Intracellular Mn(II)-associated Superoxide Scavenging Activity Protects Cu,Zn Superoxide Dismutase-deficient Saccharomyces cerevisiae against Dioxygen Stress*

Eric C. Chang and Daniel J. Kosman†

From the Department of Biochemistry, School of Medicine and Biomedical Sciences, State University of New York, Buffalo, New York 14214

Three Cu,Zn superoxide dismutase (SOD-1)-deficient Saccharomyces cerevisiae mutants do not grow in 100% O2 in rich medium and require Met and Lys when grown in air (Bilinski, T., Krawiec, Z., Liezmannski, A., and Litwinska, J. (1985) Biochem. Biophys. Res. Commun. 130, 533–539). We show herein that medium manganese(II) accumulated by the mutants rescues these O2-sensitive phenotypes; 2 mM medium Mn2+ represented the threshold required for cell growth. The accumulation of Mn2+ was not oxygen-inducible since mutants grown aerobically and anaerobically accumulated the same amount of Mn2+. Mn2+ accumulation is not unique to these mutants since wild type accumulated almost twice as much Mn2+ as did mutant. ESR spectra of the cell extracts and whole cells loaded with Mn2+ were typical of free Mn(II) ion. These spectra could not account quantitatively for the total cellular Mn2+, however.

A screen for soluble antioxidant activities in the Mn2+-supplemented cells detected O2− (superoxide) scavenging activity, with no change in catalase or peroxidase activities. This O2− scavenging activity was CN− and heat-resistant. No atherosomatic bands were revealed in nondenaturing gels of Mn2+-containing cell extracts stained for O2− scavenging activity. The Mn2+-dependant O2− scavenging activity in the cell extracts was quenched by EDTA and dialyzable. More than 60% of both the intracellular Mn2+ and the O2− scavenging activity was removed by 2-h dialysis. Dia lyzed cells were not viable in air unless resupplemented with either Met or Mn2+. Although Mn2+ supported the aerobic growth of these mutants, excess Mn2+, which correlated with an elevated O2− scavenging activity, was toxic to both mutant and wild type. The results indicate that free or loosely bound Mn2+ ion protects the mutants against oxygen stress by providing an intracellular, presumably cytosolic, O2− scavenging activity which replaces the absent SOD-1.

One-electron reduction of dioxygen leads to the generation of potentially redox active oxygen radicals. Overproduction of these species has been linked to cellular damage, termed oxygen stress (1–3). Antioxidant enzymes include the superoxide dismutases (SOD),1 the catalases and peroxidases, which scavenge the superoxide radical (O2−) and H2O2, respectively. These enzymes appear to play protective roles in aerobes (4–6).

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2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (1)
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The deletion of both Mn2+- and Fe2+-containing SODs in Escherichia coli is lethal to aerobic growth in minimal medium unless specific branched chain amino acids are added (7). The requirement for these amino acids in the double mutant can be correlated to the hypersensitivity of dihydroxy acid dehydratase, an enzyme involved in the biosynthesis of branched-chain amino acids, to hyperbaric oxygen in vivo (8) and to O2− in vitro (9). This result indicates that specific cellular proteins may be inherently sensitive to dioxygen-derived free radicals. Such sensitivity may be manifest in the requirement for SOD in aerobic growth.

The yeast, Saccharomyces cerevisiae, has two SOD proteins. SOD-1 is cytosolic and contains 1 mol each of Cu2+ and Zn2− per mol of 16-kDa monomer (10). SOD-2, a 24-kDa polypeptide, contains Mn2+ (11). SOD-2 is synthesized as a preprotein. The 27-amino acid mitochondrial targeting sequence is cleaved following import across the inner mitochondrial membrane (12). Although more than 90% of total cellular yeast O2− scavenging activity is due to SOD-1, a mutant lacking SOD-2 is sensitive to 100% oxygen and is unable to utilize a nonfermentable carbon source in air (13).

Yeast mutants lacking SOD-1 activity also are sensitive to oxygen. Three such mutants in S. cerevisiae have been isolated and partially characterized by Bilinski et al. (14). These mutants showed a nonparental Met, Lys auxotrophy when grown in air but not when grown anaerobically. All of the mutants failed to grow under 100% O2 even in rich medium. These oxygen-dependent growth defects are due to a mutation of a single nuclear gene which correlates to the lack of active SOD-1 (14, 15). This mutation has been mapped to the SOD1 locus. The activities of SOD-2 are not different from wild type. The O2− sensitivity of growth was rescued by transformation of these mutants with a plasmid containing wild type yeast SOD-1 gene. Transformation was accompanied by an increase of SOD-1 activity (15, 16). Thus, the lack of SOD-1 activity appears to be the causative factor in the mutant growth defects observed under air and oxygen.

