Oxidative Stress and Iron Are Implicated in Fragmenting Vacuoles of Saccharomyces cerevisiae Lacking Cu,Zn-Superoxide Dismutase

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The absence of the antioxidant enzyme Cu,Zn-superoxide dismutase (SOD1) is shown here to cause vacuolar fragmentation in Saccharomyces cerevisiae. Wild-type yeast have 1–3 large vacuoles whereas the sod1Δ yeast have as many as 50 smaller vacuoles. Evidence that this fragmentation is oxygen-mediated includes the findings that aerobically (but not anaerobically) grown sod1Δ yeast exhibit aberrant vacuoles and genetic suppressors of other oxygen-dependent sod1 null phenotypes rescue the vacuole defect. Surprisingly, iron also is implicated in the fragmentation process as iron addition exacerbates the sod1Δ vacuole defect while iron starvation ameliorates it. Because the vacuole is reported to be a site of iron storage and iron reacts avidly with reactive oxygen species to generate toxic side products, we propose that vacuole damage in sod1Δ cells arises from an elevation of iron-mediated oxidation within the vacuole or from elevated pools of “free” iron that may bind nonproductively to vacuolar ligands. Furthermore, additional pleiotropic phenotypes of sod1Δ cells (including increased sensitivity to pH, nutrient deprivation, and metals) may be secondary to vacuolar compromise. Our findings support the hypothesis that oxidative stress alters cellular iron homeostasis which in turn increases oxidative damage. Thus, our findings may have medical relevance as both oxidative stress and alterations in iron homeostasis have been implicated in diverse human disease processes. Our findings suggest that strategies to decrease intracellular iron may significantly reduce oxidatively induced cellular damage.

Aerobic organisms are chronically exposed to potentially harmful reactive oxygen species generated as by-products of cellular metabolism. One antioxidant enzyme Cu,Zn-superoxide dismutase (SOD1) plays an important role in detoxifying superoxide radicals (O2·−). Superoxide radicals can oxidize iron-sulfur cluster proteins liberating iron (1–4). “Free” iron can react with hydrogen peroxide (H2O2), and possibly other reactive oxygen species, to generate toxic hydroxyl radicals (OH·) by Fenton chemistry (6, 7). Hydroxyl radicals have the potential to damage proteins, nucleic acids, and membranes (4, 8–12).

In recent years, insight into oxidative defense, and SOD1 in particular, has come from studies using the unicellular eukaryote, Saccharomyces cerevisiae. Yeast lacking Sod1p grow slowly in air (13), are sensitive to superoxide generating agents (e.g. paraquat) (13, 14), and exhibit as yet poorly understood metabolic and biosynthetic defects (13–17). To further our understanding of oxidative damage to the cell, we examined the effects of superoxide dismutase deficiency on overall cell structure and organelle morphology and asked whether any such abnormalities may be tied into iron-mediated toxicity. We report here that oxidative stress to sod1Δ yeast results in fragmentation of the vacuole, an organelle analogous to the lysosome and thought to play a role in iron homeostasis (Refs. 18 and 19). Furthermore, we find lowering iron availability significantly reduces damage to the vacuole, consistent with disruption of iron handling as part of the pathway through which Sod1p deficiency causes vacuole fragmentation.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Growth Conditions.—The S. cerevisiae strains used in these studies are listed in Table I. sod1Δ strains were generated by substituting the SOD1 coding region with SOD1 replacement plasmids pK91 (sod1Δ::LEU2) or pK95 (sod1Δ::TRP1) as described previously (20). Strains for morphological analysis were propagated in YPD medium (1% yeast extract, 2% peptone, 2% glucose) or in YPG medium (1% yeast extract, 2% peptone, 2% glycerol) in air or in CO2-enriched anaerobic culture jars shaking at 100–120 rpm at 30 °C. For iron starvation studies, 50 μM of the iron chelator bathophenanthroline sulfonic acid was added to YPD, and for iron overload studies 2 mM FeCl3 was added to YPD.

