Superoxide Dismutase Activity Is Essential for Stationary Phase Survival in Saccharomyces cerevisiae

MITOCHONDRIAL PRODUCTION OF TOXIC OXYGEN SPECIES IN VIVO*

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Yeast lacking copper-zinc superoxide dismutase (CuZnSOD), manganese superoxide dismutase (SOD), catalase T, or metallothionein were studied using long term stationary phase (30–45 days) as a simple model system to study the roles of antioxidant enzymes in aging. In well aerated cultures, the lack of either SOD resulted in dramatic loss of viability over the first few weeks of culture, with the CuZnSOD mutant showing the more severe defect. The double SOD mutant died within a few days. The severity reversed in low aeration; the CuZnSOD mutant remained viable longer than the manganese SOD mutant. To test whether reactive oxygen species generated during respiration play an important role in the observed cellular death, growth in non-fermentable carbon sources was measured. All strains grew under low aeration, indicating respiratory competence. High aeration caused much reduced growth in single SOD mutants, and the double mutant failed to grow. However, removal of respiration via another mutation dramatically increased short term survival and reversed the known air-dependent methionine and lysine auxotrophies. Our results suggest strongly that mitochondrial respiration is a major source of reactive oxygen species in vivo, as has been shown in vitro, and that these species are produced even under low aeration.

Many theories of aging are based on the hypothesis that aging is caused by oxidative damage as well as other macromolecular modifications that lead to the accumulation of random intracellular molecular defects (1–4). Recent studies have supported this idea by demonstrating a correlation between increased superoxide dismutase activity, increased life-span, and decreased oxidative damage in fruit flies and nematodes (5–7). We sought to investigate this hypothesis further but in a simpler eucaryotic system. We report here the development of a new in vivo model system for the role of oxidative stress in aging based on the stationary phase of the simple eucaryote Saccharomyces cerevisiae.

A large number of in vitro studies strongly implicate the respiratory chain (8–10) as a significant source of superoxide and hydrogen peroxide in eucaryotic systems (8, 11, 12). However, model systems to investigate this issue in vivo have been lacking. The relative importance of the various known reactive oxygen species under various conditions is also often uncertain; hydroxyl radical produced from metal-catalyzed reactions of superoxide and hydrogen peroxide is frequently invoked. Recently, however, it has become abundantly clear that superoxide in some instances can have an important toxicity of its own (13–16) and that levels occurring naturally in vivo are high enough to cause damage. Hydrogen peroxide has long been known to be toxic, but the extent to which it is a naturally occurring toxin under normal conditions has also been difficult to evaluate.

S. cerevisiae, like most other eucaryotes, contains CuZnSOD1 (product of the SOD1 gene) in the cytosol and MnSOD (product of the SOD2 gene) in the mitochondria (reviewed in Ref. 14). These enzymes catalyze the disproportionation of O2

* This work was supported by a pilot research grant from the UCLA Center on Aging based on a generous gift to the center by Harold and Libby Ziff and by National Institutes of Health Grant DK-46828. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: CuZnSOD, copper-zinc superoxide dismutase; MnSOD, manganese superoxide dismutase; SOD, superoxide dismutase.
months. Many markers, such as increased heat shock resistance and glycogen accumulation, have been utilized to define stationary phase in yeast, although it appears that the best definition of stationary phase is the ability to survive for prolonged periods without added nutrients (23).

Stationary phase yeast resemble most of the cells of multicellular organisms in two important aspects: 1) most energy comes from mitochondrial respiration and 2) the cells have exited from the cell cycle, comes from mitochondrial respiration and 2) the cells have cellular organisms in two important aspects: 1) most energy comes from mitochondrial respiration and 2) the cells have exited from the cell cycle, i.e., have entered the G0 phase. In addition, damage accumulates over time in stationary phase. This damage cannot be diluted, because cell division and new synthesis are not occurring, and thus must be prevented or repaired.

Almost all published studies of yeast cells have been performed on actively growing cultures with ample nutrients and a fermentable carbon source (glucose), in spite of the fact that these conditions are relatively rare in nature and, when they do occur, do not persist indefinitely. In such log phase fermentative growth, the cells depend more heavily on glycolysis than respiration for energy and growth, and each individual cell is exposed to oxidative stress for a short period of time before it divides, allowing dilution of any accumulated damage.

