Evidence for a Novel Role of Copper-Zinc Superoxide Dismutase in Zinc Metabolism*

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The LYS7 gene in Saccharomyces cerevisiae encodes a protein (yCCS) that delivers copper to the active site of copper-zinc superoxide dismutase (CuZn-SOD, a product of the SOD1 gene). In yeast lacking Lys7 (lys7A), the SOD1 polypeptide is present but inactive. Mutants lacking the SOD1 polypeptide (sod1A) and lys7A yeast show very similar phenotypes, namely poor growth in air and aerobic auxotrophies for lysine and methionine. Here, we demonstrate certain phenotypic differences between these strains: 1) lys7A cells are slightly less sensitive to paraquat than sod1A cells, 2) EPR-detectable or “free” iron is dramatically elevated in sod1A mutants but not in lys7A yeast, and 3) although sod1A mutants show increased sensitivity to extracellular zinc, the lys7A strain is as resistant as wild type. To restore the SOD catalytic activity but not the zinc-binding capability of the SOD1 polypeptide, we overexpressed Mn-SOD from Bacillus stearothermophilus in the cytoplasm of sod1A yeast. Paraquat resistance was restored to wild-type levels, but zinc was not. Conversely, expression of a mutant CuZn-SOD that binds zinc but has no SOD activity (H46C) restored zinc resistance but not paraquat resistance. Taken together, these results strongly suggest that CuZn-SOD, in addition to its antioxidant properties, plays a role in zinc homeostasis.

With the appearance of molecular oxygen (O2) in the earth’s atmosphere, all aerobic organisms evolved methods to utilize O2 for energy production. However, reactive byproducts of O2 metabolism (also known as reactive oxygen species) can be deleterious, and aerobic life forms have developed several systems to combat metabolic and environmental sources of oxygen toxicity. One major constituent in protecting cellular components against reactive oxygen species is superoxide dismutase (SOD).1

As a part of the primary line of defense against reactive oxygen species, SODs use metal ions to catalyze the disproportionation of superoxide (O2−) to hydrogen peroxide (H2O2) and O2 (1). Eukaryotes, including Saccharomyces cerevisiae, contain two distinct SOD enzymes. Mn-SOD, encoded by the SOD2 gene, resides in the mitochondrial matrix; CuZn-SOD, encoded by the SOD1 gene, is localized mainly in the cytoplasm and nucleus as well as in the mitochondrial intermembrane space. Mutant derivatives of S. cerevisiae that lack either of the SODs exhibit phenotypic deficiencies which are particularly extreme in the sod1A mutants. Even though they are viable, sod1A strains grow poorly in air, are extremely sensitive to redox-cycling drugs, die quickly in the stationary phase, and display aerobic lysine and methionine auxotrophies. It is generally believed that the loss of superoxide-scavenging activity, which is due to the absence of SOD1 gene product, leads to a serious burden of oxidative stress and causes havoc in cellular metabolism and growth processes (2, 3).

Employing in vivo whole-cell Fe(III) EPR methodology, Keyer and Imlay (4) observed an increase in loosely bound or “free” iron2 in sod mutants of E. coli. This iron is present in the Fe(II) state, as evidenced by the fact that it is only detectable by EPR at g = 4.3 after treatment of the cells with the iron chelator desferrioxamine, which converts EPR-silent Fe(II) to EPR-detectable Fe(III). A potential source of this increased pool of “free” iron includes a class of enzymes containing exposed [4Fe-4S] clusters, which are superoxide-sensitive (5). The classic example of this type of enzyme is aconitase (6). Attack by O2− oxidizes the [4Fe-4S] cluster, resulting in the release of at least one iron ion and inactivation of the enzyme. In E. coli, this process was proposed to be the source of the “free” iron that built up in mutants lacking SOD (4). Recently, adapting the same EPR method, we demonstrated that yeast sod1A mutants also have greatly elevated levels of “free” or “EPR-detectable” iron (iron detectable at g = 4.3 by EPR). In yeast, unlike in E. coli, the iron is present in the Fe(III) state (7).

