. Oxygen consumption rates were measured using a Strathkelvin Oxygen Meter equipped with a Clark-type oxygen electrode fitted with a polypropylene capsule. The cells were harvested by centrifugation (10,000 g, 10 min) to pellet the mitochondria. The resulting supernatant, designated as the cytosolic fraction, was added to the pellet. The supernatant containing the mitochondria was then pelleted by centrifugation (23,000 g, for 10 min), resuspended in 10 mM NaPO4, pH 7.0, to a final concentration of 5–10 μg/μl, and frozen at −80 °C.

Measurements of Respiration Rates—Oxygen consumption rates in whole cells were measured using a Strathkelvin Oxygen Meter equipped with a Clark-type oxygen electrode fitted with a polypropylene capsule. The cells were harvested by centrifugation (10,000 g, 10 min) to pellet the mitochondria. The resulting supernatant, designated as the cytosolic fraction, was added to the pellet. The supernatant containing the mitochondria was then pelleted by centrifugation (23,000 g, for 10 min), resuspended in 10 mM NaPO4, pH 7.0, to a final concentration of 5–10 μg/μl, and frozen at −80 °C.

Quantitation of Carbonyl Content in Mitochondrial and Cytosolic Fractions—The protein carbonyl content was measured as described (25). Briefly, the nucleic acids were removed from the cytosolic fraction with the cytosolic fraction was added to the pellet. The supernatant containing the mitochondria was then pelleted by centrifugation (23,000 g, for 10 min), resuspended in 10 mM NaPO4, pH 7.0, to a final concentration of 5–10 μg/μl, and frozen at −80 °C.

Western blotting with a primary anti-DNP antibody, as described below. A second set of replicate gels was silver-stained for protein quantitation and identification with MALDI-TOF and MALDI-TOF mass spectrometry/mass spectrometry. Derivatization with DNP is done after gel electrophoresis as described (27), except that 100 μg of mitochondrial protein was loaded on each 13 cm Immobiline Drystrip (Amersham Biosciences). Briefly, mitochondrial proteins were first separated using isoelectric focusing and then dried down and rehydrated in an 8–18% SDS-PAGE gradient gel. One set of gels was subjected to Western blotting with a primary anti-DNP antibody, as described below. A second set of replicate gels was silver-stained for protein quantitation and identification with MALDI-TOF and MALDI-TOF mass spectrometry/mass spectrometry. Derivatization with DNP is done after isoelectric focusing because it prevents an alteration in spot migration that may result from changes in protein charge if derivatization...
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were done prior to isoelectric focusing (29).

Western Immunoblotting—The proteins were transferred to nitrocellulose membranes (Amersham Biosciences) using a Semi-Pho electroblotting apparatus ( Hoefer). Protein carbonyl groups were derivatized with DNPH as described earlier (26, 27). The resulting hydrazone was then detected with anti-DNP antibodies (Dako) at a dilution of 1:4000 and a secondary horseradish peroxidase-linked antibody (PerkinElmer Life Sciences) at a dilution of 1:25,000. Antibody binding was visualized with a chemiluminescent detection kit (PerkinElmer Life Sciences). The Western blots of gels from different strains were exposed to film for the exact same length of time. SOD proteins were detected with antibodies directed against Sod1p and Sod2p, which were kindly provided by Dr. Valeria Culotta.

Protein Identification by MALDI-TOF Mass Spectrometry—Protein spots from two-dimensional gels were prepared for MALDI-TOF as described (27) with the following changes. For low abundance proteins, as many as 16 spots were pooled together from individual gels for analysis. In addition, the proteins were digested overnight at 37 °C with sequencing grade, modified porcine trypsin (Promega) diluted in 25 mM ammonium bicarbonate to a final concentration of 0.015 mg/ml. Slices of gels from the background were excised and prepared for MALDI-TOF at the same time as protein spots. Any contaminating peaks detected in the background were deleted from the protein database searches.

Protein Sequence—Amino acid sequences were determined using an Applied Biosystems Pulsar MALDI-Q-TOF. The proteins were prepared as described above for MALDI-TOF, except that proteins were co-crystallized with 2,5-dihydroxybenzoic acid matrix (Agilent Technologies) on a stainless steel sample plate. The mass spectrometer was first calibrated with a standard of Glu-fibrinogen peptide, and then a spectra of peptide fragments was obtained for each protein. Peptides were selected for sequencing, and the resulting spectra were compared with the NCBI nonredundant data base using Mascot (Matrix Science). A protein was considered positively identified if it was identified from at least two separate protein preparations using MALDI-TOF or from at least one preparation with MALDI-TOF and sequencing of at least one peptide.