Electron Microscopy—For ultrastructural analysis of yeast by electron microscopy, strains were maintained in log phase for 24 h prior to harvesting. Cells were pelleted, washed in 0.9 mM NaCl, washed twice in 0.1 mM sodium cacodylate, pH 7.2, fixed in 3% gluteraldehyde, 0.1 M cacodylate at room temperature for 3 h, washed three times in 0.1 M cacodylate, post-fixed in 4% KMnO4, 0.1 M cacodylate for 1 h at 4 °C, washed several times in water, and incubated in 2% uranyl acetate for 1 h at room temperature. Cells were dehydrated with 10-min serial ethanol washes (35, 50, 70, 95, and four times in 100%) and infiltrated with 50% uranyl acetate, 1% osmium tetroxide, and polymerized at 65 °C for 24–48 h. Thin sections were examined using a Hitachi HU12A transmission electron microscope.

Vital Staining of Vacuoles—For analysis of yeast vacuoles by light microscopy, strains were maintained in log phase for at least 10 h prior to harvesting. Procedures for vital vacuole membrane staining using...
FM4-64 (Molecular Probes) were as described previously (21). Briefly, cells were harvested and resuspended in YPD medium + 30 μM FM4-64 for 15 min at 30 °C. Cells were pelleted, resuspended in fresh YPD medium, and incubated at 30 °C for an additional 2 h, placed on slides and viewed with standard fluorescence microscopy. Procedures for quinacrine staining were as described previously (22). Logarithmically growing cells were pelleted, resuspended in YPD, 50 mM Na2HPO4, pH 7.5, with 200 μM quinacrine and incubated at 30 °C for 5 min. Cells were pelleted, washed, and viewed on slides using standard fluorescence microscopy. All fluorescent images were photographed using a MetaMorph Imaging System (Universal Imaging Corp.) in conjunction with a CCD camera (Princeton Instruments).

Vacuolar Protein Trafficking—Trafﬁcking of carboxypeptidase Y was assayed as described previously (23). Briefly, isogenic strains 1783 (SOD1) and KS105 (sod1Δ) and isogenic strains SEY6210 (VP55) and BHY152 (eps5) were pulse labeled with Trans35S-label (ICN) for 10 min. Carboxypeptidase Y was immunoprecipitated from cell lysates, electrophoresed on an SDS-polyacrylamide electrophoresis gel, and visualized by fluorography.

\( pH, \) Metal, and Starvation Sensitivity Assays—To assay the ability of yeast to grow over a broad pH range, 1783 and KS105 yeast strains were diluted to an \( \text{OD}_{600} \) of 0.05 in minimal medium (synthetic dextrose supplemented with lysine, methionine, cysteine, leucine, tryptophan, histidine, and uracil) (24) which was adjusted to the indicated pH (covering a range from 1.0 to 8.5 in 0.5 increments). Cultures were incubated overnight, and culture growth was measured by optical density.

To assay zinc sensitivity, SOD1 and sod1Δ yeast were grown over-night in minimal medium, diluted back to an \( \text{OD}_{600} \) of 0.1 in minimal medium, inoculated with the appropriate concentration of zinc chloride, permitted to grow for 20 h in air or in an anaerobic chamber, and culture growth was measured by optical density.

Starvation sensitivity was assayed as described previously (25). 1783 and KS105 yeast were maintained in log phase for 8 h in synthetic complete medium (24). \( 10^7 \) cells were pelleted by centrifugation, washed in water, resuspended in minimal sporulation media or water, and incubated at 30 °C for 7 days. Cells were counted with a hemocytometer. 500 cells from each culture were plated out onto YPD medium and permitted to grow for 3 days at 30 °C. The number of colonies formed per 500 cells was taken as the measurement of cell survival.