We report here the results of an investigation of the roles of the antioxidant enzymes in stationary phase survival in yeast. We have found that both CuZnSOD and MnSOD play a major role in cell survival, whereas catalase T and metallothionein have little effect. In addition, studies of short term survival of respiration-deficient strains indicate that both SODs play an important role in protection against the toxic products formed during mitochondrial respiration.

**MATERIALS AND METHODS**

**Yeast Strains**—Strains used are described in Table I. The sod1- and sod2- single and double null mutants were made as described previously (18, 24). Respiration-deficient strains could be constructed using her genes and promoters (32); and YEPh WT carrying the human SOD gene and promoter region (33). SOD and catalase assays were performed for all strains (see below); levels of these enzymes were shown to be normal unless the gene had been deleted.

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**Table I**

| Yeast strain | Genotype | Reference |
|--------------|----------|-----------|
| EG103        | (DBY746) MATα leu2-3, 112 his3Δ1 trp1-289 ura3-52 GAL- | 18         |
| EG118        | EC103 with sod1Δ:URA3 | 18         |
| EG119        | EC103 with sod2Δ:TRP1 | 24         |
| EG133        | EC103 with sod1Δ:URA3 sod2Δ:TRP1 | 24         |
| EG223        | EC103 with dt1Δ:TRP1 | 24         |
| CC103        | EG103 with coq3Δ:LEU2 | 29         |
| CC108        | CC103 with coq3Δ:LEU2 sod1Δ:URA3 | 42         |
| J102         | EC103 with coq3Δ:LEU2 sod1Δ:URA3 sod2Δ:TRP1 | This study |
| DTY3         | MATα leu2-3, 112 his3Δ1 trp1-1 ura3-50 gal1 CUP15 | 30         |
| DTY4         | DTY3 with cup1Δ:URA3 | 29         |
| W303B        | MATα ade2-1, his3-11, 15 leu2-3, 112 trp1-1 ura3-1 | 26         |
| EG225        | W303B with sod1Δ:URA3 | This study |
| CC303        | W303B with coq3Δ:LEU2 | 27         |
| CC304        | W303B with atp2Δ:LEU2 | 28         |
| EG228        | W303B with coq3Δ:LEU2 sod1Δ:URA3 | This study |
| EG229        | W303B with atp2Δ:LEU2 sod1Δ:URA3 | This study |

**RESULTS**

**Yeast Strains Lacking CuZnSOD and/or MnSOD Are Respiration-competent but Oxygen-sensitive**—Growth curves were performed in SC medium with various carbon sources using yeast strains EG103 (wt), EG110 (sod2-), EG118 (sod1-), and EG133 (sod1-, sod2-). Under normal conditions, i.e., with glucose as the carbon source and high aeration (normoxia), sod1- yeast strains grew more slowly and reached a maximum density lower than that of wild type; strains lacking MnSOD grew as well as wild type. The strain lacking both SODs showed the same phenotype as the sod1- mutant (Fig. 1A).
The same strains were grown in SC with either ethanol or lactate as the carbon source under conditions of either high or low aeration (Fig. 1B). With low aeration, all three mutant strains grew nearly as well as wild type in lactate and were able to grow, but less well, in ethanol. These results indicate that the strains are able to respire and therefore that they must contain functional mitochondria; thus they are potentially competent to enter stationary phase. Under high aeration in ethanol, the mutant strains showed much reduced growth, and the double mutant was unable to grow at all. In lactate, under high aeration, the sod2 mutant also did poorly, and the double mutant did not grow at all; but, somewhat surprisingly, the sod1 strain grew nearly as well as wild type. When growth in air on rich medium plates (YPD or YPE) was tested, results similar to the high aeration liquid culture data were obtained (data not shown). The absence of cytoplasmic catalase or of metallothionein (CUP1) did not affect growth under any of these conditions (data not shown).

