A Physiological Role for Saccharomyces cerevisiae Copper/Zinc Superoxide Dismutase in Copper Buffering*

Valeria Cizewski Culotta†, Hung-Dong Joh, Su-Ju Lin, Kimberly Hudak Slekar, and Jeffrey Strain

From the Division of Toxicological Sciences, Department of Environmental Health Sciences, Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland 21205

The copper toxicity of yeast lacking the CUP1 metallothionein is suppressed by overexpression of the CRS4 gene. We now demonstrate that CRS4 is equivalent to SOD1, encoding copper/zinc superoxide dismutase (SOD). While overexpression of SOD1 enhanced copper resistance, a deletion of SOD1, but not SOD2 (encoding manganese SOD), conferred an increased sensitivity toward copper. This role of SOD1 in copper buffering appears unrelated to its superoxide scavenging activity, since the enzyme protected against copper toxicity in anaerobic as well as aerobic conditions. The distinct roles of SOD1 in copper and oxygen radical homeostasis could also be separated genetically: the pmr1, bsd2, and ATX1 genes that suppress oxygen toxicity in sod1 mutants failed to suppress the copper sensitivity of these cells. The Saccharomyces cerevisiae SOD1 gene is transcriptionally induced by copper and the ACE1 transactivator, and we demonstrate here that this induction of SOD1 promotes protection against copper toxicity but is not needed for the SOD1 protection against oxygen free radicals. Collectively, these findings indicate that copper/zinc SOD functions in the homeostasis of copper via mechanisms distinct from superoxide scavenging.

The transition metal copper represents a paradox to living organisms because trace amounts of this ion are essential to promote growth, yet concentrated levels of this same metal can drastically impair cell growth and function. Although many hypotheses have been proposed to explain copper toxicity in biological systems, a popular model exploits the ability of the metal to participate in so-called Fenton or Haber-Weiss chemistry (1–3). In this model, copper ions would catalyze the conversion of hydrogen peroxide (H₂O₂) to the powerful oxidant hydroxyl radical (OH•), which has the capacity to damage cellular components. Alternatively, copper toxicity may also be mediated through the inappropriate binding of the metal to nitrogen, oxygen, and sulfur ligands in biomolecules, thereby inactivating enzymes and disrupting cellular function (4).

In order to balance the growth inhibitory and stimulatory effect of copper ions, all organisms have evolved with various metal homeostasis factors that properly control the cellular accumulation, distribution, and detoxification of the metal. A number of copper transporters have been identified that act in the passage of copper across external membranes or in the intracellular delivery of the metal to cellular stores. For example, copper-transporting ATPases have been identified in bacteria (5), yeast (6, 7) and man (8–11). All of these ATPases share a remarkable degree of homology, exemplifying the need to tightly conserve the homeostasis of essential copper ions. Non-ATPase copper transporters have also been identified in bacteria (12) and yeast (13), and these share a common metal binding motif.

As another means of maintaining copper ion homeostasis, many eukaryotic organisms contain one or more forms of a metal binding metallothionein that acts to chelate, sequester, and thereby detoxify copper ions. In the baker's yeast, the CUP1 copper-containing metallothionein (14–16) has been demonstrated to not only protect against copper but also to guard against the oxidative damage associated with superoxide anion (17). This metallothionein of Saccharomyces cerevisiae, and another encoded by the CRS5 gene (18), are known to be transcriptionally regulated by copper ions through the action of a copper and DNA binding trans-activator, ACE1 (18–22). Interestingly, the ACE1 copper sensor factor has been shown to also induce the S. cerevisiae SOD1 gene in response to elevated copper (23, 24). SOD1 encodes a copper- and zinc-containing superoxide dismutase (SOD),† and the rationale for the coinduction of this free radical detoxifying enzyme with the metallothioneins was not completely understood (23, 24). Nevertheless, these studies on S. cerevisiae CUP1 metallothionein and SOD have substantiated the notion that considerable overlap exists between systems controlling copper ion homeostasis and oxygen radical metabolism.