Divalent manganese [Mn(II)] is known to dismutate O2− in vitro (17, 18). Lactobacillus plantarum, a facultative anaerobe which does not contain any SOD protein species, requires

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1 The abbreviations and trivial names used are: SOD, superoxide dismutase; SD, synthetic dextrose (minimal medium); YPD, yeast extract-peptone-dextrose (rich medium).

2 E. C. Chang and D. J. Kosman, unpublished observations.

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† To whom correspondence and reprint requests should be addressed.
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Mn\(^{2+}\) when grown aerobically on a nonfermentable carbon source, i.e., when it is obligately aerobic (19). This organism is also resistant to the redox-active quinone, plumbagin, which has been shown to support intracellular \( \text{O}_2 \) generation by this bacterium. Extracts of cells grown aerobically in Mn\(^{2+}\)-enriched media exhibited an EDTA-inhibitable \( \text{O}_2 \) scavenging activity which was lost slowly upon dialysis (19, 20). This activity is due to the Mn(II) accumulated by the cell which apparently is retained by a high molecular weight polyphosphate-protein ligand (20). Thus, this and other genera of the Lactobacillaceae can adapt to anaerobiosis, in part, because the species studied accumulate millimolar concentrations of Mn\(^{2+}\). This Mn(II) provides the \( \text{O}_2 \) scavenging activity provided normally in aerotolerant organisms by microorganisms of the SOD family. Lactobacillus plantarum, Mn(II) would render these mutants aerotolerant (21). Furthermore, overexpression of SOD-1 increases lipid peroxidation in cultured cells (23) and induces symptoms similar to Down's syndrome in transgenic mice (24). A yeast SOD has not yet been overexpressed in yeast, but Mn\(^{2+}\) has been shown to be mutagenic (25).

We have used Mn\(^{2+}\) as a growth supplement to investigate the relationship between cellular \( \text{O}_2 \) scavenging activity and aerobic growth of yeast using the SOD-1 yeast mutants described above, that is, we wished to test the hypothesis that as in \( L. \) plantarum, Mn(II) would render these mutants aerotolerant. The data suggest that loosely bound Mn(II) accumulated by these mutants does protect them against oxygen stress via a protein-independent \( \text{O}_2 \) scavenging activity which complements the absence of active SOD-1. On the other hand, excess Mn\(^{2+}\) inhibits growth of both mutant and wild type; the excess Mn\(^{2+}\) in the cell extracts correlates with an elevated \( \text{O}_2 \) scavenging activity.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Media components were purchased from Difco. The reagents for SOD assay were obtained from Sigma with the exception of hydroxyamine which was purchased from Gallard-Schlesinger (Analar, British Drug House). We have observed that some batches of Grade III xanthine oxidase contain more than 1 mM EDTA resulting in chelation of Mn\(^{2+}\) in the cell samples. Grade I xanthine oxidase can be used to avoid this problem. Bovine SOD-1 and catalase were obtained from Boehringer Mannheim and Sigma, respectively. (NH\(_4\))\(_2\)SO\(_4\), MgSO\(_4\), KH\(_2\)PO\(_4\), NaCl, and CaCl\(_2\) used for phosphate-free SD medium were Analar (British Drug House) reagents obtained from Gallard-Schlesinger. The Mn\(^{2+}\) standard for atomic absorption spectrophotometry was purchased from Alfa Products. All other reagents used were reagent grade. The Mn\(^{2+}\) in the dialyzed water used for growth media and reagents was below the detection limits by flameless atomic absorption spectrophotometry (0.1 pg/ml).

