Purification and Characterization of Ag,Zn-Superoxide Dismutase from *Saccharomyces cerevisiae* Exposed to Silver*

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Cu,Zn-superoxide dismutase plays an important role in protecting cells from oxygen toxicity by catalyzing the dismutation of superoxide anion into hydrogen peroxide and oxygen. In *Saccharomyces cerevisiae* Cu,Zn-superoxide dismutase is coregulated with copper-thionine by copper via the transcription factor ACE 1. We demonstrate here that presence of AgNO₃ in the culture medium leads to a five times increase of Cu,Zn-superoxide dismutase mRNA, with a concomitant six times decrease of the enzyme activity. Susceptibility of yeast to silver was apparently inversely related to Cu,Zn-superoxide dismutase activity. From silver-treated yeast a Cu,Zn-superoxide dismutase with impaired dismutation function was purified and was shown to contain silver, which was located to the copper site. These data suggest that Cu,Zn-superoxide dismutase may play an additional direct role in the defense of *S. cerevisiae* against metal stress by functioning as metal chelator.

The yeast *Saccharomyces cerevisiae* contains two species of superoxide dismutase (EC 1.15.11), i.e. the copper, zinc-, Cu,Zn-SOD [and the manganese-containing forms. The former one is localized in the cytosol while the manganese enzyme is restricted to the mitochondrial matrix. The three-dimensional structure and the mechanism of action of these enzymes are well characterized (1, 2), but their biological properties are still a matter of debate.

It has been demonstrated that these enzymes play an important role in protection against damage related to O₂ toxicity. In fact, mutants that are defective in either Cu,Zn- or manganese-SOD are not able to grow in the presence of increased oxygen concentration or of compounds that generate oxygen radical intermediates (3, 4). Both in yeast and in *Escherichia coli* manganese-SOD is able to change its level in the cell, depending on oxygen concentration of the medium (5, 6) or on the exposure to redox active molecules in the presence of oxygen (7, 8). In yeast several results indicate that Cu,Zn-SOD responds in a similar fashion to such stimuli (9, 10), in line with its proposed role as a major antioxidative enzyme of the cell.

In *S. cerevisiae* Cu,Zn-SOD and manganese-SOD behave differently in response to other stress factors such as exposure to copper (11). Cu,Zn-SOD has been reported to increase its activity upon changes of the copper concentration in the medium, whereas manganese-SOD does not. The effects on Cu,Zn-SOD cannot be explained only on the basis of oxygen-dependent redox cycling of the metal ion because a copper-dependent increase of Cu,Zn-SOD is observed also under anaerobic conditions (12).

The presence of copper at the catalytic site of Cu,Zn-SOD suggests that copper availability limits the enzymatic activity, thus exerting a regulation at the post-transcriptional level. This is the case of anaerobic cultures of *S. cerevisiae* where the presence of a copper-free proenzyme requiring copper for activation has been demonstrated (12). Furthermore, copper can modulate yeast Cu,Zn-SOD at the transcriptional level via ACE 1 (13), a transcription factor which, upon binding of Cu(I), activates the promoter of CUP 1, the gene coding for copper-thionine (14, 15). An ACE 1-binding site has been localized in the Cu,Zn-SOD promoter (13), and its ability to bind ACE 1 in vitro has been demonstrated (16).

These data point to a possible role of Cu,Zn-SOD in sharing a copper-sequestering function with metallothionein in yeast. To further investigate this aspect, we have studied the effects of exposure to silver on yeast Cu,Zn-SOD. Ag(I) has physicochemical and electronic properties similar to copper (17), but it is not capable of generating reactive oxygen species by redox cycling. Moreover, silver has been shown to be able to induce metallothionein via the ACE 1 factor to a comparable extent as copper (13, 18).

MATERIALS AND METHODS

**Chemicals**—Silver nitrate was purchased by Aldrich. Yeast extract was obtained from Difco. DE32 was from Whatman. Diphenylthiocarbazone was from Sigma.

Chelex 100, low molecular weight standard, and goat anti-rabbit horseradish peroxidase conjugate immunoblotting kit were obtained from Bio-Rad.

All other materials were of reagent grade and were obtained from the best commercial sources available.