Data Analysis and Quantification of Protein Carbonylation on Two-dimensional Gels—Two-dimensional gels and Western blots were scanned, and the resulting images were analyzed using the software Melanie 3.07 (Geneva Bioinformatics). A minimum of two replicate gels from three independent cultures (six total) were analyzed for each strain. Changes in protein level for each protein of interest were determined by quantifying spot intensity after silver staining, and changes in carbonylation level were quantitated after staining with anti-DNP antiserum. Spot intensity was measured as the volume of the spot, which is a measurement of the pixel intensities integrated over the area of the spot. The percentage change in protein carbonylation was then determined in the mutant strains, relative to the wild type. The change in carbonylation of specific proteins in the mutant strains was normalized to the amount of that protein in that strain. An increase in carbonylation was considered significant if it showed a greater than 2-fold increase relative to the wild type, and this level of increase occurred in at least two of the replicate cultures (four gels total).

Miscellaneous—Protein concentration was measured as described (30).

RESULTS

Characterization of Strains—Previous studies have shown that Sod1p is located in both the mitochondria and cytosol (11). We confirmed that this was also true of our strains by examining the levels of Sod1p and Sod2p in mitochondrial and cytosolic cell fractions (Fig. 1). As expected, Sod1p is not present in either the sod1 mutant (lanes 5 and 6) or in the sod1sod2 mutant (lanes 7 and 8), and Sod2p is absent from the sod2 mutant (lanes 3 and 4), but it showed a greater than 2-fold increase relative to the respective wild type in each of the sod1 mutants (lanes 1 and 2) in wild type and sod1 mutant cells, Sod2p is found only in the mitochondrion (lanes 1 and 5), and in wild type and sod2 mutant cells, Sod1p is found in both mitochondrial and cytosolic fractions (lanes 1–4).

Previous studies with sod mutants have used cells grown on either a defined SC growth medium (22, 31) or on the nutrient-rich YPD medium (11). Both media contain 2% glucose, which is used as the carbon source early in growth. As the glucose is consumed it is converted to ethanol, which does not repress the expression of respiratory proteins and which, in turn, can be respired. So, early in the growth cycle of a yeast culture grown on glucose, cells obtain their energy primarily by fermenting glucose, whereas late in the growth cycle they obtain their metabolic energy by respiring ethanol. The growth of cells on two different carbon sources at different times in their growth cycle has been referred to as “diauxic growth.” As observed previously, sod1 and sod1sod2 mutants grow more slowly and reach a lower cell density on SC medium than sod2 or wild type cells (Fig. 2A). The difference in growth is accentuated after 10 h of growth when cells switch from using glucose to using ethanol carbon source as their main carbon source. Growth differences between strains are less obvious on YPD medium (Fig. 2B), although, as in SC medium, there is a slowing of growth rates for the sod1 and sod1sod2 mutants, compared with wild type and sod2 mutant cells, when cells switch from using glucose to using ethanol carbon source as their main carbon source after 8 h of growth. For the experiments presented here we have chosen to grow cells on YPD medium to compare our measurements of protein oxidation with the results of a recent study that examined the level of protein carbonylation in sod1 mutants that were also grown on YPD (11).

Role of MnSOD and CuZnSOD in Protecting Proteins from Oxidative Stress in Different Phases of the Growth Cycle of a Yeast Culture—Early studies with sod mutant cells grown on glucose-containing medium have provided evidence that superoxide dismutase activity is required to protect yeast cells from oxidative stress at different times during the growth cycle. For this study sod1, sod2, and sod1sod2 mutant cells were grown on YPD and harvested in the mid-logarithmic phase after 5 h of growth and prior to the diauxic transition, in the early stationary phase after 24 h of growth, and in the late stationary phase after 5 days of growth.

To assess the degree of oxidative stress experienced by cells in these different phases of the culture growth cycle, we determined levels of mitochondrial and cytosolic protein carbonylation. Carbonyl groups (i.e. aldehyde or ketone groups) result from the oxidation of some amino acids (32) and serve as useful markers for metal-catalyzed protein oxidation that occurs under conditions of oxidative stress. Protein carboxyls are quantitated after derivatization with 2,4-dinitrophenyl hydrazine. The 2,4-dinitrophenyl hydrazine is converted to 2,4-dinitrophenyl hydrazone by interaction with carbonyl groups, and the DNP-protein conjugates are subjected to analysis by HPLC (33,
Previously, we have shown that most of the protein carbonylation of both cytosolic and mitochondrial proteins results from ROS released by mitochondrial respiration (27). From Fig. 3A, it is clear that the general levels of mitochondrial protein carbonylation for all four strains are nearly equivalent in mid-logarithmic and early stationary phases and that they increase markedly in late stationary phase cells. Interestingly, the level of mitochondrial protein carbonylation in the sod mutants is either less than, or equivalent to, the wild type strain in mid-logarithmic and early stationary phase cultures, showing that overall, mitochondrial protein oxidation does not increase in sod mutants during these phases of growth. However, the level of protein carbonylation is higher in sod1 and sod1sod2 mutants than in wild type or sod2 mutant cells during the late stationary phase. The higher level of mitochondrial protein carbonylation in sod1 and sod1sod2 mutants compared with wild type and sod2 mutants in the late stationary phase suggests that CuZnSOD is more important than MnSOD for protecting mitochondria from oxidative damage during this growth phase.