CuZnSOD Null Mutants Rapidly Lose Viability under Normal Oxygen in Stationary Phase—To determine the effect of SOD mutations on cell growth and on the transition to stationary phase, the same four yeast strains, EG103 (wt), EG110 (sod2), EG118 (sod1), and EG133 (sod1, sod2) plus EG223 (ctt1) and DTY4 (cup1) with its parental control (DTY3), were grown under conditions of high aeration (normoxia) for 48 h and then washed and resuspended in water, as described under “Materials and Methods.” (This treatment results in the longest survival for wild type yeast strains.) The viability of the cells was then determined as a function of time (Fig. 2). The wild type strain maintained viability of greater than 40% for over 30 days, whereas the SOD mutants died much sooner. The strain lacking both SODs survived only for a few days, having less than 0.01% viable cells by day 5. The single sod1 mutant was less than 0.1% viable by day 9, whereas the sod2 mutants survived as well as wild type for 5–9 days and then began dropping off, reaching less than 1% viability at day 15. The lack of the cytoplasmic catalase T in strain EG223 had a small effect on viability, in spite of the fact that cytoplasmic catalase is highly induced in stationary phase. This lack of effect was somewhat surprising, because hydrogen peroxide, which is uncharged and easily crosses biological membranes, is a likely candidate for escape from the mitochondria. Other enzymes for handling peroxides (catalase A, cytochrome c peroxidase, etc.) whose genes are still present in these cell lines may account for this protection. A leak of the charged superoxide ion seems less likely, but this evidence raises the possibility that it occurs or that superoxide is generated on both sides of the inner mitochondrial membrane. The cup1 mutation had no effect on survival under these conditions (data not shown).

Comparison between the survival rates of the sod1 and sod2 mutants (Fig. 2) indicates that there is some cross-talk between the two compartments despite the fact that the two SODs have different subcellular locations. The fact that the EG110 (sod2) strain survives as well as wild type in the early stages of this experiment indicates that the cytoplasmic CuZnSOD can at least temporarily compensate for the loss of the mitochondrial MnSOD. Nevertheless, the fact that MnSOD does itself have an important role to play is apparent from the very poor survival of EG133 (sod1, sod2) after a few days relative to EG118 (sod1).

Cells were incubated in medium containing excess amounts of lysine and methionine in order to eliminate the possibility that these known auxotrophies might be responsible for the early loss of viability seen in the sod1 null mutants. A 3-fold excess of these amino acids did not reverse the viability loss
that electron leakage from complex III via CoQ is the main source of superoxide production (8, 9). We predicted that if the same was true in vivo, then respiration-incompetent sod2 strains would perform better than their respiration-competent isogenic relatives. In order to examine this question, we obtained or constructed strains carrying a deletion in coq3 (27), as well as in sod1 or in sod1 and sod2. COQ3 is the gene for one of the enzymes required for synthesis of coenzyme Q (CoQ or ubiquinone); coq3 strains are unable to synthesize CoQ and are thus respiration-incompetent or petite (27). We measured oxygen consumption to verify that respiration was lacking. The results are shown in Fig. 5. Both EG118 (sod1) and EG133 (sod1, sod2) with wild type COQ3 showed high oxygen consumption, whereas the coq3 versions of each strain demonstrated drastically reduced consumption of oxygen.

Loss of Respiration Increases Growth and Short Term Viability of Strains Lacking CuZnSOD—Unfortunately, the long term survival experiments cannot be performed with strains that are respiration-incompetent because entry into true stationary phase is absolutely dependent on respiration. In fact, the viability of coq3 sod1 cells dropped to 10% by day 6 of incubation (data not shown). However, survival in early stages of culture is instructive and can be examined readily. The results of such experiments are shown in Fig. 6. The strains EG118 (sod1) and EG133 (sod1, sod2) reached maximum viability at 24 h, after which viability declined quickly, whereas their petite derivatives were able to survive at almost their maximum viability for at least 48 h. (Note that in this figure the y axis is a linear scale.) Thus, we can conclude that the early death of sod1 mutants in this experiment is due to oxidative damage rather than loss of respiration, because lack of respiration actually increases survival at these early stages. At later stages, our data do not allow us to determine whether loss of respiration or oxidative damage is responsible for the cell death.

Loss of Respiration Reverses Air-dependent Lysine and Methionine Auxotrophies of Strains Lacking CuZnSOD—We also carried out studies of the role of respiration in the air-dependent auxotrophies for lysine and methionine that are characteristic of sod1 strains. We found that respiration-competent COQ3 sod1 cells were able to grow only in medium supplemented with both lysine and methionine, whereas respiration-incompetent coq3 sod1 cells grew in the absence of these amino acids (Fig. 7). These results confirm the importance of
respiration in generation of reactive oxygen species and the link between such species and these amino acid auxotrophies.