**Organism and Media**—A wild type of *D. 273–10B* strain of *S. cerevisiae* was grown in a basal medium containing 1% yeast extract, 1% *NH₄*Cl, 0.03% *MgSO₄* 0.09% KH₅PO₄, 0.22% KH₂PO₄, 0.1% peptone. 0.5% glucose was added as carbon source. The copper concentration of the basal medium was 4 μM.

**Growth Conditions**—A culture grown overnight in basal medium was used as the inoculum. Cultures were grown aerobically in a rotatory shaker (Orbit Environ Shaker, LabLine Instruments) at 30 °C and 180 revolutions/min, with a flask volume/medium volume ratio of 4:1.

Ag(I) was supplied as silver nitrate to reach final concentrations of 25, 50, 100, and 200 μM from filter sterilized solution (Millipore GV 0.22-μm filter unit, Millipore Corp., Bedford, MA) freshly made each time. As control yeast were also grown in the presence of 50 μM NaNO₃.

Cells were harvested from yeast cultures grown in the late exponential phase by centrifugation at 4 °C for 20 min at 2,500 × g. Cells were washed twice with ice-cold distilled water and stored at −20 °C until needed. Cellular extracts from each culture were prepared from wet cells by vortexing (30 s for five times with chilling between passes) in the presence of glass beads. The debris and the intact cells were removed by centrifugation at 23,000 × g for 30 min.

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1 The abbreviations used are: SOD, superoxide dismutase; CUP, gene coding for copper-thionine; ACE 1, activator of CUP 1 expression; PAGE, polyacrylamide gel electrophoresis.
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FIG. 1. Growth curves of *S. cerevisiae* in the absence or in the presence of AgNO₃. ○, untreated; ▲, treated with 25 μM AgNO₃; ▼, treated with 50 μM AgNO₃.

Purification of Cu,Zn-Superoxide Dismutase from Silver-treated Yeast—Cu,Zn-SOD was purified from 500 g of yeast cells by the method of Gocsim and Fridovich (19) with some modifications. The chromatographic step was modified utilizing a linear gradient of potassium phosphate (2.5-150 mM) at pH 7.8. The enzyme was eluted at a ionic strength between 40 and 60 mM buffer as four distinct peaks, instead of 20 mM as a single peak (19). They were all active and were pooled, concentrated, and subsequently dialyzed overnight against 20 mM Tris-HCl, pH 7.8. The sample was then applied onto a Hidrol Sepharose Q 16/10 column from Pharmacia LKB Biotechnology Inc. equilibrated with 20 mM Tris-HCl, pH 7.8, and eluted with a linear NaCl gradient (0-200 mM). Fractions containing SOD activity were pooled and concentrated. Fast protein liquid chromatography was performed with a Pharmacia liquid chromatography unit.

SOD activity was measured by a polarographic method (20) with an AMEL model 466 polarographic analyzer. The determinations were conducted with sodium tetraborate buffer at pH 9.8 which allows measurement only of the Cu,Zn isoform (21). Data were expressed in arbitrary densitometric units, expressing normalized areas of integrated peaks from densitometric scanning.

Electrophoresis and Immunoblotting—Polyacrylamide gel electrophoresis was performed in a slab gel system from Bio-Rad. 7.5% acrylamide non-denaturing gels (25) were stained with Coomassie Blue R-250 and transferred in a vial with 0.5 ml of 6 M HNO₃ and hydrolyzed for 16 h at room temperature.

For zinc determination, the samples were previously treated with 0.3% diphenylcarbazone in carbon tetrachloride to remove spurious zinc (30).

RESULTS

Effect of Silver on Cell Growth—Treatment of *S. cerevisiae* with AgNO₃ produced no significant change in the growth rate or cell morphology when silver concentration into the growth medium was kept below 25 μM. When cells were grown in the presence of 25 or 50 μM silver the time required to reach the stationary phase increased from 10 h, observed for *S. cerevisiae* grown in standard conditions, to 22 and 30 h, respectively (Fig. 1). Moreover, cell growth was completely inhibited at concentrations of AgNO₃ higher than 50 μM. Resistance of yeast to silver was apparently related to Cu,Zn-superoxide dismutase activity. In fact, we found a high positive correlation between growth yield and enzyme activity (Fig. 2). Ag(I) toxicity was found to be related to the reducing power of the medium. For this reason, to obtain reproducible growth curves, we added the metal at a fixed time after medium sterilization. The effects observed in the presence of AgNO₃ were due to the Ag⁺ ions. In fact the growth curve and the Cu,Zn-SOD activity of yeast grown in the presence of 50 μM NaNO₃ were identical to those of control cells (not shown).