**Medium for Cell Growth**—The yeast extract-peptone-dextrose (YPD) medium contained 1% (w/v) yeast extract, 2% (w/v) Bactopeptom, and dextrose. The semidefined medium (SD) contained 0.67% (w/v) yeast nitrogen base without amino acids and 2% (w/v) dextrose and was buffered by 10 mM phosphate. This medium contained 9 \( \mu \)M Mn\(^{2+}\). For anaerobic growth experiments, 5 mg/liter MnSO\(_4\) did not inhibit the xanthine oxidase-catalyzed production of uric acid from hypoxanthine (assayed spectrophotometrically at 295 nm) suggesting that the Mn\(^{2+}\)-dependent \( \text{O}_2 \) scavenging activity observed was not due to an artifact of underproduction of \( \text{O}_2 \) in the SOD assay (data not shown). The \( \text{O}_2 \) scavenging activity was also determined in cellular protein samples after separation by gel electrophoresis (30). For samples containing Mn\(^{2+}\), EDTA was omitted from all the reagents used and 3 times more nitroblue tetrazolium and riboflavin were used than are suggested. The catalase activity was determined from the decrease of [H\(_2\)O\(_2\)] observed at 240 nm (31). Based on a standard catalase preparation (Sigma), 1 unit was equivalent to 861 nmol of H\(_2\)O\(_2\) min\(^{-1}\).

**Determination of Mn\(^{2+}\) and Phosphate**—Room temperature ESR spectra were obtained at 9.5 GHz using a Varian E-9 ESR spectrometer operating at a power of 30 miliwatts. The total amount of Mn\(^{2+}\) in the samples was determined by plasma atomic absorption spectrophotometry using a Perkin-Elmer model 360 equipped with model HGA 2100 graphite furnace. Phosphate analysis was described as described (32).

**Diagnosis of Cellular Mn\(^{2+}\)**—The SOD-1 mutant, Dscdl-1C, was grown to early log phase in SD medium (pH 4.5) supplemented with either Met or 4 mM Mn\(^{2+}\). Following washing, one-half of the cell pellet was resuspended in 250 \( \mu \)l of water, and an extract was prepared as described above. The remainder was resuspended in water and transferred to a dialysis bag (Spectrapor, Spectrum Medical Industries, Inc.) with a molecular mass cut-off of 10 kDa. This cell sample was dialyzed against 100 mM phosphate buffer (pH 4.5) at room temperature for 2 h prior to washing and preparation of the cell extract.

**RESULTS**

**Mn\(^{2+}\) Supplementation: Effect on Oxygen Stress**—Addition of 2 mM Mn\(^{2+}\) to YPD plates rescued growth under pure oxygen of all three SOD-1 mutants, Dscdl-1C, Dscdl-4A, and Dscdl-2C (1C, 4A, and 2C in Fig. 1). The Met and Lys auxotrophy observed under air could be rescued also if sufficient Mn\(^{2+}\) was added to the growth medium. Fig. 2A (closed circles) shows that medium Mn\(^{2+}\) abolished the Met auxotrophy in the mutant, 1C. The growth of the mutant was minimal.
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FIG. 1. The effect of Mn$^{2+}$ supplementation on the growth of SOD-1$^-$ mutants under pure oxygen. Single colonies of mutants (1C, 2C, and 4A) were streaked on YPD (left) or YPD containing 2 mM MnSO$_4$ (right). The positions of the mutants on the plates are indicated by the inset above the two plates in the figure. The plates were transferred to a desiccator which was flushed with pure oxygen for 45 min. The cultures were incubated at 30 °C for 2 days with a second $O_2$ flush after 24 h.

at medium [Mn$^{2+}$] below 2 mM, while after 48 h of growth, cultures containing 2 mM Mn$^{2+}$ did reach a stationary phase which was 63% of control (dotted line, Fig. 2A). At 4 mM medium Mn$^{2+}$, both the 20-h growth yield and the doubling time (about 2.5 h) were the same as for Met-supplemented cultures. The growth of wild type was not affected by [Mn$^{2+}$] below 4 mM (closed circles, Fig. 2B).

To test if the protective effect on $O_2$-dependent Met auxotrophy was specific for Mn$^{2+}$, Mg$^{2+}$, Cu$^{2+}$, and Zn$^{2+}$ were tested also. Magnesium chloride up to 4 mM or ZnCl$_2$ up to 1 mM in the growth medium did not provide relief from the aerobic Met/Lys auxotrophy. Addition of CuSO$_4$ up to 0.25 mM was minimally effective (data not shown). The $O_2$-dependent Lys auxotrophy in 1C and the aerobic Met/Lys auxotrophy observed for 2C and 4A were also rescued by 4 mM Mn$^{2+}$. More than 8 mM medium Mn$^{2+}$ inhibited the growth of both wild type and the mutant. This effect is discussed below.

