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 MnSO₄. Mn²⁺ retention was nearly linear in intact cells for fractions related to both low-molecular-mass and macromolecular complexes ('free' and 'bound' Mn⁺⁺, respectively). A deviation from linearity was observed in cell extracts between the control value and 0.1 mM Mn²⁺, indicating more efficient accumulation at low Mn²⁺ concentrations. The difference in slopes between the two straight lines describing Mn²⁺ retention at concentrations lower and higher than 0.1 mM, respectively, was quite large for the free Mn²⁺ 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⁺⁺ 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⁺⁺ was essentially associated with particulate structures.

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⁺⁺ 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 O₂⁻ (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⁺⁺ 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⁺⁺ 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-
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 (10000 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% NH₄Cl, 0.03% MgSO₄, 0.09% K₂HPO₄, 0.22% KH₂PO₄. MnSO₄·H₂O was added to the culture medium to obtain the desired concentrations. The Mn⁺² concentration of the medium without added MnSO₄ was 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 rotary shaker (Orbit Environment-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 10³ 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 Lazarow 1951), catalase (Luck 1963), glutathione peroxidase (Lawrence and Bark 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 Ultrasound 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 TE102 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 Mn²⁺. The total Mn²⁺ 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 Mn²⁺ concentrations. Clearly some Mn(II) is ESR-visible as low-molecular 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) Mn²⁺ in yeast as a function of the MnSO₄ 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 Mn²⁺ 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 MnSO₄ (C). Loosely packed cell pellets (10⁹ 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 MnSO₄ in 100 mM phosphate buffer pH 7.0 (D) is included. 200 G = 20 mT](image-url)