Copper content of the control cells was 0.27 ± 0.07 nmol/10⁸ cells. Silver content of acid-digested cells treated with 50 μM AgNO₃ was 0.14 ± 0.06 nmol/10⁸ cells (the value is a mean of five determinations, each one done in triplicate). This metal fraction was not removed by washing the cells either with metal chelators or with acid and was assumed to be intracel-
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Table I

Cu,Zn-superoxide dismutase in S. cerevisiae grown in the presence of AgNO3

Values reported are means ± S.D. of at least eight different yeast cultures. The mRNA values are expressed in arbitrary units from densitometric evaluation of Northern blots.

| Activity | Activity after copper addition to cell extracts | Immunoactive protein | mRNA |
|----------|-----------------------------------------------|----------------------|------|
|          | µg/mg                                          | units/10^6 cells     |      |
| Control  | 1.03 ± 0.12                                    | 1.05 ± 0.15          | 1.33 ± 0.30 |
| + Ag⁺ (50 µM) | 0.16 ± 0.09                                        | 0.47 ± 0.11          | 7.13 ± 3.02 |

Fig. 3. Native gel electrophoresis of extracts of S. cerevisiae. PAGE was stained for SOD activity. Lanes 1 and 2, purified yeast Cu,Zn-SOD, 0.5 and 0.05 µg, respectively; lanes 3 and 4, Cu,Zn-SOD from yeast treated with 50 µM AgNO3, 200 µg of total protein; lane 5, Cu,Zn-SOD from untreated yeast, 200 µg of total protein. A, in the presence of 2 mM CN⁻; B, in the absence of 2 mM CN⁻.

Fig. 4. Native gel electrophoresis and SDS-PAGE of purified Cu,Zn-SOD from S. cerevisiae. A, activity stained gel; B, Coomassie-stained gel. Lanes 1 and 3, purified Cu,Zn-SOD from yeast treated with 50 µM AgNO3, 2.5 and 10 µg, respectively; lanes 2 and 4, purified Cu,Zn-SOD from untreated yeast, 0.3 and 8.0 µg, respectively.

Fig. 5. Isoelectrofocusing of extract and purified Cu,Zn-SOD of S. cerevisiae. Activity stained gel; lane A, purified bovine Cu,Zn-SOD (pI = 4.8); lane B, purified yeast Cu,Zn-SOD (pI = 4.5), 0.2 µg; lane C, purified Cu,Zn-SOD from yeast treated with 50 µM AgNO3, 1.0 µg; lane D, extract from yeast treated with 50 µM AgNO3, 100 µg of total protein.
treated cells was similar to that of the enzyme purified from untreated cells. A lower \( k_{\text{cat}} \) was observed for the silver-containing protein, which was, however, identical to that of the native protein if expressed on the basis of the copper content (Table II). This suggests that activity is due to the presence of residual native enzyme molecules. Accordingly, the EPR spectrum of the silver-copper, zinc protein was identical to that of Cu,Zn-superoxide dismutase purified from untreated yeast (Fig. 6), although the copper signal appeared much lower than what expected on a copper/protein molar ratio 2:1, namely close to 0.4:1.

**DISCUSSION**

Ag(I) is a potent inhibitor of growth and fermentation in *S. cerevisiae*, with massive release of intracellular K+ occurring because of irreversible damage to the structure and integrity of the plasma membrane (31, 32). Under our conditions, however, *S. cerevisiae*, displayed silver tolerance even though a lag phase in growth curve was observed. Interestingly, growth yield in the presence of silver was strictly related to superoxide dismutase activity, confirming that the enzyme is necessary to yeast for proper growing in the presence of oxygen.

