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Iron storage in *Saccharomyces cerevisiae*

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A ferritin-like molecule was purified from iron-loaded cells of *Saccharomyces cerevisiae*, but its iron content was very low and was not representative of the cellular iron content. A study of the intracellular distribution of iron has shown that the vacuoles are involved in the storage of iron in the yeast cell. Moreover, it seems that this vacuolar iron can be further utilised by the cells for iron-requiring processes such as mitochondriogenesis.

Iron storage; Ferritin; Vacuole; (*Saccharomyces cerevisiae*)

1. INTRODUCTION

The chemistry of iron at physiological pH is essentially dominated by the hydrolysis and polymerisation of aqueous FeIII to insoluble and biologically inaccessible ferric hydroxides and oxyhydroxides. For this reason in all types of organism, once iron has been introduced within the cell, an intracellular storage form of iron is required which is soluble, non-toxic and bioavailable. In a wide variety of organisms the major form of iron storage is ferritin. This protein has been isolated from mammalian tissues (review [1]), plants [2], fungi [3] and more recently from bacteria [4–6]. Mammalian ferritin consists of a spherical protein shell composed of 24 structurally equivalent subunits disposed in 432 symmetry. Each subunit has a molecular mass of 20 kDa. The inner cavity contains up to 4500 iron atoms as a ferric oxyhydroxide polymer (review [7]). Bacterioferritins have a similar structure except that their subunit molecular mass is smaller (15–17 kDa) and they all contain haem groups associated with the protein [4–6].

In a previous study, we showed that iron was physiologically reduced by a transplasma membrane redox system before its uptake in the yeast cell [8]. However, nothing is known about its intracellular distribution and the way it is stored. As regards the intracellular fate of iron in fungi, in *Phycomyces* iron is stored in ferritin and this ferritin iron is in equilibrium with a soluble iron pool which may be a donor of iron for biosynthesis [9]. In other studies, it has been shown by electron microscopical observations that a ferric phosphate polymer is located on the vacuole membrane and in the vacuoles of the fungus *Penicillium* [10].

In yeast, vacuoles are the largest organelles and they are postulated to function as lysosomes and as a storage compartment [11]. A great number of divalent cations accumulate mainly in vacuoles where they are bound to polyphosphates [12,13]. This compartmentation is of the greatest importance in maintaining a cytoplasmic homeostasis for physiologically important cations such as Ca^{2+} [14], Mg^{2+} [13] and Zn^{2+} [15] and for the storage of potentially toxic cations that can be accumulated by the yeast cell (Sr^{2+}, Pb^{2+}, Cr^{3+}, ...).

In this paper, we describe two forms of iron storage in *Saccharomyces cerevisiae*: the first is a cytosolic ferritin-like molecule and the second im-

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Abbreviations: PMSF, phenylmethanesulfonyl fluoride; Mops, 3-(*N*-morpholino)propane sulfonic acid

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plies the compartmentation of iron at the vacuolar level.

2. MATERIALS AND METHODS

2.1. *Culture conditions*

Saccharomyces cerevisiae D261 was grown aerobically at 30°C in a medium containing (in 1 litre of water): 20 g yeast extract, 2 g KH_2PO_4, 2 g $(NH_4)_2SO_4$ and either 30 g glucose or 50 g glucose or 30 g ethanol plus 1 g glucose. Iron was added as ferric citrate in amounts ranging from 1 to 20 mg/l.