Studies of yeast mutants lacking the SOD1 gene (sod1A) have revealed intriguing links connecting CuZn-SOD with transition metal metabolism. Addition of manganese or copper salts to the medium in modest amounts can improve the growth of these strains (reviewed in Ref. 8). More recently, we have shown that sod1A yeast have an increased requirement for iron in aerobic growth, and under certain growth conditions, they display a moderate increase in iron uptake and accumulation (9). Moreover, a variety of second site genetic suppressors of the sod1A phenotype have been isolated (10–13), and although the gene products involved are located in different cellular compartments and organelles, they all participate in the homeo-

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§ The abbreviations used are: SOD, superoxide dismutase; WT, wild type.

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stasis of transition metal ions: copper, manganese, or iron.

Out of this scientific endeavor has also come the discovery of the copper chaperone for SOD1 (CCS). This protein is responsible for the delivery of copper to CuZn-SOD and is encoded by the LYS7 gene in *S. cerevisiae* (14, 15). Because the SOD1 polypeptide in *lys7Δ* mutants lacks copper in its active site, it is incompetent to catalyze the removal of O2



**Table I**

Yeast strains used in this study

| Strain       | Relevant genotype   | Complete genotype   | Source |
|--------------|---------------------|---------------------|--------|
| EG103        | Wild type           | MATa leu2 his3 trp1 ura3 | Ref. 3 |
| EG118        | sod1Δ               | MATa leu2 his3 trp1 ura3 sod1Δ-URA3 | Ref. 3 |
| JW101        | lys7Δ               | MATa leu2 his3 trp1 ura3 lys7Δ-LEU2 | This work |
| JW102        | lys7Δ sod1Δ         | MATa leu2 his3 trp1 ura3 lys7Δ-LEU2 sod1Δ-URA3 | This work |
| BY474        | Wild type           | MATa his3 leu2 met15 ura3 | Purchased |
| JW201        | sod1Δ               | MATa his3 leu2 met15 ura3 sod1Δ-URA3 | Purchased |
| JW202        | lys7Δ sod1Δ         | JW201 with pEmBLyex4-URA3 | This work |
| JW203        | sod1Δ/lys7Δ EMBL     | JW201 with pSALSOD-URA3 | This work |
| JW204        | sod1Δ/lys7Δ MnSOD    | JW201 with pSALSOD-URA3 | This work |
| HH101        | sod1Δ/YEp351        | EG118 with YEp351 | This work |
| HH102        | sod1Δ/lys7Δ SOD1     | EG118 with YEp600 | This work |
| HH103        | sod1Δ/lys7Δ H46C     | EG118 with YEp351-yH46C SOD | This work |

**EXPERIMENTAL PROCEDURES**

**Reagents, Media, Strains, and Cell Growth—**High purity sulfate salts of zinc, copper, and nickel were obtained from Sigma. Methyl viologen, commonly known as paraquat, and the antibiotic G418 were purchased from Research Genetics (Huntsville, AL). The yeast strains used in this study are described in Table I. Strains BY4741, JW202, and JW203 were purchased from Research Genetics (Huntsville, AL). The lys7Δ strain JW101 was obtained by deleting the chromosomal *LYS7* locus of EG103 using the *lys7Δ-LEU2* construct pLSL257 as described previously (16). Using our *sod1Δ-URA3* construct, JW102 (*lys7Δ sod1Δ*) was prepared by sequential gene deletion starting with the *lys7Δ* strain. Southern blot analysis was performed to verify the correct deletion of the *LYS7* and *SOD1* genes (data not shown). The plasmids pEMBLYex4 and pSALSOD1 (referred to herein as pMn-SOD) were kindly provided by Dr. C. Bowler (17) and introduced into JW201 (the *sod1Δ* null yeast in the presence of non-fermentable carbon source, and temperature. Parameters used for low temperature Fe(III) EPR were as follows: center field, 1500 G; sweep width, 500 G; microwave power, 20 mW; modulation frequency, 100 kHz. Further details regarding sample preparation, instrument parameters, and data analysis used for the whole-cell low temperature Fe(III) EPR measurements are delineated in our previous study (7).