However, direct effects of silver on Cu,Zn-SOD (in the absence of concomitant changes of manganese-SOD activity) were observed, and they were shown to operate at different levels. In fact, we were able to detect a specific increase in the Cu,Zn-SOD mRNA in response to silver ions, confirming that silver, like copper, can regulate Cu,Zn-SOD at the transcriptional level (17). On the other hand, a marked decrease in the enzyme activity with a concomitant slight decrease in the immunoreactive protein was observed. However, the synthesized protein is not able to perform a fully efficient catalytic function. This result was explained by the purification and characterization of a silver-containing SOD with impaired dismutase function due to the presence of silver in place of copper.

The results of analytical, spectroscopic, and catalytic determinations indicate that the majority of the protein molecules are Ag(I)-Zn-superoxide dismutase with Ag(I) most likely to be located to the copper site. This preferential binding site is in line with *in vitro* binding experiments showing that silver binds to bovine Cu,Zn-SOD with a higher affinity for the copper site than for the zinc site, and that the occupancy by zinc of the native zinc site enhances the binding of silver to the copper site (17).

As reported above, several isoforms were observed in SOD purified from silver-treated yeast. Those isoforms showed different behaviors in isoelectrofocusing and in non-denaturing acrylamide gel electrophoresis; such heterogeneity most probably reflects small differences in both net charge and overall folding, indicating a variable metal content of the various isoenzymes. The latter conclusion is supported also by the observation of a substoichiometric copper content determined in the protein purified from silver-treated yeast.

It is also interesting to note the higher antigenicity displayed by the Cu,Zn-SOD as compared either to the silver derivative obtained from silver-treated yeast or to *in vitro* prepared Ag,Zn-

![MAGNETIC FIELD (Tesla)](https://example.com/magnetic_field.png)

**FIG. 6.** Electron spin resonance spectra of purified Cu,Zn-SOD from *S. cerevisiae*: grown in the absence (b) and in the presence of 50 μM AgNO₃ (a). EPR conditions: modulation amplitude, 10 G; microwave frequency, 9.11 GHz; microwave power, 20 milliwatts; temperature, 77 K. a, 0.08 mM purified Ag,SOD; b, 0.46 mM purified Cu,SOD. Copper concentrations were 0.266 and 0.86 mM, respectively, as estimated by double integration of the spectra versus a Cu-EDTA standard.

SOD. This fact may be linked either to alteration of protein folding or to conformational masking of immunogenic regions due to the presence of silver. As a matter of fact, an altered immunoprecipitating activity respect to the metal content of the Cu,Zn-SOD was previously observed (33).

On the basis of these data, it is appealing to speculate that Cu,Zn-superoxide dismutase in *S. cerevisiae*, being an abundant, stable, high-affinity metal-binding protein, may play a role in the homeostasis of copper and other metal ions which are harmful to yeast. This role would be complementary to that of metallothionein, which is coregulated with Cu,Zn-superoxide dismutase in *S. cerevisiae* (13, 16).

A comparable situation is found in *E. coli* where the genes for iron- and manganese-superoxide dismutases are regulated by the iron responsive fur repressor protein. Furthermore, iron modulates the activities of both iron-SOD and manganese-SOD at the transcriptional and at the post-translational levels (34–36).

The physiological benefit of this type of regulation may reside in an ancestral function of the superoxide dismutase as a metal-binding protein, which develops a dismutase function when copper is bound at the solvent-accessible metal-binding site and oxygen is available.

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**TABLE II**

| Sample          | \( k_{\text{cat}} \) (M⁻¹ s⁻¹) | \( k_{\text{cat}}/\text{copper} \) (M⁻¹ s⁻¹) | \( k_{\text{cat}}/\text{copper} \) (M⁻¹ s⁻¹) | \( k_{\text{cat}}/\text{copper} \) (M⁻¹ s⁻¹) |
|-----------------|-------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|
| Control         | (1.71 ± 0.12) x 10⁶           | 0.85 ± 0.09 x 10⁶                           | 1.8 ± 0.3                                   | ND                                         |
| + Ag(1) (50 μM) | (0.30 ± 0.03) x 10⁶           | 0.80 ± 0.11 x 10⁶                           | 0.4 ± 0.1                                   | 1.5 ± 0.1                                  | 2.1 ± 0.1 |

* a ND, not detectable.
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