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Manganese accumulation in yeast cells

Electron-spin-resonance characterization and superoxide dismutase activity

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Summary. Manganese accumulation was studied by room-temperature electron spin resonance (ESR) spectroscopy in *Saccharomyces cerevisiae* grown in the presence of increasing amounts of $MnSO_4$. Mn^{2+} retention was nearly linear in intact cells for fractions related to both low-molecular-mass and macromolecular complexes ('free' and 'bound' Mn^{2+}, respectively). A deviation from linearity was observed in cell extracts between the control value and 0.1 mM Mn^{2+}, indicating more efficient accumulation at low Mn^{2+} concentrations. The difference in slopes between the two straight lines describing Mn^{2+} retention at concentrations lower and higher than 0.1 mM, respectively, was quite large for the free Mn^{2+} fraction. Furthermore it was unaffected by subsequent dialyses of the extracts, showing stable retention in the form of low-molecular-mass complexes. In contrast, the slope of the line describing retention of 'bound' Mn^{2+} at concentrations higher than 0.1 mM became less steep after subsequent dialyses of the cell extracts. This result indicates that the macromolecule-bound Mn^{2+} was essentially associated with particulate structures. In contrast to Cu^{2+}, Mn^{2+} had no effect on the major enzyme activities involved in oxygen metabolism except for a slight increase of cyanide-resistant Mn-superoxide dismutase activity, due to dialyzable Mn^{2+} complexes.

Key words: Manganese — Electron spin resonance — Superoxide dismutase — *Saccharomyces cerevisiae* — Yeast

Introduction

Manganese uptake and retention by yeast has been extensively studied as a model for cation accumulation and transport (Borst-Pauwels 1981). Manganese salts are well tolerated by yeast at concentrations as high as 3 mM (Okorokov et al. 1977). Recent studies have mostly dealt with the rate of uptake (Niewenhuis et al. 1981; Parkin and Ross 1986a, b). Although Mn(II) is a very good metal probe for ESR studies at room temperature, no report using this approach is available. On the other hand, ESR spectroscopy has been used to study copper retention by yeast (Kihn et al. 1987); however, those studies were performed at low temperature and spectra in the frozen state may be misleading as far as the identification of the metal complexes involved is concerned (Calabrese et al. 1983). A special interest in studying Mn^{2+} accumulation in yeast arises from the finding that in the bacterium *Lactobacillus plantarum*, which had been found to lack the enzyme superoxide dismutase, high intracellular levels of Mn(II) were shown to take the place of the enzyme in scavenging $O_2^{\cdot-}$ (Archibald and Fridovich 1981a).

In view of these considerations the purpose of the present paper was to obtain the following information: (a) to characterize Mn^{2+} retention in yeast by room-temperature ESR; (b) to study the effect of aerobically growing yeast in the presence of Mn(II) salts on enzyme activities that have previously been shown to be induced by high copper concentrations added to cultures of aerobically growing yeast (Galiazzo et al. 1988); (c) to establish whether the augmented Mn^{2+} concentration in yeast causes additional superoxide dismutase activity, similarly to the effect of Mn complexes seen in *Lactobacillus plantarum* (Archibald and Fridovich 1981a, b).

Materials and methods

Chemicals. Bovine serum albumin, xanthine, xanthine oxidase, cytochrome *c*, cumene hydroperoxide and 1-chloro-2,4-dini-

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trobenzene were purchased from Sigma Chemical Co., St. Louis. Sodium azide and hydrogen peroxide were from Merck, Darmstadt. Glutathione (reduced), glutathione reductase and NADPH were obtained from Boehringer Mannheim. Zymolyase (100000 U/g) was from Seikagaku Kogyo Co. Ltd, Tokyo. Potassium cyanide was from Fluka, Buchs. Yeast extract and peptone were obtained from Difco, Detroit. All other materials were of reagent grade and were obtained from the best available commercial sources.