**Assessment of Metal Ion and Paraquat Sensitivity—**Paraquat, zinc, copper, or nickel was added to the indicated medium at the indicated concentration. Experimental cultures in dextrose-containing medium were inoculated at an *A900nm* of 0.1 in 50 ml of SDC medium and grown for 72 h at 30 °C. After growth, cells were centrifuged and then briefly treated with desferrioxamine, which binds to labile iron and converts it from Fe(II) (EPR-silent) to Fe(III) prior to washing with 20 mM Tris-Cl, pH 7.4. After washing, the pellet was resuspended in 200 μl of 20 mM Tris-Cl, pH 7.4, containing 20% glycerol, and 200 μl of the resuspended cells was packed in EPR tubes. The tubes were stored at −70 °C until EPR measurements were performed. EPR spectra were recorded using a Bruker X-band spectrometer using a finger Dewar filled with liquid nitrogen attached to the cavity of the instrument to maintain low temperature. Parameters used for low temperature Fe(III) EPR were as follows: center field, 1500 G; sweep width, 500 G; microwave power, 20 mW; modulation frequency, 100 kHz. Further details regarding sample preparation, instrument parameters, and data analysis used for the whole-cell low temperature Fe(III) EPR measurements are delineated in our previous study (7).

**RESULTS**

*lys7Δ* yeast have normal SOD1 expression, but the SOD1 polypeptide lacks enzymatic activity because the copper is not incorporated into the active site (14, 15). Therefore, one would expect *lys7Δ* and *sod1Δ* strains to possess comparable antioxidant capacity and thus to share similar phenotypes. With the *lys7Δ* and *sod1Δ* strains constructed from our laboratory wild-type yeast (EG103 background), we have observed numerous similarities between these two yeast strains in terms of growth, aerobic auxotrophies for lysine and methionine, poor growth in the presence of non-fermentable carbon source, and temperature sensitivity at 37 °C. However, further characterization of the *lys7* null strain led to the discovery of certain differences between these two yeast mutants.
Resistance to Superoxide—First, we examined the effect of paraquat, a known generator of intracellular superoxide, on wild-type, lys7Δ, sod1Δ, and lys7Δsod1Δ yeast by culturing cells in the presence of varying concentrations of paraquat for 24 h. As shown in Fig. 1A, at 10 and 25 μM of paraquat, concentrations that are toxic for both sod1Δ and lys7Δsod1Δ mutant strains, lys7Δ cells still grew as well as wild-type cells, but 100 μM paraquat was toxic. In contrast, wild-type EG103 can survive in 1 to 10 mM paraquat. Identical experiments were conducted in another genetic background, BY4741, with similar results (Fig. 1B). In the BY4741 background, lys7Δ cells are resistant to the toxic effects of paraquat at concentrations as high as 100 μM (83.2 ± 2.9% control growth), whereas sod1Δ yeast exhibit significant levels of growth retardation at 25 μM (8.0 ± 4.8% control growth).

EPR-detectable Iron—Because it is postulated that an elevated superoxide level is connected to an increase in cellular “free” iron (4, 9, 22), we wonder whether the decreased sensitivity to paraquat for lys7Δ yeast correlated with a diminished activity to paraquat for wild-type lys7, sod1, and lys7sod1 derivatives. Liquid cultures inoculated at an A600 of 0.03 were grown for 24 h at 30 °C with shaking at 200 rpm. Total growth relative to that obtained in the absence of paraquat (% control growth) was determined turbidometrically at A600. Values are the means of 3 independent experiments except for lys7sod1Δ strain (n = 2). Error bars indicate 1 standard deviation.