To identify additional factors involved in copper ion homeostasis, we developed a selection strategy to isolate S. cerevisiae genes that, when overexpressed, have the ability to suppress the copper toxicity associated with loss of the CUP1 metallothionein. This strategy led to the isolation of two genes, CRS4 and CRS5, that, when present in multi-copy, conferred a relatively high degree of copper resistance to yeast lacking CUP1. In our earlier studies, we discovered that CRS5 encodes an additional yeast metallothionein, only the second to be identified in S. cerevisiae (18). Expecting to uncover a third S. cerevisiae metallothionein, we have here sequenced and characterized the CRS4 gene. Surprisingly, CRS4 was found to be identical to the S. cerevisiae SOD1 gene, encoding copper/zinc superoxide dismutase. The biological role of this enzyme in copper buffering in yeast is discussed herein.

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† To whom correspondence should be addressed: Div. of Toxicological Sciences, Department of Environmental Health Sciences, Johns Hopkins University School of Hygiene and Public Health, 615 N. Wolfe St., Baltimore, MD 21205. Tel.: 410-955-3029; Fax: 410-955-0116.

† The abbreviations used are: SOD, superoxide dismutase; kb, kilobase(s); PCR, polymerase chain reaction.
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**TABLE I**

Yeast strains used in this study

| Strains   | Genotype       | Source |
|-----------|----------------|--------|
| VC-sp6    | MATa trp1-1 ura3-52 ade- his- CAN† gal1 leu2-3,112 cup1* | (18)   |
| SL1c      | MATa trp1-1 ura3-52 ade- his- CAN† gal1 leu2-3,112 mit13 cup1Δ::URA3 | (18)   |
| EG103     | MATa leu2-3 112 his311 trp-289a ura3-52 GAL† | (26)   |
| EG133     | MATa leu2-3 112 his311 trp-289a ura3-52 sod1Δ::URA3 sod2Δ::TRP1 GAL† | (26)   |
| EG110     | MATa leu2-3 112 his311 trp-289a ura3-52 sod2Δ::TRP1 GAL† | (26)   |
| KS101     | MATa leu2-3 112 his311 trp-289a ura3-52 GAL† sod1Δ::LEU2 | (26)   |
| KS107     | MATa leu2-3 112 his311 trp-289a ura3-52 sod1Δ::TRP1 | (26)   |
| PJK1P1-2  | MATa leu2-3 112 his311 trp-289a ura3-52 pmr1Δ::LEU2 GAL† | (27)   |
| XL103b2   | MATa leu2-3 112 his311 trp-289a ura3-52 sod1Δ::TRP1 GAL† pmr1Δ::LEU2 | (28)   |
| VC119     | MATa leu2-3 112 his311 trp-289a ura3-52 sod1Δ::TRP1 GAL† bsd2Δ::LEU2 | (28)   |
| VC104     | MATa leu2-3 112 his311 trp-289a ura3-52 sod1Δ::TRP1 acet1Δ::URA3 | (29)   |
| VC121     | MATa leu2-3 112 his311 trp-289a sod1Δ::TRP1 Gal1 acet1Δ::URA3 | (29)   |

**EXPERIMENTAL PROCEDURES**

Yeast Strains, Media, and Growth Conditions—The *S. cerevisiae* strains utilized in these studies are listed in Table I. Strain 51.2C represents a cup1Δ yeast strain (18), and VC-sp6 is a cup1Δ strain containing a single chromosomal copy of CUP1 (18). The isogenic strains EG103 (SOD wild type) and EG110 (SOD mutant) were obtained by transformation of the Apal-Sad fragment from pKS1 into the Apal and Sad sites of prS540. To delete the chromosomal SOD1 gene, the pKS1 or pKS2 plasmid was linearized with PstI and used to transform haploid leu2 or trp1 yeast, respectively, by electroporation. Correct deletion of SOD1 sequences +18 to +314 was confirmed using PCR.

Identification of CRS4 as SOD1 was accomplished by sequencing both strands of p28hp and p28sas using the Sequenase method (U. S. Biochemical Corp.) modified for double-stranded DNA. Sequence information was stored by the DNA Strider software package and upon a search of the N.C.B.I data base, CRS4 was found to encompass the *S. cerevisiae* SOD1 and URA8 open reading frames. Northern and Southern blot analyses were conducted as described previously (33).