**RESULTS**

**Aberrant Vacuole Morphology in Cu,Zn-Superoxide Dismutase-deficient (sod1Δ) Yeast**—To determine effects of oxidative stress on cell morphology, aerated wild-type (1783) and sod1Δ (KS105) yeast were examined using electron microscopy (Fig. 1). Because mitochondria are thought to be a major source of superoxide radicals (e.g., Refs. 17 and 26), with leakage of electrons from the respiratory electron transport chain converting up to 5% of consumed oxygen into oxygen radicals (8), we anticipated mitochondrial abnormalities in sod1Δ yeast. However, the mitochondria of sod1Δ mutant yeast (arrowheads, Fig. 1, C and D) were indistinguishable from those in wild-type cells (arrowheads, Fig. 1, A and B). What was striking about the ultrastructure of the sod1Δ yeast was the apparent fragmentation of the yeast vacuole. While the wild-type strain (1783) typically exhibited 1–2 large vacuoles (Fig. 1, A and B), the sod1Δ mutant yeast (KS105) exhibited 5–15 smaller vacuoles in cross-section (Fig. 1, C and D). Staining such yeast with a vacuole-specific fluorescent dye quinacrine revealed a similar fragmented appearance (see Fig. 2). Imaging through multiple focal planes with a fluorescence microscope revealed that the sod1Δ yeast contained 25–50 small, individual vacuoles per cell compared with 1–3 vacuoles in wild-type yeast. Electron micrographs demonstrated that the electron densities of many smaller sod1Δ vacuoles were comparable to full-size wild-type vacuoles indicating that the vacuolar concentration of glycosylated molecules (which contribute significantly to the electron density (27)) was similar. However, some membrane-bound, non-uniform, electron transparent vesicles (arrows, Fig. 1D) were also evident in the vicinity of the vacuoles and may represent vacuole remnants or other aberrant organelles in the sod1Δ yeast.

**Vacuole Fragmentation in sod1-deficient Yeast Is Oxygen Dependent**—Because both the vacuole and Sod1p have been implicated in copper homeostasis, an initial hypothesis was that the aberrant vacuole morphology may reflect altered copper handling in sod1Δ yeast. Sod1p was identified in a genetic screen for copper-binding metallothioneins and was demon-

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**Table I**

| Yeast strain | Genotype                        | Source                          |
|-------------|----------------------------------|---------------------------------|
| 1783        | MATa leu2 his4 trp1 ura3         | Ref. 34                         |
| KS104       | MATa leu2 his4 trp1 ura3 sod1Δ::LEU2 | K. Slekar and V. Culotta, unpublished |
| KS105       | MATa leu2 his4 trp1 ura3 sod1Δ::TRP1 | Ref. 34                         |
| JS001       | MATa leu2 his4 trp1 ura3 sod1Δ::LEU2 sod2Δ::URA3 | J. Strain and V. Culotta, unpublished |
| JS002       | MATa leu2 his4 trp1 ura3 sod1Δ::URA3 | Ref. 61                         |
| KS109       | MATa leu2 his4 trp1 ura3 sod1Δ::TRP1 pmr1Δ::LEU2 | Ref. 34                         |
| SEY6210     | MATa leu2 his3 trp1 ura3 lys2 suc2 | Ref. 62                         |
| BHY152      | MATa leu2 his3 trp1 ura3 lys2 suc2 vs5Δ::HIS3 | Ref. 63                         |
| TVY1        | MATa leu2 his3 trp1 ura3 lys2 suc2 pep4Δ::LEU2 | T. Vida, unpublished          |
| SEY6210-sod1| MATa leu2 his3 trp1 ura3 lys2 suc2 sod1Δ::TRP1 | This study                      |
| TVY1-sod1   | MATa leu2 his3 trp1 ura3 lys2 suc2 pep4Δ::LEU2 sod1Δ::TRP1 | This study                      |
| YPH250      | MATa leu2 his3 trp1 lys2 ade2 ura3 | Ref. 64                         |
| YPH250-fet3 | MATa leu2 his3 trp1 lys2 ade2 ura3 fet3Δ::TRP1 | D. Kosman, SUNY, Buffalo        |
| SL202       | MATa leu2 his3 trp1 lys2 ade2 ura3 sod1Δ::LEU2 | Ref. 64                         |
| SL203       | MATa leu2 his3 trp1 lys2 ade2 ura3 sod1Δ::LEU2 sod1Δ::TRP1 | S. Lin and V. Culotta, unpublished |
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Vacuole Fragmentation Can Be Rescued by the Addition of Superoxide Radical Scavenging Mechanisms—To further elucidate the nature of the vacuole fragmentation, genetic suppressors of various sod1Δ phenotypes were employed. The first class of suppressors function in redox active metal homeostasis and apparently provide the cell with cytosolic superoxide scavenging activity. Mutations in PMR1, a Golgi P-type ATPase, lead to accumulations of manganese in the cytosol (30) which can scavenge free radicals (31–33). pmr1 mutants have been shown to rescue all known sod1Δ oxygen-dependent defects including amino acid biosynthetic deficits and paraquat sensitivity (30). As seen by fluorescent FM4-64 vacuole membrane staining in Fig. 3C and quantified in Fig. 4, the pmr1Δ mutation restores 8 wild-type vacuolar morphology to the sod1Δ mutant, suggesting that removal of oxygen-free radicals is sufficient to prevent vacuole fragmentation. Treatment of sod1Δ yeast with 1 mM Mn2+ was also sufficient to rescue fragmentation (data not shown) providing additional confirmation that cytosolic superoxide scavenging activity is sufficient to prevent vacuolar abnormalities.