Cytosolic protein carbonylation is greater in sod1 and sod1sod2 mutants compared with the wild type and sod2 mutants during early and late stationary phases of growth and is higher in sod1 mutants compared with all strains during mid-logarithmic growth (Fig. 3B). Thus, Sod1p appears to play a critical role in protecting cytosolic proteins from oxidative damage during all phases of growth. This is not surprising given that Sod1p comprises the majority of SOD in the cell and because 95–99% of Sod1p is found in the cytosol (11).

Respiration Rates Do Not Correlate with Levels of Protein Oxidation—The differences in protein carbonylation seen in the different strains used for the experiment shown in Fig. 3 may result from differences in the levels of mitochondrial respiration in these strains. To examine this possibility we measured the rates of mitochondrial respiration (cyanide-sensitive oxygen consumption) in these strains. From Fig. 4 it is clear that respiration rates increase dramatically in all strains between the mid-logarithmic and early stationary phases and then decline dramatically between the early and late stationary phases. The increase in respiration rates between the mid-logarithmic and early stationary phase cultures is a result of the depletion of glucose, and the subsequent derepression of respiratory chain proteins. It is not clear why respiration rates decline between early and late stationary phase. It is likely, however, that it is related either to the special physiology of stationary phase cultures (35) or is the result of oxidative damage to mitochondrial proteins. Respiration rates for all strains are similar in mid-logarithmic cultures. Each of the sod mutants have lower rates of respiration than wild type cells in early stationary phase cultures, and the sod1 and sod1sod2 mutants have significantly lower respiration rates than the wild type or sod2 mutant in late stationary phase cultures. By comparing these results with those shown in Fig. 3, two interesting conclusions emerge. First, despite the large increase in respiration in all strains between the mid-logarithmic and early stationary phases, there is no corresponding increase in

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**Fig. 2. Growth curves for S. cerevisiae strains.** The cells were grown at 28 °C and 200 rpm in SC medium with 2% dextrose (A) or YPD (B). The plots are representative of at least two cultures grown per strain. Culture density was monitored with a Klett Summerson colorimeter equipped with a No. 54 green filter.
Mitochondrial Protein Carbonylation in sod Mutants—To determine whether the two forms of SOD protect similar or different proteins within mitochondria from oxidative damage, we subjected mitochondrial proteins to two-dimensional electrophoresis and identified carbonylated proteins with anti-DNP antibodies followed by MALDI-TOF analysis. Initially, we attempted to use mitochondrial proteins from cells grown to the late stationary phase. However, they were greatly reduced in the mutant cells and had lower levels of protein spots 18, 19, 24, 26, and 27. Indeed, all strains from late stationary phase lack spots 21–23 and have lower levels of protein spots 18, 19, 24, 26, and 27. Together, these findings provide clear support for protein degradation in late stationary phase cells from all strains and make it difficult, if not impossible, to interpret changes in the protein carbonylation of specific proteins in late stationary phase cells.

An alternative way of asking about the relative roles of CuZnSOD and MnSOD in protecting mitochondrial proteins from oxidative stress is to use mid-logarithmic phase cells and induce oxidative stress by treating them with the electron donor paraquat (36). Treatment with 1 mM paraquat for 2 h did not increase protein oxidation in wild type cells (Fig. 6), nor did it slow the growth rate of these cells (Table I). During a 2-h period, wild type cell density (i.e. cells/ml) increased by about 76% in the presence of paraquat. In contrast, 1 mM paraquat increased protein carbonylation in the sod1sod2 mutant (Fig. 6) and slowed its growth during the 2-h treatment period. sod1sod2 mutant cells incubated without paraquat had a cell density increase of about 85%, but sod1sod2 mutant cells incubated with paraquat increased by only 47%. This effect of paraquat on slowing the growth of sod1sod2 mutants has been observed previously (36). Paraquat also inhibited the growth of sod2 and sod1 mutants (Table I). This indicates that although wild type cells have the necessary antioxidant defenses to protect themselves from the oxidizing effects of 1 mM paraquat, the mutant cells are deficient in them.