Zinc Sensitivity—One of the more puzzling aspects of the phenotypes of sod1Δ mutants has been their heightened sensitivity to extracellular zinc. We discovered this effect several years ago, and since then, we have sought for an explanation. Wild-type cells tolerate 5 or even 10 mM zinc in the medium (data not shown). On the other hand, cells lacking CuZn-SOD exhibit growth retardation at zinc levels as low as 1 mM. Thus, we decided to assess the ability of excess zinc ions to inhibit growth in lys7Δ yeast as well. In the experiment shown in Fig. 3A, lys7Δ, sod1Δ, lys7sod1Δ, and the parental wild-type strain (EG103) were cultured in the presence of increasing concentrations of ZnSO4. Unlike the sod1Δ mutant, which displayed marked growth retardation at 0.5 mM ZnSO4 (~73% control growth), lys7Δ cells behaved like wild type at all concentrations of ZnSO4 tested (up to 10 mM, Fig. 3 and data not shown). To ascertain that this phenotypic difference between lys7Δ and sod1Δ null strains is not a strain-dependent phenomenon, we tested the effect of excess zinc ions on these two mutants made in the BY4741 background. As shown in Fig. 3B, the resistance of lys7Δ to elevated levels of ZnSO4 mirrored that observed for the EG103 background.

In two genetic suppressors of sod1Δ mutants, pmr1 and bsd2, increased sensitivity toward manganese and copper ions was found to correlate with the accumulation of intracellular manganese and copper, respectively (11, 12). Thus, we proceeded to explore whether the sensitivity to excess zinc in the sod1Δ cells was associated with elevated levels of total cellular zinc. The total cellular level of zinc in the sod1Δ mutant was compared with those in isogenic lys7Δ and wild-type strains. In the experiment shown in Fig. 4, zinc levels were measured in cells after 72 h of growth in SDC medium. The sod1Δ, as well as the lys7Δsod1Δ, mutants accumulate ~50% higher levels of zinc than do the wild-type and lys7Δ strains, further implicating SOD1 in the regulation of heavy metal metabolism.

Specificity of the Extracellular Metal Ion Sensitivity—To evaluate the effects of other transition metal ions on wild-type, lys7Δ, and sod1Δ yeast, the growth of these strains was tested in the presence of CuSO4 and NiSO4. As shown in Fig. 5A, the extent of growth inhibition is comparable for all three strains.
supplemented with the specified concentration of ZnSO$_4$. Liquid cul-

tures were inoculated at an $A_{600}$ of 0.05, and total cell growth following a 24-h incubation at 30 °C was determined by measuring OD at 600 nm ($A_{600}$). For A and B, the means of three independent experiments are shown; error bars indicate standard deviation.

FIG. 3. Effect of excess zinc on the growth of WT, lys7Δ, and sod1Δ yeast. Growth of the designated yeast strains (circle, WT; inverted triangle, lys7Δ; square, sod1Δ; diamonds, lys7Δsod1Δ) in either EG103 (A) or BY4741 (B) background was tested in SDC medium supplemented with the specified concentration of ZnSO$_4$. Liquid cultures were inoculated at an $A_{600}$ of 0.05, and total cell growth following a 24-h incubation at 30 °C was determined by measuring OD at 600 nm ($A_{600}$). For A and B, the means of three independent experiments are shown; error bars indicate standard deviation.

FIG. 4. Total cellular iron and zinc content. Iron and zinc accumulation levels in the specified yeast strains (dark gray bar, WT; light gray bar, lys7Δ; white bar, sod1Δ; hatched bar, lys7Δsod1Δ) were measured by an inductively coupled plasma-atomic emission spectrometer as described under “Experimental Procedures.” Each value represents the standard error of the means of at least 15 independent measurements. (For WT, n = 16; for lys7Δ, n = 20; for sod1Δ, n = 15; for lys7Δsod1Δ, n = 16.) As has been observed previously, low concentrations of CuSO$_4$ (0.5 mM) enhanced the growth of sod1Δ and lys7Δ mutants, but higher concentrations were equally toxic to mutant and wild-
type cells. In medium supplemented with [Ni$^{2+}$] ranging from 0 to 1.0 mM, lys7Δ and sod1Δ demonstrate similar growth potential (Fig. 5B). Interestingly, low medium [Ni$^{2+}$] seemed to have a more pronounced effect on the growth of wild-type (EG103) yeast. It is possible that the weakened antioxidant capacity of lys7 and sod1 null mutants have caused a constitutive up-regulation of various cellular stress genes, thus render-

FIG. 5. Effect of excess medium [Cu$^{2+}$] and [Ni$^{2+}$] on growth. Growth of the indicated yeast strains (circle, WT; triangle, lys7Δ; square, sod1Δ; diamonds, lys7Δsod1Δ) in the EG103 background was tested in SDC medium supplemented with the indicated concentrations of either CuSO$_4$ (A) or NiSO$_4$ (B). Liquid cultures were seeded at an $A_{600}$ of 0.05, and total cell number was evaluated after a 24-h growth period at 30 °C. The means of 4 independent experiments are shown; the error bars indicate standard deviation.