**RESULTS**

Suppression of Copper Toxicity by the *S. cerevisiae* CRS4 Gene—Copper toxicity in yeast cells lacking a functional CUP1 metallothionein can be suppressed by overexpression of the *S. cerevisiae* CRS4 gene (Copper-resistant suppressor) gene (18). As shown in Fig. 1, yeast lacking CUP1 metallothionein (cup1Δ) were unable to grow on plates containing 25 \( \mu \)M copper whether cultured aerobically or anaerobically. When the cup1Δ yeast strain was transformed with either the multi-copy CRS4 or CRS5 gene, cells exhibited a strong resistance to 25 \( \mu \)M copper under both aerobic and anaerobic conditions (Fig. 1). Hence, overexpression of CRS4 or CRS5 can alleviate copper toxicity in cup1Δ yeast in a manner that is not dependent upon atmospheric oxygen.

To examine the extent to which CRS4 protects cells from copper toxicity, a liquid culture test was utilized to obtain measurements of the mean inhibitory concentration for copper (concentration required to inhibit total cell growth by 50%). On the YeP13 yeast epimisal plasmid, CRS4 is amplified to 5–10 copies/cell (Southern blot analyses, not shown), and in cup1Δ yeast, this elevation in CRS4 gene dosage increased the mean inhibitory concentration for copper by approximately 400% (Fig. 2A). To test whether this increased resistance to copper is specific to cup1Δ strains, we also assayed for CRS4 function in a cup1Δ strain harboring a single copy of the metallothionein gene. As shown in Fig. 2B, these strains are normally more resistant to copper toxicity than are cup1Δ yeast, and this resistance was increased further by overexpression of CRS4; however, the CRS4-containing plasmid only increased the mean inhibitory concentration by approximately 35% in cup1Δ cells (Fig. 2B). The apparent nonadditive effects of CRS4 and CUP1 suggested that these two genes may play functionally redundant roles in copper detoxification.

Identification of CRS4 as the *S. cerevisiae* SOD1 Gene—Two identical isolates of CRS4 (p12 and p28) were obtained from the genomic library, and these comprise a 6.0-kb fragment of
genomic DNA (Fig. 3). To identify the CRS4 gene in this fragment, segments of p28 were sub-cloned and tested for the ability to confer copper resistance. A 1.5-kb Sphl-HpaI segment of this DNA was found to suppress copper toxicity in yeast lacking CUP1 (Fig. 3). Sequence analyses of this region revealed the presence of two previously cloned genes, the S. cerevisiae URA8 (34) and SOD1 (35) loci. Of these, isolated sequences of SOD1 were found to be necessary and sufficient to suppress the copper toxicity of cup1Δ yeast (Fig. 3), demonstrating that CRS4 is equivalent to SOD1.

The S. cerevisiae SOD1 gene (35) encodes a copper and zinc containing SOD that is known to scavenge superoxide anion (O2−) radicals and is thought to play a critical role in protecting cells against oxidative damage. Yeast strains containing mutations in the SOD1 gene exhibit a number of aerobic defects including a deficiency in lysine biosynthesis and in sulfur metabolism and a sensitivity toward atmospheric oxygen and paraquat, a O2−-generating agent (25, 26, 35–39). All of these defects are dependent upon the presence of oxygen, indicating that SOD plays a pivotal role in protecting cellular constituents against oxygen-related toxicity.

To explore the role of SOD1 in copper buffering, we compared the growth of a sod1Δ mutant lacking the endogenous SOD1 gene to that of a SOD1 wild-type strain. Resistance to copper was also tested in a sod2Δ mutant lacking the mitochondrial manganese containing SOD and a sod1Δ sod2Δ double mutant strain lacking both forms of SOD. The strains utilized for these studies also contain a wild-type tandemly amplified CUP1 locus and are considerably more resistant to copper toxicity than are cup1Δ and cup1Δ yeast strains. As shown in Fig. 4, the strains containing a sod1Δ single or sod1Δ sod2Δ double mutation exhibited an increased inhibition of growth relative to wild-type yeast in 60 μM copper medium, and this increased sensitivity toward copper was not observed with sod2Δ single mutants.