Cellular Accumulation of Mn$^{2+}$.—To investigate if the rescue of Met auxotrophy correlated with accumulation of soluble Mn$^{2+}$ in the mutants, the amount of Mn$^{2+}$ in the mutants was determined. All of the mutants accumulated 14 (2C)- to 59 (4A)-fold more Mn$^{2+}$ than controls in which Met was added to support growth (column 3, Table I). In order to show quantitatively if this Mn$^{2+}$ accumulation was typical for these mutants only or if it was characteristic of wild type as well, a comparison of the accumulation of Mn$^{2+}$ in a wild-type yeast and in 1C grown in air and in nitrogen was made. Wild type cells accumulated 35 pmol of Mn$^{2+}$/10$^8$ cells under aerobic conditions. The mutant 1C accumulated 39 and 28 pmol/10$^8$ cells aerobically and anaerobically, respectively. Thus, Mn$^{2+}$ accumulation was essentially independent of strain genotype or $pO_2$. A detailed analysis of the relationship between medium [Mn$^{2+}$] and cell growth with wild type and mutant (1C) is presented below.

Manganese-associated $O_2$ Scavenging Activity in Cell-free Extracts.—The growth defect in these mutants correlates to the lack of SOD-1 (14). In addition, transformation of any of the mutants using a plasmid containing yeast wild type SOD-1 gene rescues the aerobic growth much as Mn$^{2+}$ supplementation does (15, 16). Therefore, the $O_2$ scavenging activity in extracts prepared from Mn$^{2+}$-grown mutant cell cultures was measured. Generally, there are at least three components in yeast which can exhibit $O_2$ scavenging activity: SOD-1, which is sensitive to 1 mM CN$^-$; SOD-2, which is resistant to CN$^-$ at 1 mM; and transition metals, whose scavenging activities are typically quenched by EDTA (33). A SOD assay without added CN$^-$ or EDTA yielded the total $O_2$ scavenging activity. A CN$^-$-resistant $O_2$ scavenging activity was found in all extracts made from cells pregrown in 4 mM Mn$^{2+}$ (Table I). This CN$^-$ resistance suggests that the elevated $O_2$ scavenging activity was not due to SOD-1. Cyanide-resistant $O_2$ scavenging activities ranged from 101 to 390 units/mg of protein in 1C and 4A, respectively. This was approximately a 25-fold increase of $O_2$ scavenging activity (cf. 2C) over the Met-grown control under the same assay conditions. The $O_2$ scavenging activity appeared to correlate to the amount of Mn$^{2+}$ in the cell extract, but was independent of the protein concentration (data not shown). Bovine SOD-1 (10 units/ml of culture) in the medium did not abolish the Met auxotrophy indicating the protection of cell growth by Mn$^{2+}$ arose intracellularly.

The CN$^-$-resistant $O_2$ scavenging activity in the cell ex-
were washed twice with water and then diluted into fresh SD medium (pH 4.5) to $A = 0.1$. The SD medium contained all required nutrients and either Met or 4 mM MnCl$_2$. Cell extracts were prepared from cultures in midlog phase ($A = 1.5$). The $O_2$ scavenging assay was performed as described under "Methods." EDTA (1 mM) was either added or omitted as indicated. Grade III xanthine oxidase (Sigma) was used in this experiment. The average $O_2$ scavenging activity in the absence of EDTA and CN$^-$ for the Mn$^{2+}$-grown samples was 4.2 ± 1.1 units/nmol of Mn$^{2+}$. The specific scavenging activity of aqueous MnSO$_4$ was 6.1 (Grade I xanthine oxidase) or 5.3 (Grade III xanthine oxidase). Note that addition of CN$^-$ in this assay generally enhances dye formation and thus the apparent specific activity of Mn$^{2+}$ and SOD-2. ND, not determined.

To explore the possibility that this Mn$^{2+}$-dependent $O_2$ scavenging activity might be associated with some other cellular protein(s), cell extracts from IC grown in Mn$^{2+}$ (100 units of $O_2$ scavenging activity/mg of protein, Table I) were analyzed by 7% non-denaturing polyacrylamide gel electrophoresis, and a SOD assay was done in the gel in the absence of EDTA. As controls, equal amounts of protein from cell extracts of wild type and of mutant 2C transformed with a SOD-1-containing plasmid were also analyzed. The two control samples had specific SOD-1 activities of 144 and 22 units/mg of protein, respectively. Achromatic bands corresponding to SOD-1 were seen in the two controls, but none was observed in all cell samples from IC (data not shown).