Organism and media. The yeast *Saccharomyces cerevisiae*, strain D273-10B, was used for the experiments. The basic culture medium contained (mass/vol.): 0.5% glucose, 1% yeast extract, 0.1% peptone, 1% NH_4Cl, 0.03% $MgSO_4$, 0.09% K_2HPO_4, 0.22% KH_2PO_4. $MnSO_4 \cdot H_2O$ was added to the culture medium to obtain the desired concentrations. The Mn^{2+} concentration of the medium without added $MnSO_4$, measured by ESR (see below), was <50 nM. A 17-h culture grown in the basal medium was used as the inoculum. Yeast was grown in a rotatory shaker (Orbit Environ-shaker, Lab-Line Instrument, Melrose) at 30° C and 180 rpm.

Preparation of cell extracts. Cells were harvested from yeast cultures in the late exponential phase by centrifugation for 20 min at 2500 *g* and washed twice with cold distilled water. A fraction of the washed cells was used for the ESR experiments. Cells from 1 l of culture were incubated for 20 min at 22° C in 50 ml of a solution containing 0.64 M 2-mercaptoethanol and 0.025 M EDTA. Cells were then washed in 70 ml cold sorbitol medium (1.1 M sorbitol, 0.05 M potassium phosphate buffer, pH 7.7). The washed cells were incubated for 40 min at 37° C in 100 ml sorbitol medium containing 1.5 mg zymolyase/g wet cells. Spheroplasts were collected by centrifugation at 6000 *g* for 15 min and washed with sorbitol medium. The pellet was suspended in 3 ml cold solution containing 0.6 M sorbitol and 0.01 M Tris HCl (pH 7.4) and the suspension was sonicated in an ethanol/ice bath with a Branson model B-12 sonicator at 40-W power for 3 min in 30-s intervals. Cell debris was removed by centrifugation at 7800 *g* for 15 min. The supernatant was dialyzed once or twice against 10^3 vol. of the same buffer for 7 h and assayed for cytochrome oxidase activity. A fraction of the dialyzed extract was then clarified at 35000 *g* for 20 min and used for other enzyme determinations and ESR experiments. Protein was determined by the method of Bradford (1976).

Enzyme assays. Cytochrome oxidase (Cooperstein and Lazarrow 1951), catalase (Luck 1963), glutathione peroxidase (Lawrence and Burk 1976), glutathione transferase (Habig et al. 1974) and fumarase (Massey 1955) activities were determined as previously reported. Superoxide dismutase activity was assayed according to the cytochrome *c*/xanthine oxidase/xanthine method (Crapo et al. 1978). The test was performed in the presence of 0.01 mM KCN to inhibit cytochrome oxidase and peroxidase activities. The relative amount of Cu,Zn- and Mn-superoxide dismutase was analyzed in the presence of 3 mM cyanide, which suppresses the Cu,Zn-superoxide dismutase activity in both solution and activity-stained gel electrophoresis (Crapo et al. 1978; Beauchamp and Fridovich 1971). An LKB UltroScan XL laser densitometer was used to quantify superoxide dismutase isoenzymes on gels, in the presence of KCN (Hassan and Fridovich 1977). Enzyme activities were assayed using a Lambda 9 Perkin-Elmer spectrophotometer.

Electron spin resonance. Room-temperature ESR measurements were measured with a Bruker ESP 300 spectrometer using a standard TE_{102} cavity. The six-line manganese spectrum was recorded with 100-mT scans using 1-mT modulation, 25-mW incident microwave power and a scan time of 42 s; normally four scans were accumulated to improve the signal-to-noise ratio. Samples of yeast extracts or intact cells were placed directly in the flat ESR room-temperature quartz cell to measure the signal of the ESR-visible Mn^{2+}. The total Mn^{2+} content was determined by addition of 0.1 M HCl to liberate the manganese bound to macromolecular structures and thus ESR-invisible (Miller and Cox 1982).