Fig. 4. Quantitative effect of sod1Δ, sod2Δ, pmr1Δ, and TKL1 on vacuole fragmentation. Strains (all isogenic to 1783) were maintained in logarithmic phase of growth in rich media (YPD) shaking at 120 rpm for 8 h, then stained with FM4-64, and the number of vacuoles per cell counted. Values represent 300 cells for each genotype scored in three independent experiments (~100 cells scored per genotype per experiment). Standard deviations between experiments were less than 10%.

A second class of suppressors alleviates only a subset of sod1 null deficiencies that apparently arise from a decreased level of NADPH in sod1Δ yeast (34). sod1Δ yeast exist in a more oxidized state than wild-type yeast presumably due to utilization of cellular reserves of reductants (such as GSH and NADPH) for the spontaneous reduction of reactive oxygen species (5). Overexpression of transketolase (TKL1) stimulates generation of NADPH by the pentose phosphate pathways and thus rescues the methionine auxotrophy and slow growth defect of the sod1Δ yeast (34). Despite the ability to rescue these aspects of the sod1Δ phenotype, overexpression of TKL1 does not rescue the vacuole fragmentation (Fig. 3D). Quantification (Fig. 4) confirms that, for the most part, vacuoles remain fragmented with increased Tk1p, suggesting that the fragmentation is not related to lowered NADPH levels.

Vacuole Fragmentation Is Specific to the Cytosolic Cu,Zn-Superoxide Dismutase Deficiency—In addition to Cu,Zn SOD1 which is predominantly cytosolic, eukaryotes also have a nuclear-encoded manganese SOD2 localized within the mitochondrial matrix. To determine whether vacuole fragmentation is a general defect of any superoxide dismutase deficiency, vacuole morphologies of aerated wild-type (1783), sod1Δ (KS105), sod2Δ (JS002), and sod1Δ sod2Δ (JS001) strains were com-
pared using FM4-64 (Fig. 5), as well as by electron microscopy (data not shown). Yeast deficient for the mitochondrial manganese SOD2 (JS002) had vacuole morphologies indistinguishable from those of wild-type yeast (1783), while sod1Δ (KS105) and the double mutant sod1Δsod2Δ (JS001) yeast exhibited vacuolar fragmentation (Fig. 5; quantified in Fig. 4). This provides evidence that vacuole aberrations are specific to Sod1p deficiency and suggests that the free radicals responsible for vacuole fragmentation are cytosolic or vacuolar in location.

Vacuole Function Is Modestly Compromised in sod1Δ Yeast—To determine the potential metabolic consequences of the aberrant vacuoles in sod1Δ yeast, we examined various vacuole-related functions. The yeast vacuole, like the mammalian lysosome, is an acidic compartment containing abundant hydrolases responsible for macromolecular degradation. In addition, the yeast vacuole plays a role in metabolite storage, pH homeostasis, and sequestration of potentially toxic substances such as metals (for review, see Ref. 35).

Staining with the pH-sensitive vital dye, quinacrine, indicated that the sod1Δ vacuoles were appropriately acidified (Fig. 2C). However, yeast lacking sod1 were unable to grow at a pH below 3.0 or above 7.0, whereas the wild-type yeast maintained viability over a broader pH range of 2.5 to 7.5 (Fig. 6A). This would suggest that sod1Δ yeast may not be able to regulate the cytosolic [H+] when extracellular proton levels are particularly high or low. Indeed, other known yeast mutants with a sensitivity to pH are vacuolar mutants (27), suggesting that this sod1Δ pH sensitivity is vacuole related.