2.2. *Ferritin purification*

The cells were grown in the culture medium described above with 30 g/l glucose and 4 mg/l iron and harvested in the stationary phase. The cell paste was resuspended in 0.03 M phosphate buffer, pH 7.5, containing 0.001 M PMSF and disrupted in a Dyno-Mill grinder. The cell debris were eliminated by centrifugation (20000 × *g*/30 min) and a 2% (w/v) protamine sulfate solution was added to the supernatant in order to remove nucleic acids. The suspension was clarified by centrifugation and dialysed against 0.03 M phosphate buffer, pH 7.5. The sample was applied to a DEAE-cellulose column equilibrated with the same buffer. After extensive washing of the column with the equilibration buffer, ferritin was eluted by a linear gradient NaCl from 0 to 0.5 M in the same buffer. The purification was pursued on a FPLC chromatographic system (Pharmacia, Uppsala, Sweden) using a Mono Q column. Ferritin was eluted with the same gradient as above. Thereafter, it was concentrated by ultrafiltration and applied to a column of Sephacryl S300 (105 × 4 cm). The elution was performed with 0.1 M Tris-HCl buffer, pH 8.3. Ferritin was finally purified on a CsCl density gradient: 0.5 ml of concentrated sample was layered on 4.5 ml of 65% (w/v) CsCl and centrifuged for 20 h at 48000 rpm (rotor SW65). Ferritin sedimented as a brown band at a density of 1.33 g/ml. After extensive dialysis, purity was checked by PAGE and SDS-PAGE. Ferritin iron was determined as in [16]. Spectral studies were carried out on an Aminco DW2A spectrophotometer (American Instruments, Silver Springs, USA).

2.3. *Cellular distribution of iron*

The cells were grown as described above with either 5% glucose or 3% ethanol plus 0.1% glucose and 1 mg/l ^{59}FeIII-citrate. The cells were harvested in exponential phase and one part of these cells was incubated at 100 mg/ml with 20 mg/l ^{59}FeIII-citrate and 5% glucose for 2 h at 30°C. The intracellular iron distribution was compared between these cells. For the ethanol adaptation experiments, the cells were grown with 5% glucose and 20 mg/l ^{59}FeIII-citrate, harvested in exponential phase, washed 4 times with 2% EDTA and transferred to an iron-deprived medium [17,18] with 3% ethanol and 0.1% glucose. Samples were taken at different times and the intracellular iron distribution was investigated. For studies of iron uptake by protoplasts, protoplasts (100 mg/ml) were preincubated for 30 min at 30°C with 5% glucose and then iron was added (0.36 mM ^{59}FeIII-citrate). Samples were taken at the indicated times and the intracellular iron distribution was determined. In all cases, the cells were washed 4 times with 2% EDTA prior to their transformation into protoplasts in order to remove iron non-specifically bound to the cell walls.

Protoplasts were prepared according to [19]. They were washed 3 times with buffer A (1 M sorbitol, 0.5 mM EDTA, 10 mM imidazole, pH 6.4). A moderate osmotic shock was realised by resuspending them in the same medium with 0.3 M sorbitol. After 2 min of stirring, the suspension was passed 5 times in a potter homogenizer. After 5 min, the homogenate was diluted twice with buffer A in order to increase the osmotic pressure. The homogenate was then centrifuged 3 times at 50 × *g*/5 min. The pellet containing unbroken cells was discarded and the supernatant was centrifuged at 3000 × *g*/30 min. The pellet (P3000) was resuspended in minimum buffer B (0.7 M sorbitol, 10 mM Mops-Tris, pH 7) and layered on top of a discontinuous gradient made of buffer C (0.7 M sucrose, 10 mM Mops-Tris, pH 7), buffer C + 2.5% Ficoll and buffer C + 5% Ficoll. The centrifugation was carried out at 10000 × *g*/10 min. Vacuoles which sedimented on top of the buffer C layer were then diluted with buffer B and centrifuged at 3000 × *g*/30 min. The pellet was resuspended in minimum buffer B and recentrifuged in the Ficoll gradient. The vacuoles were then pure enough. The supernatant S3000 was centrifuged at 17000 × *g*/15 min and the resulting supernatant (S17000) was recentrifuged at 100000 × *g*/30 min to yield the cytosolic fraction. The pellet P17000 was resuspended in buffer B and centrifuged at 1100 × *g*/10 min. The pellet was discarded and the supernatant was centrifuged at 17000 × *g*/10 min. The resulting pellet was the mitochondrial fraction. The temperature was kept at 4°C throughout the operations.

