PARTICLE-INDUCED X-RAY EMISSION (PIXE): A PRACTICAL APPROACH TO DETERMINE THE MANGANESE ACCUMULATION BY YEAST MITOCHONDRIA[a]

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abstract: In this study, particle-induced X-ray emission (PIXE) is used to monitory the Mn^{2+} accumulation by yeast mitochondria, under conditions of Mn^{2+} stress. We found that mitochondria accumulates the cation, leading to a steady-state level in the first 20 minutes of incubation under high Mn^{2+}.

Introduction

Manganese is an essential metal for all organisms and has been implicated in regulation of growth and metabolism of fungi, mainly due to its influence on several key enzymes. Mn^{2+} has been proposed as an important cell regulator [1] and it may, under some circumstances, substitute for calcium [2]. In yeast, it appears to be preferentially located in the vacuole, where it is bound to polyphosphate; this organelle is thought to be the main determinant for Mn^{2+} detoxification [3-7]. Mn^{2+} is also present in other sucellular compartments. Thus, the cytosol contains Mn^{2+} as well as Mn^{2+}-dependent enzymes, which include pyruvate decarboxylase, glutamine syntethase, and arginase [8]. In the Golgi apparatus, Mn^{2+} activates glycosyltransferases that are involved in the processing of secreted proteins [8,9]. In the mitochondria, Mn^{2+} is required by mitochondrial superoxide dismutase (SOD), by enzymes of the citric acid cycle [8] as well as by proteases involved in mitochondrial protein import. As most of the trace metals, manganese in toxic when present in high concentrations, having growth-inhibitory and mutagenic properties in yeast [10].

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The bakers' yeast *Saccharomyces cerevisiae* represents an ideal eukaryotic system in which to unravel metal trafficking pathways. This organism has been extremely powerful for elucidating the manganese acquisition pathway and a number of candidates for manganese trafficking have already been identified. The transport of manganese (and calcium) into the secretory pathway is accomplished by a Golgi-localized P-type ATPase known as Pmr1p [11-14]. Atx2p is another manganese homeostasis factor that localizes to intracellular vesicles [15] whereas the uptake of manganese into yeast vacuoles is accomplished by vacuolar Ccc1p [16, 17]. Ahp1p is another protein involved in trafficking manganese, apparently transporting this cation from cytosol to mitochondria [18].

When studying metal trafficking, assessment of intracellular distribution of cations can be enlightening. Particle-induced X-ray emission (PIXE) is a multielement analysis technique that is useful for applications in biological sciences. PIXE was used to monitory the uptake or efflux rates of individual trace elements [19,20] as well as the intracellular distribution of manganese [18]. In this article, we use PIXE to monitory the manganese accumulation by yeast mitochondria, under conditions of manganese stress.

Experimental

Yeast strains, growth conditions, and isolation of mitochondria

The brewer's yeast *Saccharomyces cerevisiae*, strain W303 1A (*MAT* **a** *trp1 leu2 ade2 ura3 his3 can1-100*) was used for our experiments. Cells were grown in synthetic medium containing 0.67% yeast nitrogen base without amino acids, amino acids mixture, 0.1% glucose, and for mitochondria proliferation, 3% glycerol. Cell growth was assessed by measuring OD_{600}. For isolation of mitochondria, 100 ml-cultures were used (cell density, $5x10^7$ cells/ml). Mitochondria were isolated from a homogenate of yeast spheroplasts as described by Yaffe [21], including the purification in a Percoll gradient step. Succinate dehydrogenase (SDH) was used as a mitochondrial marker and its activity was measured using the method described by Munujos *et al*. [22]. Mitochondrial extracts were obtained by permeabilizing organelle membranes with digitonin (1mg/mg protein).