To ask whether the lack of CuZnSOD or MnSOD affects the carbonylation of specific mitochondrial proteins, we compared the pattern of mitochondrial protein carbonylation in sod1 and sod2 mutants with the pattern of mitochondrial protein carbonylation from wild type cells. This was approached by first identifying protein spots whose carbonylation level increased in each mutant relative to the wild type and then normalizing the level of carbonylation to the level of the protein as determined by quantitating the spot intensity after silver staining.
proteins in each mutant treated with paraquat was significant, we set the threshold for protein carbonylation (normalized to protein level) at a 2-fold change, relative to the levels seen in wild type cells treated with paraquat.

Silver-stained two-dimensional gels of mitochondrial proteins from wild type cells treated with paraquat reveal about 400 protein spots (Fig. 7B), which represents over half of known yeast mitochondrial proteins (37, 38). The pattern observed is similar to that reported recently for JM43, another wild type yeast strain (27). Overall, the pattern of spots seen for

![Image of SDS-PAGE and two-dimensional electrophoresis](http://www.jbc.org/content/early/2018/07/18/jbc.M118.789931/Fig5.large.jpg)
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FIG. 6. Western blot showing the level of protein carbonylation in wild type and sod mutants treated with 1 mM paraquat. The cells were grown to early mid-logarithmic phase in YPD medium at 28 °C and 200 rpm. Paraquat was then added to a final concentration of 1 mM, and the cultures were grown for an additional 2 h. The cells were then harvested, and whole cell extracts prepared for gel electrophoresis. 5 μg of protein was separated on a 10% SDS-polyacrylamide gel and then electroblotted onto nitrocellulose. Carbonyl groups were detected by Western blotting with a primary antibody directed against the DNPH-derivatized carbonyl groups. Lanes 1 and 2 represent carbonylated proteins from wild type cells, untreated (lane 1) and treated (lane 2) with 1 mM paraquat. Lanes 3 and 4 represent carbonylated proteins from sod1sod2 mutants, untreated (lane 3) and treated (lane 4) with 1 mM paraquat. The arrows indicate protein bands that are more highly carbonylated in the sod1sod2 mutants treated with paraquat compared with the untreated cells.

The values given are the averages of two independent cultures as the percentage of increase in cell density after 2 h.

|                      | Control | 1 mM paraquat |
|----------------------|---------|---------------|
| Wild type            | 76.8    | 75.4          |
| sod2−                | 89.2    | 69.5          |
| sod1−                | 85.1    | 36.5          |
| sod1− sod2−          | 82.9    | 46.79         |

The effects of the sod1 and sod2 mutants is similar to that observed for the wild type. However, the carbonylation levels of some proteins clearly differ among wild type, sod1 and sod2 mutant cells. Using the criteria established above, we find that 19 protein spots, representing 11 unique proteins, are at least twice as carbonylated in the sod2 mutant as in the wild type (Fig. 7). These proteins were identified by MALDI-TOF analysis and in some cases from sequencing peptide fragments using MALDI-TOF mass spectrometry/mass spectrometry (Table II). Most of these proteins are located within the mitochondrial matrix, where MnSOD is located (Table II). The exceptions are glyceraldehyde-3-phosphate dehydrogenase (Tdhp), which is found in the cytosol, and porin1 (Por1p), which is located in the outer mitochondrial membrane. The presence of glyceraldehyde-3-phosphate within the mitochondrial fraction may represent contamination of this fraction with cytosolic proteins, or alternatively, glyceraldehyde-3-phosphate dehydrogenase may be an enzyme like fumarase that is distributed between both the mitochondria and cytosol (39). It was not possible to determine which glyceraldehyde-3-phosphate isofrom, Tdhp2 or Tdh3p, is carbonylated because these two proteins in yeast overlap slightly on two-dimensional gels (40). In addition, both proteins become carbonylated in S. cerevisiae when cells are treated with hydrogen peroxide (41). Spot 12 was identified as a mixture of both the pyruvate dehydrogenase E1 component and the core 1 protein of the ubiquinol-cytochrome c reductase complex (complex III). This mixture, initially identified with MALDI-TOF, was confirmed by sequencing four peptides. Two peptides were identified as fragments from pyruvate dehydrogenase, and the other two were identified as fragments from the core 1 protein. It was not possible to determine whether one or both of these proteins are carbonylated. The level of expression for nearly all carbonylated proteins is similar in the sod2 mutant and wild type cells, except for Ilv5p (spot 9), Lpd1p (spot 18), and Ilv2p (spot 24), which are lower in the mutant compared with the wild type.