In comparison, strains containing a mutation in SOD2 exhibited an elevated sensitivity toward 30 μM cadmium that was not observed with sod1Δ mutants (Fig. 4). In other studies, the sod1Δ and sod2Δ mutants consistently exhibited impaired growth relative to wild-type strains at 30–90 μM CuSO4 and 5–50 μM CdCl2, respectively (not shown). The increased copper sensitivity of sod1Δ mutants was also observed with anaerobically grown cells (not shown), consistent with our observation that overexpression of SOD1 suppressed copper toxicity in both aerobic and anaerobic conditions (Fig. 1). Thus the SOD1 protein appears to play a role in copper buffering in addition to oxygen radical scavenging.

Copper Toxicity and the Genetic Suppressors of SOD1 Deficiency—We have previously shown that the oxygen-dependent defects of sod1Δ mutants (lysine and methionine auxotrophies, sensitivity toward atmospheric oxygen and paraquat) can be suppressed by alterations in the expression of three nuclear genes, ATX1 (40), PMR1 (27), and BSD2 (28). Overexpression of the ATX1 gene, or mutational inactivation of either PMR1 or BSD2, will suppress to some degree all of the aerobic defects of yeast containing a sod1Δ mutation. In the present studies, we have tested whether these suppressors of oxygen toxicity can also reverse the copper sensitivity associated with loss of the copper/zinc SOD. These experiments would additionally address the potential overlap between the apparent roles of SOD1 in copper buffering and in oxygen radical detoxification.

As shown in Fig. 5A, sod1Δ mutants still exhibited a striking sensitivity toward copper toxicity when the anti-oxidant gene ATX1 was overexpressed. Furthermore, a pmr1 mutation was also incapable of reversing the copper toxicity of sod1Δ cells, since the sod1Δ pmr1Δ mutant still exhibited impaired growth in 30–70 μM concentrations of copper compared with SOD1 wild-type yeast containing a pmr1Δ mutation (Fig. 5B). A mutation in BSD2 was likewise ineffective in suppressing the
copper sensitivity associated with loss of SOD1 (Fig. 5C). BSD2 gene mutations are themselves associated with an increase in sensitivity toward copper (28), and an additive effect on copper toxicity was observed with the combination of bsd2 and sod1 mutations (Fig. 5C). Hence, perturbations in the function of ATX1, PMR1, and BSD2 can suppress only the oxidative defects and not the copper toxicity of sod1 mutants. These results provide genetic evidence that SOD1 plays separate physiological roles in copper ion buffering and oxygen radical detoxification.

The Role of ACE1 in SOD1-mediated Protection against Copper—It has previously been demonstrated by Tamai et al. (17) and by Ciriolo et al. (42) that the S. cerevisiae SOD1 gene is positively regulated at the level of transcription by copper ions and ACE1, the same copper sensor trans-activator that regulates the yeast CRS5 and CUP1 metallothionein genes (23, 24). The rationale for this curious co-regulation of SOD1 and the metallothioneins by copper was not well established (23, 24). In the present studies, we utilized two approaches to test whether the induction of SOD1 by copper ions is essential to its role in protecting yeast against the toxicity of oxygen radicals and/or copper. In the first line of study, the ACE1 gene was deleted in the background of both sod1Δ and SOD1 wild-type yeast, and cells were tested for sensitivity toward paraquat (a generator of O2·−) and copper. As shown in Fig. 6A, SOD1 wild-type cells exhibited a striking resistance toward paraquat over sod1Δ yeast, and this SOD1-mediated protection against free radical damage was not altered in strains containing an ACE1 gene deletion. In contrast, the functional SOD1 gene did not efficiently protect against copper toxicity in strains lacking ACE1 (Fig. 6B). This finding indicates that the ACE1 factor is needed for the SOD1 protection against copper but not oxygen free radicals.