The Mn$^{2+}$-dependent $O_2$ scavenging activity was also heat-resistant. An extract made from mutant cells (1C) pregrown in Mn$^{2+}$-medium to early log phase was boiled for 15 min, then centrifuged. The supernatant was removed, and the precipitate was washed in 10 × the original extract volume. The initial extract, supernatant, and precipitate were analyzed as shown in Table II. Boiling precipitated 83% of the total cellular protein in the initial extract; 14% of the Mn$^{2+}$ in the extract co-precipitated with this protein. The $O_2$ scavenging activity in the three fractions directly correlated to the amount of Mn$^{2+}$ present; that is, the specific $O_2$ scavenging activity in each of the three fractions in Table II was 4.5 ± 0.5 units/nmol of Mn$^{2+}$, essentially the same specific activity exhibited by MnSO$_4$. This result also indicates that the $O_2$ scavenging activity in the cell extract was not functionally associated with specific protein(s).

**Table I**

| Yeast strain | Medium addition | Mn$^{2+}$ | $O_2$ scavenging activity | protein | Total cellular protein | Mn$^{2+}$ content |
|-------------|-----------------|----------|--------------------------|--------|-----------------------|-----------------|
| IC          | Met             | 0.8      | 2.4                      | 1.0    | 4.2                   | 4.2 ± 1.2       |
|             | Mn$^{2+}$       | 20.0     | 101.1                    | 0.3    | 108.2                 | 1.3             |
| 4A          | Met             | 0.5      | 2.8                      | 0.3    | 4.9                   | 0.2             |
|             | Mn$^{2+}$       | 28.0     | 391.0                    | 0.6    | ND                    | ND              |
| 2C          | Met             | 0.6      | 4.2                      | 0.5    | 6.8                   | 0.2             |
|             | Mn$^{2+}$       | 8.4      | 101.3                    | 2.6    | 177.5                 | 2.9             |

The relationship between medium [Mn$^{2+}$], total $O_2$ scavenging activity, and cell growth is shown in Fig. 2. The $O_2$ scavenging activity was measured in these experiments in the absence of EDTA and CN$^-$.

**Table II**

| Sample | Mn$^{2+}$ | Protein | $O_2$ scavenging activity |
|--------|-----------|---------|---------------------------|
| Extract | nmol | % total | µg | % total | units/mg | % total |
| Original | 28.1 | 100 | 905 | 100 | 111 | 100 |
| Sup     | 15.8 | 56 | 155 | 17 | 77 | 69 |
| Ppt     | 3.8  | 14 | 460 | 51 | 18 | 16 |
on the inhibition of cell growth due to Mn^{2+} (data not shown). This result suggests the toxic effect caused by Mn^{2+} is not due to extracellularly produced H_2O_2. However, Mn^{2+}-dependent inhibition of cell growth in wild type yeast correlated with an increase in total O_2 scavenging activity. For example, at 8 mM medium Mn^{2+}, the cell growth was inhibited by 25% and ~750 units of O_2 scavenging activity/mg of protein were detected in that cell extract. This was a 5-fold increase of scavenging activity compared to cells grown at 4 mM Mn^{2+}. At 16 mM Mn^{2+}, at which [Mn^{2+}] the cell growth was completely inhibited, the scavenging activity increased by 15-fold (Fig. 2B). The activities of SOD-1 and SOD-2 made little contribution to this increase (legend, Fig. 2).

On the other hand, the overnight growth of the mutant (1C) was minimal at medium [Mn^{2+}] up to 2 mM, while 4 mM Mn^{2+} supported the cell growth at control level (solid line, closed circles, Fig. 2A). At 2 mM Mn^{2+}, the mutant cell accumulated 5.7 ± 0.6 pmol of Mn^{2+}/μg of protein (n = 2, data not shown); the O_2 scavenging activity in this cell extract was 55 units/mg of protein (open circles, inset, Fig. 2A). In the absence of added Mn^{2+}, the O_2 scavenging activity in mutant cell extract was ~2 units/mg (Table I). Therefore, addition of 2 mM Mn^{2+} to the medium induced a 26-fold increase in O_2 scavenging activity. Although this activity level did not support a wild type doubling time by the mutant, it was sufficient for the cells to reach a stationary phase which was 83% of control (dotted line, Fig. 2A). In contrast to wild type, inhibition of mutant growth was not observed at 8 mM Mn^{2+} (O_2 scavenging activity, 310 units/mg of protein). At 16 mM Mn^{2+}, however, mutant cell growth was inhibited by more than 75%. This inhibition correlated with a marked increase in scavenging activity from 310 (8 mM) to more than 1000 units/mg of protein.