Only six protein spots, representing five unique proteins, are more than twice as carbonylated in the sod1 mutant compared with the wild type cells. Some of these proteins are also carbonylated in the sod2 mutant, suggesting that there is some overlap in function between sod1 and sod2. Interestingly, none of the carbonylated proteins from the sod1 mutant are found within the mitochondrial intermembrane space where CuZn-SOD is localized. Moreover, two of the carbonylated proteins, Tdh2/3p and Eno2p, are cytosolic proteins. The presence of these two proteins in the mitochondrial fraction may be due to partial contamination of the mitochondrial fraction with cytosol because Tdh3p and Eno2p are two of the most abundant proteins in the yeast proteome and thus difficult to eliminate from the mitochondrial fraction (40). Alternatively, like fumarase, they may be capable of localizing to both the mitochondrial and cytosolic compartments (39). Spot 6 was identified as keto-acid reductoisomerase in the sod2 mutant but Tdh2/3p in the sod1 mutant. This spot may represent a mixture of the two proteins that is enriched for one or the other, depending on the genetic background. Most protein spots have the same level of expression between the mutant and wild type. Only spot 30 (Tdh2p) decreases in expression in the sod1 mutant compared with the wild type. There are several other proteins that decrease in level (Ilv5p, Aco1p, and Ilv2p) compared with the wild type, but they do not show an increase in carbonylation.

**TABLE I**

Growth of wild type and SOD mutants in the presence and absence of 1 mM paraquat

|          | Control | 1 mM paraquat |
|----------|---------|---------------|
| Wild type| 76.8    | 75.4          |
| sod2−    | 89.2    | 69.5          |
| sod1−    | 85.1    | 36.5          |
| sod1− sod2− | 82.9 | 46.79         |

**DISCUSSION**

The results presented here provide interesting new insight concerning the relative roles of CuZnSOD and MnSOD in protecting mitochondria from oxidative stress. By quantitating the overall level of protein carbonylation in sod1, sod2, and sod1sod2 mutants with an HPLC assay, we have found that the absence of CuZnSOD or MnSOD has little, if any, effect on the overall level of mitochondrial protein carbonylation during mid-logarithmic or early stationary phase growth and that mitochondrial protein carbonylation increases only in late stationary phase cultures. We have also found that the absence of CuZnSOD has a larger effect on both mitochondrial and cytosolic protein carbonylation than the absence of MnSOD and that overall levels of oxidative protein damage are not correlated with the levels of mitochondrial respiration. In addition, MALDI-TOF analysis of paraquat-treated cells shows that specific mitochondrial proteins become carbonylated in sod2 but not sod1 mutants and that some proteins show increased levels of carbonylation in both sod1 and sod2 mutants.

The Effects of Respiration on Protein Carbonylation in Sod Mutants—Interestingly, mitochondrial protein carbonylation in wild type or mutant cells is not correlated with the level of mitochondrial respiration during any phase of growth, so it is clear that the increase in protein carbonylation observed in late stationary phase cultures cannot be explained simply by the switch from fermentative to respiratory growth that cells experience during stationary phase (35). The finding that overall level of protein carbonylation is not correlated with mitochondrial respiration rates is consistent with the results from recent
FIG. 7. Two-dimensional gel electrophoresis of mitochondrial proteins from cells treated with the electron donor paraquat. The cells were grown to the early mid-logarithmic phase in YPD medium at 28 °C and 200 rpm. Paraquat was then added to a final concentration of 1 mM, and the cells were incubated at 28 °C and 200 rpm for 2 h. The cells were then harvested, and mitochondria were isolated as described under "Experimental Procedures." Mitochondrial proteins were separated by isoelectric focusing followed by SDS-PAGE on an 8–18% gradient gel. Total protein was...
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studies with yeast and other eukaryotes (42–44). These studies have shown that ROS levels are affected by the degree to which mitochondrial electron transport is coupled to ATP synthesis and not by the rate of mitochondrial electron transport per se. Low levels of ROS are produced during state 3 respiration and are increased during a switch from state 3 to state 4 respiration, brought about by an increase in the ATP/ADP + P_i ratio. This transition from state 3 to state 4 respiration is thought to result in an increase in mitochondrial membrane potential, which can prolong the lifespan of the ubisemiquinone within the Q cycle and attenuate the production of superoxide (45, 46). Aguilanu et al. (43) have found that either carbon or nitrogen starvation in wild type yeast cells results in a decrease in respiration rate and an increase in protein oxidation. Carbon or nitrogen starvation favors state 4 respiration, a more reduced respiratory chain, and hence higher ROS production. These findings are interesting in the context of the late stationary phase data reported here because late stationary phase cells experience both nitrogen and carbon starvation (35).