As has been observed previously, low concentrations of CuSO$_4$ (0.5 mM) enhanced the growth of sod1Δ and lys7Δ mutants, but higher concentrations were equally toxic to mutant and wild-type cells. In medium supplemented with [Ni$^{2+}$] ranging from 0 to 1.0 mM, lys7Δ and sod1Δ demonstrate similar growth potential (Fig. 5B). Interestingly, low medium [Ni$^{2+}$] seemed to have a more pronounced effect on the growth of wild-type (EG103) yeast. It is possible that the weakened antioxidant capacity of lys7 and sod1 null mutants have caused a constitutive up-regulation of various cellular stress genes, thus render-

ing these cells more resistant to other types of environmental stresses. Overall, the experimental evidence depicted in Fig. 5 suggests that the ability of both lys7Δ and sod1Δ cells to sequester other transition metal ions probably remains intact on a gross level and that the sensitivity of sod1Δ mutants to excess metal ions is specific to zinc.

Replacement of CuZn-SOD with Bacterial Mn-SOD—To assess the relative contributions of the enzymatic superoxide dismutase activity and the zinc binding capacity of CuZn-SOD, we restored the superoxide resistance of sod1Δ yeast by over-expressing a bacterial Mn-SOD in their cytoplasm. Because Mn-SOD is expected to restore SOD activity but not zinc binding capacity in vivo, we were interested to see whether its expression would have any effect on the zinc sensitivity of the sod1Δ mutant.

The sod1 null mutant in the BY4741 background was transformed with either pSALSOD (pMn-SOD), a yeast expression vector containing the B. stearothermophilus Mn-SOD gene under the control of the GAL-CYC1 promoter, or pEMBLyex4 (pEMBL), the corresponding vector control (17). Growth of the sod1Δ/pMn-SOD strain in galactose resulted in the appearance of Mn-SOD activity and loss of the aerobic lysine auxotrophy (data not shown and Ref. 17). We tested the paraquat sensitivity of the plasmid-containing strains and found that sod1Δ/pMn-SOD cells behave very much like wild-type yeast, resisting paraquat concentrations as high as 1.0 mM (41.1 ± 11.4% control growth for sod1Δ/pMn-SOD compared with 52.8 ± 2.2% for wild type (WT)), whereas the sod1Δ/pEMBL strain was much more sensitive (Fig. 6A). Possibly because of the galactose in the medium, somewhat higher levels (0.2 and 0.5 mM) of paraquat are required to cause toxicity in the sod1Δ/pEMBL as compared with the non-plasmid-containing sod1Δ
strains. Nevertheless, we can conclude that the prokaryotic Mn-SOD efficiently strengthened the antioxidant capacity of the sod1Δ yeast as evidenced by greatly increased resistance to paraquat, a superoxide-generating chemical.

We then tested whether the transformed strains were resistant to zinc (Fig. 6B). For medium [Zn^{2+}] below 2.0 mM, all four strains behaved similarly (data not shown). Thus, higher concentrations of ZnSO_{4} than were previously used were required to evaluate the ability of these transformants to resist zinc toxicity, again possibly because of the use of galactose-based medium. As can be seen in Fig. 6B, expression of cytoplasmic Mn-SOD does not restore zinc resistance to wild-type levels, supporting a role for CuZn-SOD in zinc homeostasis.