**Dialyzable Mn^{2+}-dependent O_2 Scavenging Activity**—The physiological significance of the dialyzable Mn^{2+} in cells was investigated. Mutant strain 1C was grown to A_600 nm ~ 0.9 in a SD medium (pH 4.5) containing 4 mM Mn^{2+} or Met as control. Both cell samples were washed with distilled water and then dialyzed against 100 mM phosphate buffer. Cell extracts were made at the start of the dialysis (t = 0) and after 2 h. At that time, the Mn^{2+} content of the Mn^{2+}-pregrown culture decreased from 6.9 to 2.5 pmol of Mn^{2+}/μg of protein or a loss of 63% of the initial cell-associated Mn^{2+}. This loss of Mn^{2+} was accompanied by a 79% decrease in the total EDTA-inhibitable O_2 scavenging activity in the extract (from 42.7 to 8.8 units/mg of protein) suggesting that the dialyzable Mn^{2+} made the major contribution to the Mn^{2+}-dependent cellular O_2 scavenging activity. Significantly, after removal of this fraction of intracellular Mn^{2+} by dialysis, when resuspended in growth medium, the cells needed at least 2 mM medium Mn^{2+} (or Met) to continue aerobic growth, e.g. the pattern of overnight growth response was the same as that shown in Fig. 2A. The accumulated Mn^{2+} in wild type cells was lost readily upon dialysis, also, consistent with the result that the accumulation of Mn^{2+} in yeast is independent of genotype (data not shown). This result suggests that the Mn^{2+} which provides a diffusible O_2 scavenging activity is responsible for protecting against the O_2-induced growth defects in the mutants.

**Investigation of the State of Mn^{2+} in Cell Extracts**—The state of the Mn^{2+} in the cell extracts and the fractions after heat treatment (Table II) was analyzed by electron spin resonance spectroscopy (ESR). The original cell extract (Table II) which contained 56 μM Mn^{2+} exhibited an ESR spectrum equivalent to the spectrum of the 9 μM MnSO_4 standard made in water shown in Fig. 3a. After heat treatment of the extract, 23 μM ESR-detectable Mn(II), or 50% of the total initial Mn^{2+}, was found in the supernatant (data not shown). The precipitate contained 8 μM total Mn^{2+} (Table II), but this Mn^{2+} was ESR-silent. These results suggest that 16% of the total Mn^{2+} in the original cell extract was free Mn(II) or bound Mn(II) within a virtually symmetric ligand field shielded from solvent H_2O. That is, most of the Mn^{2+} in the soluble cell fraction, about 84%, appeared to be associated with some cellular component(s) that eliminated the ESR spectrum typical of free Mn(II) or was present in another ESR-silent redox state. Following heat treatment, a portion of this Mn^{2+} was released into the supernatant: essentially all Mn^{2+} present therein was ESR-detectable (cf. Table II).

Phosphate is known to abolish the ESR signal typical of hexaquo-Mn(II) by shortening the electron spin relaxation time (20), thus the relationship between the amount of ESR-detectable Mn(II) and phosphate in cell extracts was investigated. Mutant 1C was grown in 30, 200, and 400 μM medium phosphate, and the cell extracts were assayed for Mn^{2+} and phosphate and examined by ESR. The amount of ESR-detectable Mn(II) in a cell extract containing 15 μM total Mn^{2+} and 132 μM phosphate (Fig. 3b) was equivalent to the 9 μM Mn(II) standard (Fig. 3a). The extract examined in Fig. 3c (sample contained 622 μM phosphate) contained 3 times more [Mn^{2+}] (48 μM) than did the sample in Fig. 3b; the ESR spectra had essentially the same amplitudes, however. Similarly, the ESR spectrum shown in Fig. 3d (sample contained 725 μM phosphate) was one-half of that in Fig. 3a, although the sample contained 4-fold more Mn^{2+} (35 versus 9 μM). Thus, the amplitudes of the Mn(II) ESR spectra were inversely proportional to the cellular [phosphate].