Iron in the cellular fractions was measured by counting the radioactivity of the samples in a scintillation counter (Packard Instruments). Alkaline phosphatase [20], α-mannosidase [21], cytochrome oxidase [22] and glucose-6-phosphate dehydrogenase [22] activities were determined by published methods. Protein concentration was determined according to [23] with bovine serum albumin as standard. Ficoll 400 was purchased from Pharmacia.

3. RESULTS

3.1. *Ferritin*

A ferritin-like molecule was purified from iron-loaded cells of *S. cerevisiae*. This protein gave a single band on PAGE stained for proteins but also a positive Prussian blue reaction indicative of its iron content. Its molecular mass was estimated to be 274 kDa, as determined by gel filtration using a superose 12 column (Pharmacia) and horse spleen ferritin, catalase, aldolase, bovine serum albumin and ovalbumin as molecular mass markers. The subunit molecular mass was ~11 kDa, as determined by SDS-PAGE with horse spleen ferritin, myoglobin, lysozyme, cytochrome *c* and insulin as molecular mass markers.

As shown in fig.1, the visible absorption spectrum of this protein gave a maximum at 411 nm in the oxidized state. In the reduced spectrum, the

main band shifted from 411 to 420 nm and two other bands appeared at 525 and 555 nm. This is a typical cytochrome *b* spectrum which was also reported for different bacterioferritins [5,6]. It should be noted that when ferritin was purified by another method, including ammonium sulfate precipitation and thermic denaturation, it did not show this cytochrome spectrum anymore (unpublished). The iron content of yeast ferritin was found to be very low: typically 50 to 100 iron atoms per molecule (assuming a molecular mass of 270 kDa). Moreover, when we increased the iron concentration in the culture medium (20 mg/l or more), the iron content of the cells increased but not the iron content of ferritin (not shown).

3.2. *Cellular distribution of iron*

A moderate osmotic shock (0.3 M sorbitol) was found to be the most reproducible method for obtaining well-preserved intracellular structures. The purity of the cellular fractions was tested (tables 1 and 2) and agreed well with published data [24]. Moreover, the integrity of the purified vacuoles was confirmed microscopically by their ability to accumulate neutral red and their bursting in distilled water.

From the data presented in tables 1 and 2, it appears that the cytosolic fraction contains little iron either with cells showing a fermentative metabolism (glucose-grown cells) or a respiratory metabolism (ethanol-grown cells). An interesting point is that the iron content of this cellular fraction does not increase and even decreases when the iron concentration in the culture medium is raised from 1 to 20 mg/l. In contrast, there is a clear iron accumulation in the vacuolar fraction with both types of metabolism: the vacuolar iron concentration increases 8.8 times with ethanol-grown cells and 5.9 times with glucose-grown cells. Finally, the amount of iron in the mitochondrial fraction seems to be relatively constant: the mitochondrial iron concentration in ethanol-grown cells increases only 1.3 times when the extracellular iron concentration increases 20 times. It should be noted here that glucose-grown cells do not possess fully developed mitochondria [25]: the 'mitochondrial' fraction in table 2 is probably derived from vacuolar contamination. However, this fraction is quantitatively negligible.

In order to determine whether or not vacuolar

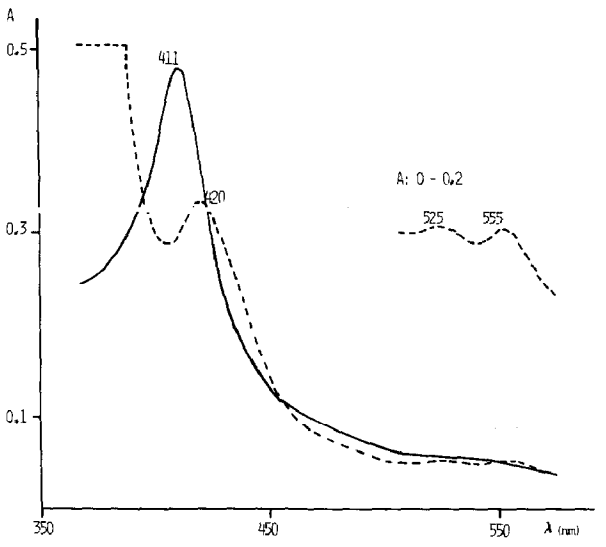

Fig.1. Visible absorption spectrum of yeast ferritin: (——) oxidised state; (– – –) reduced state (with a few grains of dithionite).