The finding that overall levels of mitochondrial protein carbonylation levels are equivalent in mid-logarithmic and early stationary phase wild type and sod1 or sod2 mutant cells indicates that neither CuZnSOD nor MnSOD are required for protecting the majority of mitochondrial proteins from oxidation during these growth phases, as previously concluded by Fortuniak et al. (47). Using a spectrophotometric assay for measuring protein carbonyls, this study found that the level of protein carbonylation was not increased in S. cerevisiae mutants lacking sod1 or both sod1 and sod2 compared with wild type cells (47). Similarly, Escherichia coli mutants lacking MnSOD and FeSOD do not show an increase in protein carbonylation during mid-logarithmic growth, relative to wild type cells (48). Carbonylation only increases in these mutants during the stationary phase. Our carbonylation results, however, do not agree with those of Sturtz et al. (11), who have found an increase in mitochondrial protein carbonylation in sod1 mutants compared with wild type cells during the mid-logarithmic phase. These differences may be attributable to differences in the strains used or to the different methods used to detect carbonyl groups in the two studies. In our study, protein carbonylation was quantified by HPLC. This method provides a direct measure of mmol of carbonyl/m protein because it normalizes the carbonyl signal to the amount of protein loaded on the column. In contrast, Sturtz et al. (11) measured protein carbonylation using one-dimensional Western blotting with an antibody directed against the derivatized carbonyl group.

Our finding that the overall level of mitochondrial protein carbonylation is unaffected during early growth phases in sod1, sod2, or sod1sod2 mutants does not preclude the possibility that CuZnSOD and MnSOD are critical for protecting specific proteins during these growth phases. Given that there are ~700 proteins associated with the mitochondrion in yeast (37, 38), an increase in carbonylation of a few proteins could easily go undetected. A case in point is aconitase and homoaconitase, which previous studies have shown are protected from oxidative stress by both CuZnSOD and MnSOD (24, 49). Moreover, it is important to note that protein carbonylation is only one form of protein oxidation and that MnSOD and CuZnSOD may play a role in protecting mitochondrial proteins from other types of oxidative or nitrosative damage during early growth phases. Aconitase, as well as other mitochondrial proteins, are inactivated by peroxynitrite which forms 3-nitrotyrosines in proteins (50, 51). Peroxynitrite (ONOO^-) is formed by the reaction of superoxide with nitric oxide, and thus levels of ONOO^- are likely higher in mutants lacking SOD compared with wild type cells.

Specific Proteins Are Carbonylated in sod1 and sod2 Mutants Exposed to Paraquat—By identifying carbonylated proteins in sod1 and sod2 mutants exposed to paraquat, it is clear that a relatively small number of proteins are affected by the absence of either MnSOD or CuZnSOD. This suggests that these superoxide dismutases protect specific proteins from paraquat-generated oxidants. Three conclusions can be reached by comparing those proteins that become carbonylated in sod1 and sod2 mutants. First, both CuZnSOD and MnSOD are required to protect some mitochondrial proteins. These include: one outer mitochondrial membrane protein (porin), one matrix protein (substrate 2 of isocitrate dehydrogenase), and one inner membrane protein (the core 1 protein of ubiquinol cytochrome c reductase). Second, by itself, CuZnSOD protects only one protein, enolase 2, found in our mitochondrial preparations. Interestingly, enolase 2 is a cytosolic protein. Insofar as CuZnSOD resides in both the cytosol and intermembrane space, it seems likely that the enhanced carbonylation of enolase 2 in the sod1 mutant results from the cytosolic and nonmitochondrial activity of CuZnSOD. Third, by itself, MnSOD protects six mitochondrial proteins (substrate 1 of keto-acid reductoisomerase, substrate 1 of pyruvate dehydrogenase, the α subunit of ATP synthase, aconitase, ace-tolactate synthase, and YHb flavohemoglobin). As expected, all of these proteins reside in the matrix, where MnSOD itself is located. When considered together, these findings indicate that MnSOD protects some mitochondrial matrix proteins from enhanced levels of carbonylation brought about by exposure to paraquat. They also indicate that CuZnSOD, by itself, does not protect any mitochondrial proteins from paraquat-induced oxidative stress. Instead, CuZnSOD works together with MnSOD to protect a small number of mitochondrial proteins. Our results agree with those recently reported by Wallace et al. (49), who determined that Sup1p is required for protecting two matrix enzymes, homoaconitase and aconitase, from inactivation. It is not clear why both CuZnSOD and MnSOD are required to protect some mitochondrial proteins. One possibility is that superoxide becomes protonated in the intermembrane space and diffuses into the matrix. The phospholipids of the mitochondrial inner membrane impart a negative charge to the membrane that attracts protons. This results in a decrease in the pHe near the membrane that in turn facilitates the protonation of superoxide and formation of the perhydroxyl radical (HO^·). Unlike superoxide, this ROS can diffuse across the inner membrane and into the matrix (52). This process is likely enhanced in cells lacking sod1, and the influx of ROS may overwhelm the capacity of Sod2p to protect matrix proteins from oxidative damage. Alternatively, these two superoxide dismutases may work at different times during the biogenesis of these proteins. For example, because of its location in the intermembrane space and cytosol, CuZnSOD may act while the protein is being targeted to the mitochondrion and imported across the inner and outer mitochondrial membranes, whereas MnSOD, which is located in the matrix, may act after the protein has been imported. A third possibility is that these two superoxide dismutases function in protecting different amino acid side chains on each protein. These possibilities are currently under study.