We have obtained similar results with strains constructed in another genetic background, the strain EG225, an sod1' derivative of W303B (data not shown). In this strain, in addition to petites generated by the coq3 deletion, we had available strains that were respiration-incompetent due to the deletion of the ATP2 gene, which codes for the beta subunit of the mitochondrial ATPase (28). Either kind of petite mutation improved the survival and allowed growth without lysine or methionine, compared with the respiration-competent parent (data not shown). These experiments confirmed that our results were not peculiar to one strain.

DISCUSSION

Our results show that superoxide dismutase is a major antioxidant in yeast stationary phase, playing a very important role in its survival under nongrowing conditions and implying that the superoxide ion is a major damaging species. Additionally, we show that mitochondrial respiration is a primary source of superoxide and other reactive oxygen species in vivo. Previously this conclusion was based on studies done in vitro.

Studies in isolated mitochondria showed that the majority of the superoxide in eucaryotic cells was produced in the mitochondria by electron leakage at the QH2:cytochrome c segment (complex III) (8); it has been estimated that over 80% of the superoxide in S. cerevisiae is produced in this manner (9). We were interested to note that the sod1' strains grew much better on lactate than on other nonfermentable carbon sources. In yeast, through the action of lactate:cytochrome c oxidoreductase, lactate can pass electrons directly to cytochrome c, thus bypassing coQ (38). This observation supports the idea that much of the cytoplasmic superoxide generated by respiration in vivo comes from complex III or before (as has been previously noted in vitro). Alternatively, it may indicate that steps after CoQ are less sensitive to oxidative damage; the ability to bypass complex I and III and send electrons to complex IV by another route would then lead to better growth.

Another study by the same group (40), reported that strains of S. cerevisiae deficient in both CuZnSOD and electron transport showed a substantial growth defect in 21% oxygen compared with the respiration-competent sod1' parent and concluded that respiration reduced cytosolic oxidant stress in vivo. Again, results in our system were different. In two different sod1' strains, removal of respiration improved survival and prototrophic growth relative to similar respiration-competent strains, leading us to conclude that respiration increased oxidant stress. Differences in strains used or in the end point measured may account for these discrepancies.

The differences observed between the single and double sod' mutants reinforce the hypothesis that despite the strong compartmentalization of these two enzymes, there is significant overlap between their functions. Apparently the two superoxide dismutase enzymes cooperate but cannot fully replace each other. Even though the most dramatic defects have been ob-
served for the cells lacking the cytoplasmic CuZnSOD, the MnSOD is nonetheless essential in its compartment. The fast loss of viability of CuZnSOD null mutants under normal but not low aeration may indicate that the mitochondrial enzymes are able to prevent migration of reactive oxygen species to the cytoplasm under low but not normal environmental oxygen levels. Interestingly, the survival rate for the sod2- mutant was not changed by alteration of the state of aeration nearly as much as was the survival rate for the sod1- mutant, such that MnSOD was more important than CuZnSOD for survival in low oxygen. This result has implications for higher organisms, because the dissolved oxygen concentration that most mammalian cells are exposed to is close to our low aeration conditions, making it likely that MnSOD plays a more important role than has been previously realized, in spite of its lower abundance.

Our results confirm roles of considerable importance for both SODs in stationary phase survival and agree with recently reported results in prokaryotes in E. coli lacking intracellular sod (41). In that work, the stationary phase loss of viability was dependent on the presence of oxygen and could be prevented by a synthetic SOD mimic.

In summary, we have developed a simple in vivo model system using long term survival of S. cerevisiae maintained in stationary phase in which to investigate the interplay between damaging and protective mechanisms in nongrowing cells. We believe this is a good model for mammalian cells and will lead to better understanding of the oxidative processes involved in cancer, neurological diseases, and aging.

Acknowledgments—We thank Dr. Catherine Clarke for plasmids and strains, Kaoru Goshima and Vanessa Moy for strain construction and excellent technical assistance, and Masis Babajanian for catalase deletion strains.

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