The vacuole is responsible for the storage of a subset of amino acids and serves as a nitrogen and phosphate reserve (35). When yeast are exposed to adverse starvation conditions, such as that which occur when yeast sporulate or enter stationary phase, they normally up-regulate vacuolar hydrolases to turnover macromolecules to recycle necessary nutrients (36–38). Consistent with previous reports (16, 17), we found that when starved for nitrogen, phosphate, or amino acids, the sod1Δ yeast exhibited decreased viability (Fig. 6B). This suggests that the fragmented vacuoles may not provide adequate stores of nutrients for starvation conditions or that vacuole-dependent macromolecular turnover processes may be compromised. This is similar to pep vacuolar mutants which also exhibit a sporulation defect and inability to survive starvation conditions (39).

The vacuole (like the mammalian lysosome) additionally plays a role in the sequestration of metals. Mutations in vacuolar components Pep3p and Pep5p result in aberrant vacuole morphology and increased sensitivity to iron, cadmium, and zinc (28). Similarly, sod1Δ yeast not only have aberrant vacuoles but also an increased sensitivity to iron (40), cadmium (41), and zinc (Fig. 6C), and like the vacuole abnormalities, the sensitivity to these metals is oxygen-dependent. Thus, metal homeostasis and/or sequestration may be altered in these sod1Δ cells. Altogether, the evidence presented here suggests that oxidatively damaged sod1Δ vacuoles may be compromised in several aspects of function.

Novel Mechanism of Vacuolar Fragmentation in sod1Δ Yeast—Because aberrant vacuole morphology has been described in a number of known yeast mutants, we examined whether the sod1Δ fragmentation may be occurring through a previously identified pathway. Vacuole fragmentation has been described in yeast mutants, known as Class B vps mutants, which exhibit aberrant trafficking of vacuole proteins (27). To investigate whether loss of Sod1p affects vacuolar protein trafficking, the maturation of the vacuolar protease carboxypepti-
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Fig. 7. Aberrant sod1Δ vacuole morphology does not arise from vacuole protein mistrafficking or increased autophagy. A, carboxypeptidase Y (Cpy) forms (69-kDa Golgi precursor and 61-kDa mature vacular product) immunoprecipitated from 35S-pulse labeled yeast lysate of strains SOD1 (1783), sod1Δ (RS105), VP55 (SEY6210), and vps5 (BHY152) visualized by autoradiography after separation on an SDS-polyacrylamide electrophoresis gel. B, FM4-64 vacuolar membrane staining of SOD1 and sod1Δ yeast with (PEP4) or without (pep4Δ) the Pep4p protease.

Fig. 8. Vacuole fragmentation is iron-dependent. FM4-64 vacuolar membrane staining of isogenic SOD1 fet3Δ (YPH250-fet3) and sod1Δ fet3Δ (SL203) yeast grown in low-iron media (A and B) (YPD + 50 mM bathophenanthrolinedisulfonic acid), SOD1 FET3 (YPH250), and sod1Δ FET3 (SL202) yeast grown in standard media (C and D) or iron-rich media (E and F) (YPD + 2 mM iron).

Iron Is Implicated in Aberrant Vacular Morphology—Because iron homeostasis is altered in sod1Δ-deficient cells and subcellular fractionation of iron-loaded cells suggest the vacuole is a major site for iron sequestration (18, 19), one additional explanation for vacuole fragmentation in sod1Δ yeast may be that the vacuole is particularly prone to damage due to higher levels of adverse iron-mediated reactions. Superoxide has been demonstrated to react with labile iron-sulfur clusters of dehydratase enzymes such as aconitase and dehydrogenases (3, 46, 47) to liberate iron, which may then be trafficked to the vacuole/lysosome. Free iron can react readily with the reactive oxygen species to generate toxic hydroxyl radicals which have an estimated lifetime of ~2 ns and radius of diffusion of ~20 Å in aqueous solution (reviewed in Ref. 48). Thus, if hydroxyl radicals were generated by vacuolar iron pools, they would induce damage primarily within the vacuole.