visualized by silver staining. Carbonylated proteins were detected by Western blotting using a primary antibody directed against DNPH-derivatized carbonyl groups. A, carbonylated proteins on Western blots. Labeled spots represent all spots that increase in each mutant at least 2-fold, relative to the wild type. B, silver-stained gels, corresponding to the Western immunoblots shown in A. Labeled spots on the silver-stained gels represent all of the spots that were analyzed for changes in protein and carbonylation level.
### Identification of carbonylated proteins by MALDI peptide mass fingerprinting

| Spot I.D. | Protein Identification | Average increase in carbonylation | Apparent mass kDa | Calculated mass kDa | PL<sub>app</sub> | PL<sub>calc</sub> | Number of peptides matched | Percent protein coverage | Number of peptides sequenced | Representative peptide sequence | Location |
|-----------|------------------------|-----------------------------------|-------------------|---------------------|-----------------|----------------|-----------------------------|--------------------------|-----------------------------|--------------------------------|----------|
| sod2 mutants |
| 1 | Outer mitochondrial membrane protein porin 1 | 2.6 | 30 | 30 | 9.3 | 6.8 | 7 | 28.3 | 1 | LEFAHLPGLK | Mitochondrial outer membrane |
| 3 | Outer mitochondrial membrane protein porin 1 | 4.1 | 30 | 30 | 7.6 | 6.8 | 8 | 32.2 | | | Mitochondrial outer membrane |
| 5 | Glyceraldehyde-3-phosphate dehydrogenase 2/3 | 3.0 | 34 | 36 | 7.3 | 6.5 | 12 | 28.1 | | | Cytosol |
| 6 | Chain 1: ketol-acid reductoisomerase | 8.7 | 34 | 39 | 7.0 | 6.3 | 9 | 30.2 | 1 | GALIDYPIFK | Mitochondrial matrix |
| 7 | Chain 1: ketol-acid reductoisomerase | 12.7 | 35 | 39 | 6.7 | 6.3 | 6 | 22.4 | 2 | QINFGTIVETYER | Mitochondrial matrix |
| 9 | Chain 1: ketol-acid reductoisomerase | 9.7 | 39 | 39 | 7.1 | 6.3 | 5 | 15.2 | 1 | SLEFNSQPDRYR | Mitochondrial matrix |
| 10 | Chain 1: ketol-acid reductoisomerase | 4.3 | 39 | 39 | 6.9 | 6.3 | 6 | 19.0 | | | Mitochondrial matrix |
| 12 | Chain 1: pyruvate dehydrogenase E1 component and ubiquinol-cytochrome reductase complex core protein 1 | 4.3 | 44 | 43/47 | 6.6 | 6.2/5.9 | 11/15 | 28.2/45.5 | 4 | PDA1: GPLvLEYETYR | PDA1: mitochondrial matrix |
| 16 | ATP synthase α chain | 11.7 | 50 | 55 | 7.0 | 6.7 | 17 | 38.0 | | | Mitochondrial matrix |
| 17 | ATP synthase α chain | 11.3 | 50 | 55 | 7.3 | 6.7 | 16 | 34.1 | | | Mitochondrial matrix |
| 18 | Chain 1: dihydrolipoamide dehydrogenase | 8.4 | 54 | 52 | 7.1 | 6.5 | 10 | 23.4 | 1 | QENLEADVLLVAVGR | Mitochondrial matrix |
| 19 | Chain 1: dihydrolipoamide dehydrogenase | 3.7 | 54 | 52 | 7.2 | 6.5 | 8 | 21.5 | 2 | RPYIAGLAER | Mitochondrial matrix |
| 21 | Acotinate | 3.7 | 83 | 82 | 7.6 | 7.1 | 21 | 32.5 | 3 | SMIEYLEATGR | Mitochondrial matrix |
| 22 | Acotinate | 4.0 | 83 | 82 | 7.8 | 7.1 | 8 | 12.0 | | | Mitochondrial matrix |
| 23 | Acotinate | 3.8 | 83 | 82 | 8.2 | 7.1 | 6 | 7.3 | | | Mitochondrial matrix |
| 24 | Acetolactate synthase | 5.9 | 67 | 65 | 7.0 | 6.6 | 9 | 22.9 | | | Mitochondrial matrix |
| 29 | Chain 1: ketol-acid reductoisomerase | 3.6 | 39 | 39 | 6.5 | 6.3 | 9 | 28.7 | 1 | SLEFNSQPDRYR | Mitochondrial matrix |
| 30 | Isocitrate dehydrogenase subunit 2 | 15.3 | 38 | 38 | 7.8 | 7.2 | 5 | 40.4 | 1 | TTYENVDLVLIR | Mitochondrial matrix |
| 31 | Flavohemoprotein | 3.6 | 45 | 45 | 6.4 | 5.8 | 10 | 36.1 | | | Mitochondrial matrix |
| sod1 mutants |
| 1 | Outer mitochondrial membrane protein porin 1 | 2.6 | 30 | 30 | 9.3 | 6.8 | 7 | 28.3 | | | Mitochondrial outer membrane |
| 6 | Glyceraldehyde-3-phosphate dehydrogenase 2/3 | 8.3 | 34 | 36 | 7.0 | 6.5 | 10 | 29.6 | | | Cytosol |
| 13 | Enolase 2 | 11.3 | 44 | 47 | 5.9 | 5.7 | 10 | 28.9 | | | Cytosol |
| 14 | Enolase 2 | 4.2 | 44 | 47 | 6.0 | 5.7 | 17 | 45.0 | | | Cytosol |
| 30 | Isocitrate dehydrogenase subunit 2 | 5.7 | 38 | 38 | 7.9 | 7.2 | 5 | 40.4 | | | Mitochondrial matrix |
| 33 | Ubiquinol cytochrome c reductase complex core protein 1 | 4.3 | 45 | 47 | 6.6 | 5.9 | 10 | 28.5 | 2 | DSGLWGFSTATR | Associated with mitochondrial inner membrane, facing the matrix |
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REFERENCES