Mn^{2+} determination. Yeast cells were grown $5x10^7$ cells/ml, then Mn^{2+} was added to the desired concentration. Cells were incubated with Mn^{2+} for various times. Mn^{2+}-loaded cells were rapidly harvested and transferred to centrifuge tubes that contained 6 ml silicone oil (550; Dow Corning) on top of which had been layered 8 ml of 10 mM MES-Tris buffer (pH 8) with 20 mM EDTA, vortexed briefly and let 10 seconds for phase separation. Cells were harvested by centrifugation through the silicone layer, while the externally-bound ions remained complexed with EDTA. The buffer and the silicone oil were aspirated off, and cells were used to isolate the mitochondria that were subjected to PIXE analysis for Mn^{2+} determination.

Preparation of PIXE samples. 5 μl of purified mitochondria were placed on polycarbonate membranes (Nucleopore Corp., U. S. A., pore size 0.1 μm, thickness ~ 6 μm) and let dry overnight in a desiccator containing silicagel. The Mn^{2+} in the mitochondrial material was determined using a Van de Graaff accelerator (Nissin High Voltage Co. Ltd., model AN-2500). The proton energy used was 2 MeV. The Mn^{2+}

determined was expressed as μg/mg total cellular protein. Proteins were measured using the method described by Bradford [23]. All measurements were done three times, and the results were similar. Unless otherwise stated, all manipulations were done at 4°C.

Results and discussion

The number of individual mitochondria per yeast cell varies from 1-2 in early logarithmic phase on glucose. When grown on nonfermentable carbon sources (*e.g.*, glycerol, ethanol, lactic acid, etc.) the number of mitochondria is higher compared to the same stage of growth on glucose. In this study, we chose glycerol as a carbon source for the yeast cells used to determine the mitochondrial accumulation of Mn^{2+}. Glycerol induces mitochondria proliferation and the yield in mitochondria isolation can be therefore increased.

Mn^{2+} concentration in conventional yeast media is about 3 μM [24], but *S. cerevisiae* cells are viable in environments containing much higher levels. Thus, the wild-type strain W303 1A that was used in our experiments could grow on glycerol agar plates containing $MnCl_2$ up to concentrations as high as 8-10 mM (data not shown). We also determined the growth of yeast cells in liquid glycerol media containing various concentrations of $MnCl_2$ (Fig. 1).

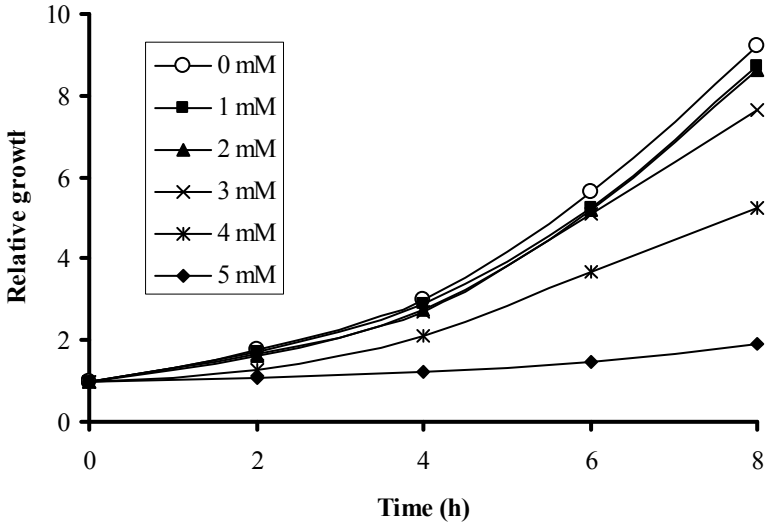

Fig.1. ***Influence of Mn^{2+} on growth.*** *Yeast cells were grown in synthetic media containing 3% glycerol up to 1 x10^6 cells/ml, before various concentrations of $MnCl_2$ were added. Growth was assessed by measuring OD_{600}.*