We also tested the significance of ACE1 in SOD1 function by inactivating the ACE1 binding site in the SOD1 promoter. A pair of 2-μm-containing constructs were made that contain

**Fig. 3. Identification of the CRS4 gene.** Plasmid p28 was isolated from a yeast genomic YEp13 library as a clone that confers copper resistance to cup1Δ yeast. The remaining constructs represent fragments of the p28 insert subcloned into the 2-μm yeast vectors YEp13, pRS423, or pRS425 as described under “Experimental Procedures.” The constructs were used to transform strain 51.2c, and all were found to support growth of this cup1Δ strain on minimal medium containing 25 μM CuSO4 (as in Fig. 1). Positions and directions of the SOD1 and URA8 open reading frames are indicated. Arrows on the pVC736 fragment indicate primers used for PCR amplification. RI, EcoRI; Sp, SphI; S1, Sad; H1, Hpal.

**Fig. 4. Effects of sod1Δ and sod2Δ gene deletions on tolerance to heavy metals.** The indicated strains of yeast were cultured as described in Fig. 2 in minimal medium that was either untreated (control) or supplemented with 60 μM CuSO4 (copper) or 30 μM CdCl2 (cadmium). Total growth was measured as in Fig. 2, and results represent the averages of two to four independent trials (the complete range of values obtained are represented by error bars). Strains tested are as follows: SOD1+, EG103; sod1Δ, KS101; sod2Δ, EG110; sod1Δ sod2Δ, KS100. All strains contain an amplified CUP1 locus and normally exhibit a high level of copper resistance.

**Fig. 5. Suppressors of oxidative damage and the effect on copper toxicity in sod1Δ yeast.** The indicated yeast strains were cultured in liquid minimal medium supplemented with the indicated concentrations of CuSO4 as in Fig. 4. % Control Growth was obtained by dividing the A600 value obtained with copper-treated yeast over that of control untreated cells. Strains of yeast utilized are as follows: A, sod1Δ-pATX1, KSI07 transformed with the p18 construct harboring ATX1 (40); SOD1-ATX1, EG103 transformed with p18; B, sod1Δ pmr1Δ, VC119; SOD1-ATX1, EG103 transformed with p18; B, sod1Δ pmr1Δ, VC119; C, sod1Δ bsd2Δ, VC120; SOD1+ bsd2Δ, XL103Δbsd2Δ. All strains contain an amplified CUP1 locus. Results represent averages of two to three independent trials where range ±10%. In the various experiments, control untreated cells grew to an A600 of 2.5–4.0.

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Fig. 6. Paraquat and copper toxicity in yeast lacking SOD1 and ACE1. The indicated strains of yeast were cultured in a minimal medium supplemented with the indicated concentrations of either paraquat (A) or CuSO4 (B), and total cell growth was measured as in Figs. 4–5. % Control Growth was obtained by dividing the A600 value obtained with paraquat treated yeast over that of control untreated cells. The strains utilized are as follows: SOD+, EG103; sod1Δ, KS105; SOD+ ace1Δ, VC104; sod1Δ ace1Δ, VC121. All strains contain an amplified CUP1 locus; the ace1Δ strains exhibit a striking copper sensitivity due to the loss of CUP1 gene induction by copper.

SOD1 sequences –257 to +586, with respect to the translational start site. These constructs, pVC733 and pVC736, were identical, except pVC733 contained a CGC to TAT substitution made in pVC733. Both plasmids were used to transform a sod1Δ strain and were monitored for copper induction by Northern blot analysis. As shown in Fig. 7A, the two SOD1 constructs were expressed to equal degrees in the absence of copper; however, only the pVC736 construct containing the wild-type ACE1 binding site responded to copper through induction of SOD1 mRNA. To test whether this increase in SOD1 mRNA is essential to its role in buffering oxygen radicals versus metals, sod1Δ strains harboring the pVC733 mutated, and the pVC736 wild-type constructs were tested for sensitivity to both oxygen and copper. Lysine auxotrophy is an excellent marker for oxygen toxicity in yeast lacking copper/zinc SOD (26, 35, 36, 38, 39), and both pVC733 and pVC736 were fully capable of complementing the lysine auxotrophy of sod1Δ cells (not shown). The wild-type and mutant constructs were also equally effective in complementing the paraquat sensitivity of sod1Δ yeast (Fig. 7B). Hence, the ACE1 binding site does not appear to impact on the ability of SOD1 to protect against oxidative damage. However, in contrast to results obtained with paraquat (Fig. 7B), the pVC736 wild-type construct was substantially more effective in complementing the copper sensitivity of sod1Δ yeast than was the pVC733 mutant construct, and this result was observed in the background of both sod1Δ and sod1Δ sod2Δ yeast (Fig. 7C). Identical experiments were conducted with the same SOD1 wild-type and mutant constructs expressed on a yeast CEN vector, and although the CEN constructs were less efficient at complementing the sod1Δ mutation, the same qualitative results were obtained; the wild-type SOD1 construct was considerably more effective in suppressing copper toxicity (data not shown). Together with the studies on ace1Δ yeast (Fig. 6), these findings strongly indicate that the role of SOD1 in copper buffering, but not in oxygen radical detoxification, is dependent upon the ACE1 trans-activator and a functional ACE1 binding site in the SOD1 promoter.
DISCUSSION