**DISCUSSION**

This study demonstrates that addition of Mn^{2+} protects yeast SOD-1^{-} mutants against O_2-induced auxotrophy for Met. The original cell extract (Fig. 3a) was grown in 1 mM phosphate SD medium (pH 4.5) was washed twice with water, then inoculated into fresh SD medium containing different [phosphate], 4 mM Mn^{2+}, and all amino acids required for growth except Met. The inocula were adjusted to achieve a turbidity of 1.0 for all cultures after overnight growth. Cell extracts were prepared in H_2O. The medium [phosphate] was 30 (b), 200 (c), or 400 (d) μM. The measured [Mn^{2+}] (atomic absorption) and [phosphate] (μM) in the samples were: 15 and 132 for b, 48 and 622 for c, and 35 and 725 for d, respectively. The sample in a was a 9 μM MnSO_4 standard in water. The parameters for the ambient temperature, constant gain ESR spectra were: microwave frequency, 9.519 GHz (Hewlett-Packard frequency meter); microwave power, 30 milliwatts; modulation amplitude, 5 G; scan range, 1000 G, with the field centered at 3200 G (arrow, marked by reference to diphenylpicrylhydrazyl).
and Lys and relieves their inability to grow in 100% oxygen in rich medium. This rescue correlates with the intracellular accumulation of Mn^{2+}, and with elevated Mn^{2+}-dependent O_2 scavenging activity in cell extracts. This O_2 scavenging activity was not due to SOD-1 because: (a) the activity was EDTA-inhibitable, but that of SOD-1 is not; (b) the activity was Mn^{2+}-dependent and in the cell is due to the presence of loosely bound Mn(I). The evidence for this conclusion is summarized as follows. First, the O_2 scavenging activity was inhibited by EDTA. Second, the O_2 scavenging activity was heat-resistant. This result together with the negative result of the SOD gel assay argue against the involvement of any specific protein(s) in this O_2 scavenging activity. Third, the specific O_2 scavenging activities of cell extracts from all mutants and wild type preloaded with Mn^{2+}, and of the supernatants and precipitates of cell extracts after heat treatment were essentially the same as that of free Mn^{2+} (around 5 units/nmol of Mn^{2+}). Fourth, the Mn^{2+} in IC and wild type cells was readily lost by dialysis. Finally, Mn^{2+}-depleted mutant cells, which had lost 79% of the accumulated O_2 scavenging activity, could not grow in air unless Met or Mn^{2+} was resupplemented. The reason that less than 20% of the accumulated Mn^{2+} exhibited an observable Mn(II) ESR spectrum is probably due to the presence of low molecular weight Mn^{2+}-protein and/or orthophosphate complexes in which the Mn(II) was readily accessible to H_2O. Water in fast exchange at Mn(II) significantly shortens the electron spin relaxation time resulting in ESR line broadening (38). These complexes and/or the Mn(II) they contain could be readily dialyzable and are capable of scavenging O_2 since, among other characteristics, they do appear to have an accessible coordination site for O_2 (17, 18, 35). Nevertheless, the possibility that the intracellular Mn^{2+} exists in another, ESR-silent oxidation state cannot be ruled out. Although Mn^{2+} may have a cellular function(s) which can ameliorate the toxicity of dioxygen other than supporting an O_2 scavenging activity, our results indicate that it is this Mn^{2+}-dependent activity which is essential to the aerobic growth of these mutants. First, quantitatively, the protection of aerobic growth by Mn^{2+} correlated with an apparent Mn^{2+}-dependent O_2 scavenging activity. Second, the addition of Mg^{2+}, which can substitute for Mn^{2+} in many enzymatic reactions, did not rescue cell growth in these mutants. This result suggests that the rescue due to Mn^{2+} is associated with its redox chemistry specifically. Cu^{2+}, which is also redox-active and capable of dismutating O_2 (37), did provide some supplementation at 0.25 mM. Since Cu^{2+} was toxic to the strains used at concentrations above 0.5 mM, adding more Cu^{2+} did not provide more protection. Finally, transformation of all the mutants carrying a scdl allele by a plasmid containing the yeast wild type SOD-1 gene rescued the O_2-dependent growth defects. The transformation and the accumulation of intracellular Mn^{2+} both resulted in an increase of O_2 scavenging activity in these mutants while having no effect on the activities of other anti-oxidant enzymes (15).  