Results and discussion

Figure 1 shows representative room-temperature ESR spectra of intact yeast cells grown at different Mn^{2+} concentrations. Clearly some Mn(II) is ESR-visible as low-moleular mass Mn(II) complexes. Control spectra, such as the one included in the figure, suggest they might be Mn(II)-phosphate. Figure 2 reports a plot of an ESR-visible (i.e. low-molecular-mass complex) and ESR-silent (that is bound but acid-labile) Mn^{2+} in yeast as a function of the $MnSO_4$ added to the growth medium. The retention is approximately linear for both forms in the range reported in the figure and is still linear up to 15 mM. At the latter concentration inhibition of the cell growth occurred. However some deviation from linearity is evident between the control value and the values recorded above 0.1 mM Mn. It has in fact been established that the accumulation is faster at low Mn^{2+} concentrations (Parkin and Ross 1986b). This trend was seen much better in measurements on yeast extracts (Fig. 3). From the data shown in the figure it can be suggested that: (a) there is a fraction

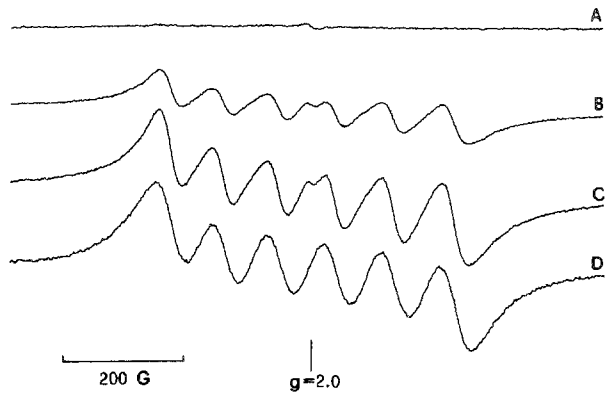

Fig. 1A–D. ESR spectra of yeast cells grown without additional manganese **(A)**, in the presence of 0.5 mM **(B)** or 1.0 mM $MnSO_4$ **(C)**. Loosely packed cell pellets (10^9 cells/ml) were transferred directly to a standard quartz flat ESR cell and measured as described in Materials and methods. For comparison, a spectrum of 1 mM $MnSO_4$ in 100 mM phosphate buffer pH 7.0 **(D)** is included. 200 G ≡ 20 mT

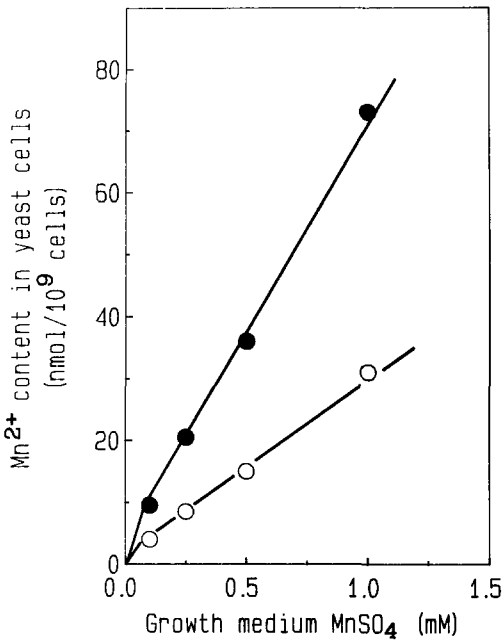

Fig. 2. Manganese content in yeast grown in the presence of different Mn^{2+} concentrations. ESR-visible (○) Mn^{2+} was calculated from the spectra obtained from the pellet of washed cells, whereas total Mn^{2+} (●) was obtained after release of bound Mn^{2+} by treatment with HCl

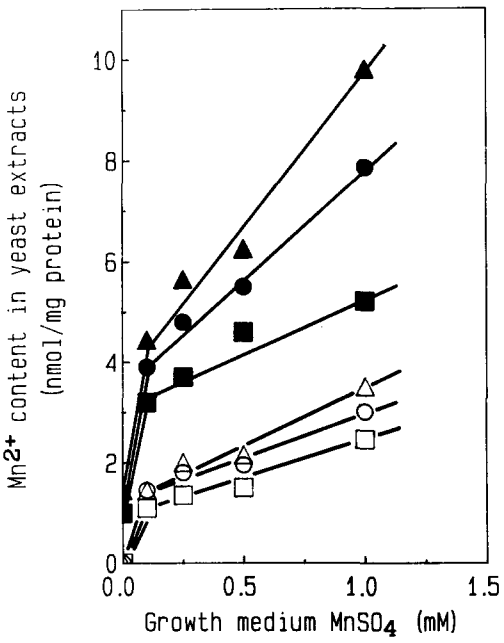

Fig. 3. ESR measurements of manganese content in extracts from yeast grown at different Mn^{2+} concentrations. Results were obtained from undialyzed (△, ▲), once-dialyzed (○, ●) and twice-dialyzed (□, ■) extracts. Visible manganese (△, ○, □) and total manganese (▲, ●, ■) were determined as described in Materials and methods