We found that Mn^{2+} did not impair cell growth at concentrations up to 3 mM. At 4 mM, Mn^{2+} reduced growth rate considerably, but the cells could still undergo division. At 5 mM,

Mn^{2+} induced growth arrest (Fig. 1). To determine the Mn^{2+} accumulation by mitochondria, we used the 3 mM $MnCl_2$ concentration in the incubation medium. At this concentration, Mn^{2+} accumulation did not interfere with mitochondria integrity and proliferation (as seen by microscopic analysis and SDH activity, data not shown), and at the same time it was high enough to allow detection of Mn^{2+} dynamics in mitochondria. We found that Mn^{2+} accumulated in the mitochondria in the first 20 minutes of exposure of cells to the cation, before reaching a saturation level (Fig. 2A).

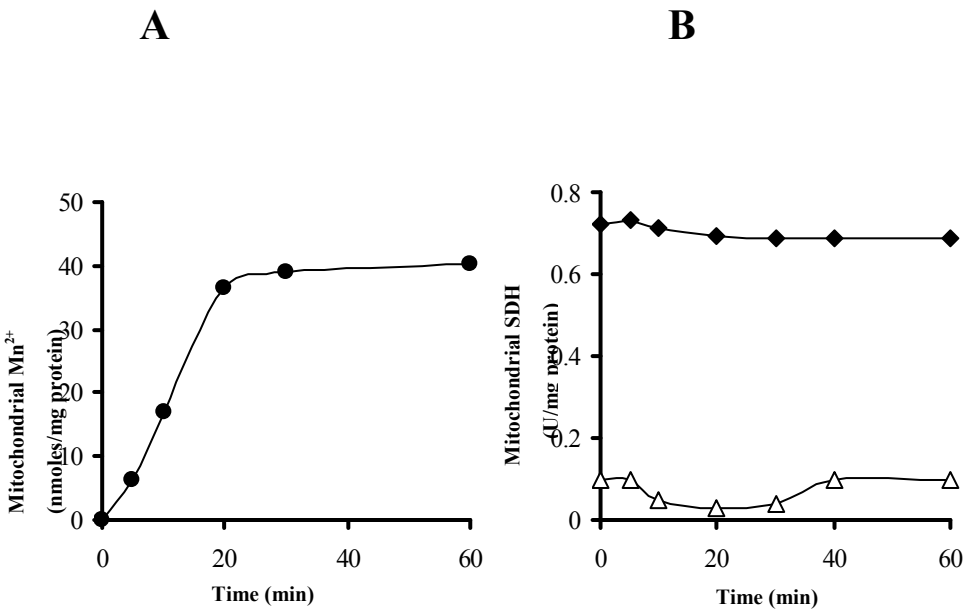

Fig. 2. ***A. Mn^{2+} accumulation in yeast mitochondria.*** *Yeast cells were grown in synthetic media containing 3% glycerol to 5 x10^7 cells/ml, before $MnCl_2$ was added (3 mM final concentration). Mitochondria were isolated and their Mn^{2+} content was determined by PIXE.* ***B. Succinate dehydrogenase (SDH) activity*** *was monitored as a mitochondrial marker. SDH activity was measured in mitochondrial extracts (closed diamonds) and non-mitochondrial extracts (opened triangles).*

The activity of mitochondrial enzyme succinate dehydrogenase was measured as mitochondrial marker in the extracts obtained from the same pool that was used for Mn^{2+} detection by PIXE (Fig. 2B, closed diamonds). The non-mitochondrial fraction was also tested for SDH activity and was found to be almost free of mitochondrial contamination (Fig. 2B, opened triangles). The mitochondrial fractions used for Mn^{2+} detection were also tested for contamination by other subcellular components, but we practically found no activity of vacuolar or cytosolic enzymatic markers (data not shown). Although we cannot completely rule out the possibility of contamination with other subcellular compartments, we consider that the approach we used offers a possibility to monitor cation accumulation by yeast mitochondria.

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