1. Boveris, A., Oshino, N., and Chance, B. (1972) Biochem. J. 128, 617–630
2. Turrens, J. F. (1997) Biochim. Biophys. Acta. 525–325
3. McCord, J. M., and Fridovich, I. (1969) J. Biol. Chem. 244, 6049–6055
4. Costa, V., and Moradas-Ferreira, P. (2001) Mol. Aspects Med. 22, 217–246
5. Turrens, J. F., and Boveris, A. (1980) Biochem. J. 191, 421–427
6. Poyton, R. O. (1998) Nat. Genet. 20, 316–317
7. Geller, B. L., and Winge, D. R. (1982) J. Biol. Chem. 257, 8945–8952
8. Chang, L. Y., Slot, J. W., Geuze, H. J., and Crapo, J. D. (1988) Biochemistry 27, 2689–2693
9. Keller, G. A., Warner, T. G., Steimer, K. S., and Hallewell, R. A. (1991) J. Biol. Chem. 266, 3942–3947
10. Gralla, E. B. (1997) in Oxidative Stress and the Molecular Biology of Antioxidant Defenses (Scandalios, J. G., ed) pp. 495–525, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
11. Sturtz, L. A., Diekert, K., Jensen, L. T., Lill, R., and Culotta, V. C. (2001) J. Biol. Chem. 276, 38084–38089
12. Okado-Matsumoto, A., and Fridovich, I. (2001) J. Biol. Chem. 276, 38368–38373
13. Higgins, C. M., Jung, C., Ding, H., and Zaozhang, X. (2002) J. Neurosci. 22, 1–6
14. Macmillan-Crow, L. A., and Cruthirds, D. L. (2001) Proc. Natl. Acad. Sci. U. S. A. 88, 7381–7385
15. Julien, J. P. (2001)
16. Wiedau-Pazos, M., Goto, J. J., Rabizadeh, S., Gralla, E. B., Roe, J. A., Lee, M. K., Valentine, J. S., and Bredesen, D. E. (1996) J. Biol. Chem. 271, 20473–20479
17. Yim, M. B., Kang, J. H., Yim, H. S., Kwak, H. S., Chock, P. B., and Stadtman, E. R. (1996) J. Biol. Chem. 271, 34773–34784
18. Beckman, J. S. (1996)
19. Fortuniak, A., Jakubowski, W., Bilinski, T., and Bartosz, G. (1996) Biochem. Mol. Biol. Int. 38, 1271–1276
20. Wallace, M. A., Liou, L., Martins, J., Clement, M., Bailey, S., Longe, V., Valentine, J. S., and Gralla, E. B. (2004) J. Biol. Chem. 279, 32055–32062
21. Castro, L., Rodriguez, M., and Radi, R. (1994) J. Biol. Chem. 269, 29409–29415
22. Koeck, T., Po, X., Hazen, S. L., Crab, J. W., Stuehr, D. J., and Aulak, K. S. (2004) J. Biol. Chem. 279, 27257–27262
23. Hawkins, C. L., and Davies, M. (2001) Biochim. Biophys. Acta. 1504, 196–219
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Kristin M. O'Brien, Reinhard Dirmeier, Marcella Engle and Robert O. Poyton

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