of manganese that is lost upon centrifugation of cell debris and which apparently provides a major contribution in smoothing out the dual-slope character of the plot in Fig. 2; (b) subsequent dialyses emphasize the difference between the two slopes and lead to a final situation in which the acid-labile and free Mn^{2+} increase with parallel trends.

The presence of manganese in the medium had practically no inductive effect on enzyme activities related to oxygen metabolism (Table 1), contrary to the effects observed with copper salts (Galiazzo et al. 1988). For some activities the effect was inhibitory, although growth was unaffected. Glutathione peroxidase acting on H_2O_2 as substrate was significantly decreased at higher Mn^{2+} concentrations; this result is in contrast with the induction observed in the presence of copper (Galiazzo et al. 1987). No effect on enzyme activities was seen in control experiments where Mn^{2+} was added directly to the various enzymes. Superoxide dismutase activity (Fig. 4) increased as a function of increasing Mn^{2+} concentration and the effect was shown to be related to a 60% increase of the CN-insensitive fraction (which in yeast is due to Mn-superoxide dismutase). In contrast the Cu,Zn-superoxide dismutase activity given by the CN-inhibited fraction was unaffected.

However, performance of the assay on dialyzed extracts (Table 2) suggested that the putative Mn-superoxide dismutase activity was actually due to dialyzable Mn^{2+} complexes. This was confirmed by activity-stained gel electrophoresis of extracts which did not show any substantial change of intensity of the band corresponding to Mn-superoxide dismutase in cells grown at high Mn^{2+} concentration (Table 3).

Conclusions

1. Yeast cells retain manganese up to concentration as high as 15 mM without any deleterious effect on cell growth; slight inhibition of some oxygen-related enzyme activities is observed, is contrast to the effects of copper. This result supports the suggestion that the effects of copper are related to specific mechanisms of induction of this class of enzyme activities (Galiazzo et al. 1987, 1988).
2. Parallel increases of low-molecular-mass and macromolecular complexes are observed by ESR in dialyzed extracts, suggesting the existence of an equilibrium between the different fractions. Dia-

Table 1. Effect of addition of manganese to yeast enzymes

$[Mn^{2+}]$ (mM)	Cytochrome oxidase	Catalase	Fumarase	GSH peroxidase (H_2O_2)	GSH peroxidase (cumene)	GSH transferase
	[μmol min^{-1} (mg protein)$^{-1}$]			[nmol min^{-1} (mg protein)$^{-1}$]		
0 (control)	0.29±0.01	23.6±2.1	0.22±0.04	1.89±0.38	4.6±1.3	1.9±0.1
0.10	0.31±0.03	16.2±0.8	0.11±0.02	0.89±0.15	5.2±0.3	1.3±0.1
0.25	0.29±0.01	12.7±0.5	0.16±0.03	1.20±0.24	5.4±0.1	1.7±0.2
0.50	0.31±0.02	13.7±1.2	0.11±0.01	0.95±0.08	5.0±0.6	1.7±0.2
1.00	0.28±0.02	16.4±0.6	0.12±0.01	0.16±0.10	4.7±0.1	1.5±0.1

lyzed extracts retain both fractions in considerable amounts, although less than undialyzed extracts, and in a different ratio than in undialyzed extracts. It is clear from Figs 2 and 3 that the bound Mn^{2+} is essentially associated with particulate material, since the increase of the corresponding fraction is a function of added Mn^{2+} is less steep in extracts and dialyzed extracts, becoming nearly parallel to that of free Mn^{2+} in twice-dialyzed extracts.