The rescue of the Mst auxotrophy in mutant (IC) by Mn^{2+} exhibited a "delayed-threshold" in that medium [Mn^{2+}] below 2 mM supported no or little growth in 24 h, while cultures grown in 2 mM Mn^{2+} did approach stationary phase after 48 h (Fig. 2A, dotted line). As indicated in Fig. 1, 2 mM Mn^{2+} in rich medium supported mutant growth under 100% O_2. The 2 mM threshold could be associated with the Mn^{2+}-dependent O_2 scavenging activity which was 55 units/mg of protein under these conditions. However, optimal growth did require 80 units of O_2 scavenging activity/mg of protein. This activity is higher than the log phase SOD-1 activity in two of the pJW3-SOD1 transfectants of these mutants (15), ~15 units of O_2 scavenging activity/mg of protein. This latter activity level is the lowest we have observed in an aerotolerant yeast except for the strains which carry physiological suppressors of the scdl allele. These revertants exhibited wild type O_2 scavenging activity which was essentially the same as that of free Mn^{2+} (around 5 units/nmol of Mn^{2+}).

An optimal amount of Mn^{2+} in SOD-1 mutants supported aerobic growth, but excess Mn^{2+} inhibited cell growth in both wild type and mutant. Excess Mn^{2+} in humans induces symptoms similar to Parkinson's disease (39) and in yeast is mutagenic (24). Although the mechanism of the toxicity in mammals is not known, the involvement of dioxygen radicals has been shown to be relevant in vitro (40). The Mn^{2+} toxicity observed here was more apparent in air than in N_2 suggesting that oxygen is at least partially responsible for the toxicity of Mn^{2+} in yeast as well. Nevertheless, 16 mM Mn^{2+} did inhibit anaerobic cell growth to 60% of an anaerobic control; therefore, above 8 mM, Mn^{2+} toxicity was clearly O_2-dependent (data not shown).

As shown in Fig. 2, inhibition of growth was correlated with an increase of intracellular O_2 scavenging activity. The overproduction of SOD-1 in mice has been shown to induce symptoms similar to Down's syndrome, a process probably mediated by the overproduction of H_2O_2 from the SOD reaction (23, 24). Overexpression of Fe^{2+}-SOD in E. coli, which apparently leads to an increase of intracellular H_2O_2, is also toxic (22). Since Mn^{2+} exhibits an O_2 scavenging activity in the cell extracts, our results indicate a correlation between elevated O_2 scavenging activity and cytotoxicity in yeast. The effect of overexpression of yeast SOD-1 gene in yeast is under investigation.

Although the accumulation of Mn^{2+} in both SOD-1" yeast mutant and L. plantarum is not O_2-inducible, the latter accumulates Mn^{2+} more effectively. The Mn^{2+} in yeast was readily dialyzable while only 40% of the Mn^{2+} in L. plantarum was lost in 48 h (20). The difference appears related to the co-accumulation of polyphosphate in L. plantarum. The Mn^{2+}-polyphosphate-protein complex in this bacterium is stable and nondialyzable (20). The effect of phosphate on the Mn^{2+}-induced rescue of cell growth in the yeast mutants was investigated (data not shown). The mutant (IC) was grown in SD medium containing a defined amount of phosphate in the presence of either Mn^{2+} (4 mM) or Met. The growth yield of mutant (and wild type) leveled off at 200 μm medium phosphate independent of whether Met or Mn^{2+} was added as supplement. Although the accumulation of Mn^{2+} by the
mutant in early log phase leveled off at 200 μM medium phosphate, the accumulation of phosphate did not. At 200 μM medium phosphate, the mutant accumulated 0.3 fmol of phosphate/cell. We estimated from published data (41) that at the end of log phase, yeast accumulates polyphosphate in the range of a pmol/10⁶ cells, or 0.1% of the orthophosphate detected in our experiments. In contrast, high molecular weight polyphosphate accounts for ~80% of the total phosphate in L. plantarum (20). The data suggest that accumulation of phosphate (or polyphosphate) by yeast is not a prerequisite for the O₂ resistance provided by Mn^{II}.

In summary, we infer that intracellular, loosely bound Mn(II) protects these yeast mutants by providing a cytosolic phosphate/cell. We estimated from published data (41) that at the end of log phase, yeast accumulates polyphosphate in the weight polyphosphate accounts for ~80% of the total phosphate. We infer that intracellular, loosely bound Mn(II) protects these yeast mutants by providing a cytosolic phosphate/cell. We estimated from published data (41) that at the end of log phase, yeast accumulates polyphosphate in the range of a pmol/10⁶ cells, or 0.1% of the orthophosphate detected in our experiments. In contrast, high molecular weight polyphosphate accounts for ~80% of the total phosphate in L. plantarum (20). The data suggest that accumulation of phosphate (or polyphosphate) by yeast is not a prerequisite for the O₂ resistance provided by Mn^{II}.

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