To determine the degree to which iron contributes to vacuole damage, sod1Δ yeast were either starved for iron or loaded with excess iron and vacuolar morphologies were examined. By deleting Fet3p oxidase (which is thought to convert Fe2+ to Fe3+ for import by the Ftr1p iron transporter (49–51)), high-affinity iron uptake was impaired in both SOD1 fet3Δ and sod1Δ fet3Δ yeast. As seen in Fig. 8B, absence of the Fet3p transport component ameliorated fragmentation (Fig. 8B). fet3Δ did not rescue other oxygen-dependent sod1Δ phenotypes (such as the lysine biosynthetic defect) suggesting that fet3Δ acts by decreasing intracellular (and vacuolar) iron, thereby diminishing the iron catalyst necessary for adverse chemistry rather than simply acting globally to reduce oxygen radicals in sod1Δ yeast. Furthermore, growth in iron-rich media increased sod1Δ fragmentation (Fig. 8F), again consistent with reactive oxygen species reacting with vacuolar iron to provoke local damage.

DISCUSSION

Because mitochondria are the primary intracellular sources of superoxide radicals, we anticipated mitochondrial damage in sod1Δ yeast. However, the most obvious sod1Δ defects were not in the mitochondria, but rather in the vacuole. Oxidative stress to Sod1p-deficient yeast leads to vacuolar fragmentation through an iron-dependent process. Evidence that the vacuole fragmentation is oxygen (presumably superoxide radical) mediated includes the findings that only aerobically grown sod1Δ...
yeast exhibit aberrant vacuoles and genetic suppressors (such as pmr1Δ) capable of rescuing all other known aerobic sod1Δ deficits can also rescue this fragmentation phenotype. Iron deprivation (as in fet3Δ sod1Δ strains) also alleviates the fragmentation without providing resistance to O$_2$ generating reagents or ameliorating other oxygen-dependent phenotypes of sod1Δ yeast. Thus, reactive oxygen species arising in the absence of Sod1p, in combination with iron, provoke damage to vacuoles.

The notion of iron exacerbating damage under conditions of oxidative stress has been raised previously in other contexts. Fe$^{2+}$ can react with physiological levels of H$_2$O$_2$ (and possibly other reactive oxygen species) to produce toxic hydroxyl radicals (reviewed in Ref. 48). In vivo, iron is not found typically in a free state but is sequestered as iron complexes or is bound protectively to enzymes, proteins, or iron carriers (for review, see Refs. 48 and 52). However, superoxide can alter this cellular iron homeostasis. Several groups have found in vivo evidence that superoxide radicals oxidize labile iron-sulfur clusters to liberate iron (1, 2, 3, 46, 47) although the fate and redox state of the “liberated” iron is not known. Additionally, genetic screens have found that mutations in iron-sulfur cluster assembly proteins lessen oxidative damage in sod1Δ yeast, suggesting that reducing the number of iron-sulfur targets for oxygen radical damage lessens damage in these oxidatively stressed cells (53). Similarly, superoxide radicals have been shown to oxidize iron storage components to liberate iron which is released in a form capable of catalyzing hydroxyl radical production (54). More generally, Wisnicka et al. (40) have suggested that superoxide radicals (in sod1Δ yeast) may influence the redox status of cellular iron pools by reducing Fe$^{3+}$ to the more reactive Fe$^{2+}$ state. Although total iron is not elevated in sod1Δ yeast, the cellular distribution of iron, the ratio of ferrous to ferric iron, and the level of free iron may be altered. In any case, much evidence does suggest that oxygen radicals liberate iron which in turn may exacerbate oxidative damage.