3. Superoxide dismutase activity is unaffected by growth in the presence of high Mn^{2+} concentrations. The apparent increase of CN-insensitive activity was found to be due to dialyzable complexes, while low-molecular-mass complexes retained after dialysis do not contribute to the total Mn-superoxide dismutase activity (Table 2).

In relation to the data obtained with *L. plantarum*, these results show that formation of enzymatically active Mn^{2+} complexes is dependent on the special environment created by cell metabolism and should actually be considered an evolutionary adaptation rather than just an epiphenomenom of the accumulation of high Mn(II) concentrations.

As a general conclusion of this work it may be stated that, in comparison with copper or cobalt (Galiazzo et al. 1988), Mn^{2+} accumulation is harmless to yeast, but at the same time devoid of any regulatory effect, at least as far as oxygen-related enzymes are concerned.

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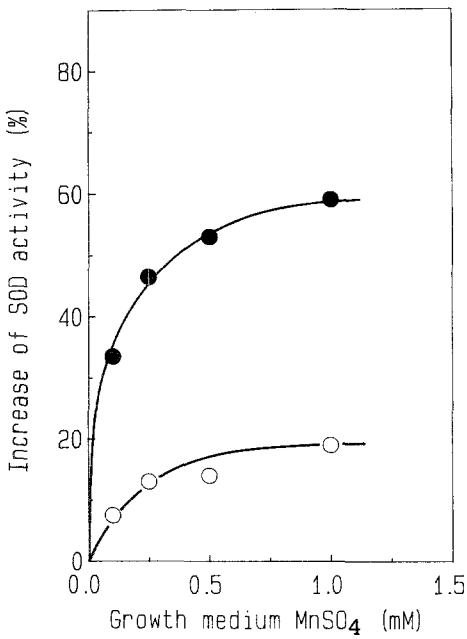

Fig. 4. Effect of Mn^{2+} on total superoxide dismutase (○) and Mn-superoxide dismutase (●) activities in once-dialyzed yeast extracts. Activities were measured as described in Materials and methods

Table 2. Superoxide dismutase (SOD) activity in yeast grown in the presence of different Mn^{2+} concentrations

Extract	$[Mn^{2+}]$ (mM)	Total SOD (U/mg protein)	Mn-SOD
Non-dialyzed	0 (control)	28.3±2.5	15.3±1.3
	0.10	29.3±2.1	18.9±0.8
	0.25	36.2±2.5	24.7±1.5
	0.50	31.1±1.9	21.1±0.6
	1.00	32.2±1.5	24.7±1.0
Once-dialyzed	0 (control)	28.7±2.4	8.8±1.2
	0.10	29.3±1.9	10.7±1.1
	0.25	30.7±2.5	11.0±1.7
	0.50	30.8±2.0	11.8±1.5
	1.00	31.4±2.3	12.0±1.4
Twice-dialyzed	0 (control)	28.8±1.7	9.7±0.9
	0.10	28.9±1.8	8.7±0.4
	0.25	29.6±1.9	9.6±0.2
	0.50	28.8±2.1	10.1±0.3
	1.00	28.0±2.5	9.2±1.3

The activity of the non-dialyzed extract was measured with the omission of EDTA from the reaction mixture

Table 3. Relative amounts of superoxide dismutase (SOD) isoenzymes in yeast grown in the presence of different Mn^{2+} concentrations

$[Mn^{2+}]$ (mM)	Cu,Zn-SOD/ Mn-SOD	Cu,Zn-SOD (% of the total SOD activity)	Mn-SOD
0 (control)	2.4	70.5	29.5
0.10	2.5	71.9	28.1
0.25	2.3	69.9	30.1
0.50	2.2	68.8	31.2
1.00	2.2	69.1	30.9

Cell extracts of *S. cerevisiae* were subjected to disc electrophoresis on 12.5% polyacrylamide slab gel. After electrophoresis the gel was stained for superoxide dismutase activity (Beauchamp and Fridovich 1971) and photographed. The distribution of the isoenzymes was recorded on the negative film by scanning at 633 nm and the Cu,Zn- and Mn-superoxide dismutase activity were estimated from the areas corresponding to the absorbance peaks and expressed as percentage of the total activity in each sample

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