Replacement of CuZn-SOD with a Mutant SOD1 Polypeptide, yH46C—In a converse experiment, to provide further mechanistic insight into the zinc sensitivity observed in sod1Δ cells, we overexpressed yH46C (a mutant SOD1 protein lacking catalytic activity but capable of binding metal ions) in sod1Δ yeast. Growth of the sod1Δ/yH46C strain was tested in medium supplemented with ZnSO_{4}. As shown in Fig. 7, sod1Δ/yH46C grew as well as sod1Δ/ySOD1 in the presence of 1.0 and 2.0 mM ZnSO_{4}, but sod1Δ/YEp351 (vector control) exhibited significant growth inhibition at those concentrations. Expression of this mutant SOD had no effect on the resistance to paraquat, confirming that it is catalytically inactive. (At a concentration of 25 μM paraquat, growth of sod1Δ/yH46C was just 2.1 ± 0.5% control growth.)

DISCUSSION

In this study, we demonstrate that certain differences exist between lys7Δ and sod1Δ yeast. lys7Δ yeast are not as sensitive to the redox-cycling drug paraquat as the sod1Δ mutant, although they are still much less resistant than the isogenic wild-type cells. In addition, lys7Δ cells exhibit wild-type levels of "free" iron as measured by Fe(III) EPR, whereas sod1Δ mutants display a 5-fold increase (Fig. 2). Moreover, the lys7Δ strain grows as well as wild type in elevated zinc, while the sod1Δ strain fails to thrive (Fig. 3). The increased zinc sensitivity of the sod1Δ strain may be due to the fact that it accumulates excess zinc relative to the wild-type and lys7Δ strains (Fig. 4). To explain the phenotypic differences between sod1Δ and lys7Δ yeast, two different (albeit not mutually exclusive) hypotheses have been considered. The first hypothesis is that the lys7Δ strain retains an extremely small amount of SOD activity, below the detection limit of currently available assays. The second is that the copper-free SOD1 protein, which is present in lys7Δ but not in the sod1Δ strain, plays a role in zinc metabolism either by acting as a depot for zinc ions or through some other mechanism. To distinguish among these possibilities, we performed experiments designed to separate SOD enzymatic activity from the zinc binding activity. We found that cytoplasmic expression of a bacterial Mn-SOD fully restored the paraquat resistance of sod1Δ strains but did not fully restore the zinc sensitivity (Fig. 6). Conversely, expression of yH46C, a mutant CuZn-SOD with no enzymatic activity, fully restored zinc resistance (Fig. 7) but had no effect on paraquat resistance.

The first hypothesis (that there may be a residual amount of SOD activity in lys7Δ yeast) is supported by the facts that the lys7Δ strains appear slightly more resistant to oxidative stress (e.g. paraquat resistance is a little higher in the lys7Δ mutants than in the sod1Δ strains) and that the zinc sensitivity of sod1Δ strains is only observed in aerobic growth. However, another possible explanation for these phenotypic differences is that paraquat resistance is conferred by superoxide dismutase activity plus some other antioxidant function of the SOD1 polypeptide. For example, inactive apoSOD1 polypeptide might be able to physically protect some vulnerable site(s). (If this is the case, the SOD activity is clearly the more important function because expression of active SOD in the cytoplasm increases paraquat resistance 100-fold or more, while expression of SOD1 made inactive by the absence of Lys7 only increases paraquat resistance a few fold.) The main evidence against the residual activity hypothesis is the fact that SOD activity is

![Fig. 6. Effect of expression of bacterial Mn-SOD on resistance of sod1Δ strains to paraquat and zinc. Growth of the indicated yeast strains (open circle, WT; open triangle, sod1Δ; closed circle, sod1Δ/mMnSOD; closed triangle, sod1Δ/pEMBL) in the BY4741 background was tested in SGA medium supplemented with the indicated concentrations of either paraquat (A) or ZnSO_{4} (B). Liquid cultures were inoculated at an A_{600} of 0.05 (A) or 0.07 (B), and total cell number was evaluated after growing at 30 °C with shaking at 200 rpm for 24 h. The means of at least 4 independent experiments are shown; error bars indicate standard deviation.](http://www.jbc.org/)