In yeast, the vacuole plays a role in iron homeostasis. Subcellular fractionation of iron-loaded cells suggests that the vacuole is the major site of iron sequestration (18) and yeast lacking a recognizable vacuole structure are unable to accumulate high levels of iron (19). Thus, an iron-enriched yeast vacuole may be particularly susceptible to iron-mediated damage in superoxide-rich sod1Δ yeast. One possibility is that the vacuole damage may result from a disruption of the iron storage state in the vacuole. Under the influence of increased superoxide levels (which perhaps may be found throughout the Sod1p-deficient cell), vacuolar iron pools may be converted to a more accessible and active Fe$^{2+}$ state that can catalyze hydroxyl radical production and damage macromolecules in the immediate vicinity of the vacuole. A second, not mutually exclusive, possibility is that alterations in iron handling and homeostasis within the cell may result in an increased trafficking of free iron to the vacuole. Increased vacuolar iron may result in increased iron-mediated oxidative reactions within the vacuole or alternatively, the increased iron may simply bind nonproductively to various sites in the vacuole (i.e. sulfur, nitrogen, or oxygen containing ligands). We cannot rule out the possibility that the primary damage actually occurs to cytosolic components that then secondarily afflicts vacuolar structure and function. Indeed, we should stress, however, that there is not a global defect in trafficking to the vacuole or vacuole assembly as judged by normal carboxypeptidase Y trafficking results. In any case, both reactive oxygen species and iron contribute to vacuole changes.

Iron-mediated damage occurs not just in the yeast vacuole but also in the mammalian analogue, the lysosome. Similar to the yeast vacuole, the lysosome plays a role in intracellular iron storage, homeostasis, and detoxification (55, 56). Iron overload in the rat liver has been shown to increase lysosomal fragility (56). Although lysosomal fragility has been partially attributed to lipid peroxidation, we should stress that similar lipid membrane damage is not the cause of sod1Δ vacuole abnormalities because S. cerevisiae do not synthesize the polyunsaturated fatty acids that are susceptible to lipid peroxidation (57). Damage to vacuolar membrane proteins (as well as lysosomal membrane proteins) is a more likely possibility.

By examining structural changes in cellular architecture caused by Sod1p deficiency, we have uncovered a plausible explanation for various puzzling defects in the sod1Δ yeast: some of the pleiotropic sod1Δ phenotypes may arise as secondary consequences of a compromised vacuole. For example, sod1Δ yeast have a sporulation defect (16) and an increased death rate upon entering stationary phase (17). Previously, this sensitivity to adverse conditions was attributed to mitochondrial insufficiency (16, 17). However, although sod1Δ yeast do not grow as robustly on non-fermentable carbon sources as wild-type yeast, they nevertheless are respiration proficient (e.g. Ref. 15). Instead, we suggest these sod1Δ abnormalities may be, in part, vacuole related. When yeast are exposed to such adverse conditions, they normally up-regulate vacuolar hydrolases to turnover intravacuolar reserves and cytosolic macromolecules in order to recycle necessary nutrients (36–38). The fact that the sod1Δ yeast cannot survive through such starvation conditions may be an indication that either vacuolar nutrient storage or the vacuolar-dependent macromolecular turnover processes are compromised. Indeed, many known vacuolar mutants also cannot survive adverse conditions (39). Furthermore, we demonstrate that sod1Δ yeast have a sensitivity to pH which cannot easily be explained without invoking the role of the vacuole in pH homeostasis. Likewise, an increased sensitivity to transition metals may be directly or indirectly related to vacuole abnormalities. Thus, some of the curious metabolic and biochemical abnormalities in sod1Δ yeast may in fact be attributable to vacuolar aberrations.

In summary, we demonstrate that oxidative stress to sod1Δ yeast results in vacuolar fragmentation and either removal of oxygen or iron can ameliorate the damage. This may have medical relevance in that oxidative damage and alterations in iron homeostasis have been implicated in a number of disease states including atherosclerosis, neurodegeneration, arthritis, and aging (for review, see Ref. 48). In some cases oxidative insult is thought to be the primary cause of damage. For example, brain and spinal cord deterioration after ischemic or traumatic injury often appears excessive for the level of trauma (58). One explanation put forth is that iron-binding capacity in the central nervous system and in the cerebral spinal fluid bathing the central nervous system is particularly low. Thus, release of iron by oxidatively or mechanically damaged cells, organelles, and proteins may catalyze toxic oxidative side reactions causing further cell injury. Consistent with this concept, preliminary trials of treatment with iron-chelating agents have had success in diminishing post-traumatic degeneration of brain and spinal cord (59, 60). Thus, iron-mediated oxidative damage is likely a common event in aerobic organisms, and dissection and understanding of the process (and ways of prevention) in yeast may give insight into human disease.

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\*V. C. Culotta, unpublished results.
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