The studies presented here strongly suggest that copper/zinc SOD serves a physiological role in copper ion buffering. We demonstrate that the S. cerevisiae SOD1 gene can act as a multicopy suppressor of copper toxicity. SOD1 was only one of two loci found to have the capacity to suppress the strong copper sensitivity associated with loss of the S. cerevisiae CUP1 metallothionein. The other, CSR5, encodes an additional metallothionein (18). SOD1 not only protects against copper toxicity as a multicopy locus, but the single copy chromosomal gene also participates in copper buffering since a sod1Δ gene deletion was associated with an increased sensitivity toward copper. Of the two forms of SOD, copper buffering appears specific to the copper/zinc containing enzyme; a null mutation in the SOD2 gene encoding the mitochondrial manganese SOD was not associated with an increased sensitivity toward copper.

We propose several lines of evidence that the apparent role of copper/zinc SOD in copper buffering is distinct and perhaps unrelated to the well established role of this enzyme in free radical detoxification. First, the previously reported defects associated with loss of S. cerevisiae SOD1 (aerobic growth inhibition, methionine and lysine auxotrophy, increased mutation rate) were absolutely dependent on the presence of oxygen (25, 28, 35–39). In contrast, the copper sensitivity associated with sod1Δ mutants was observed independent of atmospheric oxygen. The roles of SOD1 in copper and oxygen radical homeostasis can also be separated at the genetic level. A mutation in the PMR1 or BSD2 genes or overexpression of ATX1 will suppress the oxygen-related defects associated with loss of SOD1 function (26–28, 40); however, these same genetic perturbations failed to overcome the increased copper sensitivity of sod1Δ mutants. We initially found this result surprising, since all three of these suppressors are themselves involved in copper homeostasis. PMR1 encodes a P-type ATPase homologue that is believed to function in the delivery of manganese, copper, and calcium ions into the Golgi (27); BSD2 is involved in the homeostasis of copper and cadmium (28), and ATX1 appears to encode a small copper carrier (40). Evidently these suppressors influence the local concentration and availability of redox active transition metals but do not offer any metal buffering function.

It has been proposed that SOD can circumvent the Haber-Weiss reaction (the two-step copper or iron catalyzed production of OH−) by scavenging the O2•− that would normally provide scavenging activity (45–50). The SOD1 protein also participates in copper buffering since a sod1Δ deletion and with SOD1 genes lacking a functional ACE1 binding site. In contrast, the functional ACE1 factor and ACE1 binding sequences were found to be necessary for the protection against copper toxicity afforded by SOD1. We therefore propose that the constitutive level of copper/zinc SOD is sufficient to protect cells against oxidative damage by efficiently scavenging O2•−. However, when exposed to toxic copper levels, the cell responds by further increasing SOD1 levels. This elevation in SOD1, together with the induction of the metallothioneins, provides an effective method for metal buffering.

It is noteworthy that mutations in the human SOD1 gene have been associated with a familial form of amyotrophic lateral sclerosis (FALS), a fatal neurodegenerative disorder affecting the motor neurons (44). All FALS mutations are dominant, and in certain instances, the disease cannot be explained simply by a loss of SOD1 O2•− scavenging activity (45–50). The apparent role of copper/zinc SOD in copper buffering may in fact have important implications with regard to the etiology of FALS (47).

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