![Fig. 7. Effect of expression of yH46C, a catalytically inactive SOD1 point mutant, on zinc tolerance in sod1Δ yeast. Yeast strain EG118 (sod1Δ) was transformed with empty vector (YEp351), with a plasmid directing the expression of WT ySOD1 (YEp600), or with a plasmid directing the expression of H46C-SOD1, an enzymatically inactive mutant SOD1 protein. Growth was tested in the presence of various concentrations of ZnSO_{4} (black bars, 0 mM; gray bars, 1.0 mM; white bars, 2.0 mM). Liquid cultures inoculated at an A_{600} of 0.05 were grown for 24 h at 30 °C with shaking at 200 rpm. Total growth relative to that obtained in the absence of ZnSO_{4} (% control growth) was determined by measuring turbidity at A_{600}. Values are the means of 6 independent colonies; error bars indicate standard deviation.)](http://www.jbc.org/)
ions, including copper, nickel, and zinc (28, 29). Because vacuole function or acidification are sensitive to various metal stress component that is exacerbated when SOD activity is restored. This may indicate that zinc toxicity has an oxidative response experimentally.

Another level of complexity is that zinc resistance of the sod1Δ strain was somewhat improved by cytoplasmic expression of the heterologous Mn-SOD, although it was not fully restored. This may indicate that zinc toxicity has an oxidative stress component that is exacerbated when SOD activity is missing. It is possible that this is related to the differences in “free” iron shown in Fig. 2. Alternatively, it may simply be that a SOD-competent cell is better able to defend itself against other stresses or that cellular antioxidant defenses assist in managing disturbances in metal homeostasis.

Numerous studies have implicated the vacuole in metal ion storage and homeostasis (24–27), and mutants defective in vacuole function or acidification are sensitive to various metal ions, including copper, nickel, and zinc (28, 29). Because sod1Δ mutants display aberrant vacuolar morphology (22), the possibility remained that the zinc sensitivity was caused by vacuole malfunction in the sod1Δ yeast. We reasoned that if the zinc sensitivity in our strain was due to defective vacuolar function, the ability of the cells to handle elevated levels of transition metal ions other than zinc would be affected as well. The experimental results depicted in Fig. 5 clearly demonstrate that sod1Δ cells are as resistant as wild-type cells to increased levels of Cu²⁺ or Ni²⁺. That is, their ability to detoxify metal ions other than zinc remains intact, and the defect responsible for the zinc sensitivity in sod1Δ strains is not one of general vacuolar function. Taken together, these data strongly suggest that the copper-free SOD present in lys7Δ cells as well as the normally metallated CuZn-SOD present in WT cells participate in zinc ion homeostasis.

A relationship between zinc metabolism and iron metabolism is also possible. The zinc sensitivity of sod1Δ mutants may be an indirect consequence of their aberrant iron metabolism. Yeast sod1Δ mutants exhibit increased levels of “free” iron, which may also come from the destruction of vulnerable [Fe-S] clusters (7), and its accumulation may be exacerbated by the increased cellular transport of iron under conditions of superoxide stress (9). Because iron(II) and Zn(II) are similar chemically with respect to their coordination chemistry, they are transported by some of the same cellular transporters and can be bound by some of the same cellular proteins. Increased iron uptake may result in increased zinc uptake as well (30, 31). Thus, attempts by the sod1Δ yeast to increase iron may backfire in two ways: increased formation of hydroxyl radical due to increased Fenton chemistry (32) and the accumulation of toxic intracellular levels of zinc. Here we report that lys7Δ yeast, unlike sod1Δ yeast, do not exhibit altered iron metabolism as evidenced by their wild-type-like level of EPR-detectable “free” iron (Fig. 2), and they do not accumulate increased levels of zinc inside the cells. Further work will be required to determine whether increased levels of “free” iron and of zinc are a result of the same process or of different ones, but the relationship is intriguing.

In summary, our work has revealed unexpected differences between lys7Δ and sod1Δ mutant yeast that imply connections between superoxide toxicity, zinc metabolism, and iron metabolism. Our results indicate that the SOD1 polypeptide participates somehow in zinc homeostasis and that superoxide stress clearly involves iron metabolism. Continued investigations will shed more light on these relationships.

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