iron can be further utilised by the cells, iron-rich cells which did not contain active mitochondria (glucose-grown cells) were adapted on an iron-poor ethanol medium which stimulates mitochondriogenesis and the evolution of iron distribution was investigated (table 3). The increasing activity of cytochrome oxidase, a mitochondrial marker enzyme, indicates the progressive appearance of mitochondria in the cells during the adaptation to a respiratory metabolism. In parallel, we observe the decrease of the vacuolar iron content. The iron values reported for the mitochondrial fraction must be corrected because at the beginning of the experiment the major part of the iron is not due to the few mitochondria already present in the fraction but more probably to contaminations (see above). After 20 h of growth in ethanol, the cytochrome oxidase reaches a plate of maximum activity (not shown) indicating that the mitochondria have reached their full development at that time. Therefore we can consider that the major part of the iron in that fraction is associated with active mitochondria and then, on the basis of the cytochrome oxidase activity, we can estimate the part of iron associated with active mitochondria throughout the experiment as in table 4. These corrected values show an increase in the mitochondrial iron concentration during the ethanol

Table 1

Intracellular iron distribution of cells grown on 3% ethanol and enzymic activities of the cellular fractions

		Cellular fractions			
		Protoplasts	Vacuoles	Mitochondria	Cytosol
Specific enzymatic activities (nmol·min^{-1}·(mg protein)$^{-1}$) and purification factors	alkaline phosphatase	27.8 ± 5.7 (1)	1720 ± 280 (62)	5.5 ± 2.1 (0.2)	14.4 ± 2.6 (0.5)
	α-mannosidase	0.036 ± 0.01 (1)	0.67 ± 0.04 (19)		
	cytochrome oxidase	248.7 ± 46.8 (1)	49.5 ± 8.6 (0.2)	1090 ± 184 (4)	28.3 ± 5.4 (0.1)
	glucose-6-phosphate dehydrogenase	270 ± 25 (1)			283 ± 38 (1)
Iron content (μg Fe·(mg protein)$^{-1}$) and enrichment factor	cells grown with 1 mg Fe/l	0.04 ± 0.01 (1)	0.23 ± 0.06 (5.7)	0.10 ± 0.03 (2.5)	0.016 ± 0.002 (0.4)
	cells incubated with 20 mg Fe/l	0.15 ± 0.03 (1)	2.06 ± 0.61 (14)	0.13 ± 0.02 (0.9)	0.024 ± 0.002 (0.2)

Mean ± deviations of three experiments

Table 2

Intracellular iron distribution of cells grown on 5% glucose and enzymic activities of the cellular fractions

		Cellular fractions			
		Protoplasts	Vacuoles	Mitochondria	Cytosol
Specific enzymatic activities (nmol·min^{-1}·(mg protein)$^{-1}$) and purification factors	alkaline phosphatase	22.8 ± 3.5 (1)	1038 ± 142 (45)		8.5 ± 2.7 (0.4)
	α-mannosidase	0.08 ± 0.01 (1)	1.01 ± 0.03 (13)		
	cytochrome oxidase	23.5 ± 2.3 (1)	15.8 ± 3 (0.7)	11.8 ± 4.7 (0.5)	
	glucose-6-phosphate dehydrogenase	59.9 ± 2.3 (1)			81.8 ± 4.1 (1.4)
Iron content (μg Fe·(mg protein)$^{-1}$) and enrichment factor	cells grown with 1 mg Fe/l	0.20 ± 0.02	1.19 ± 0.48	0.07 ± 0.01	0.08 ± 0.01
	cells incubated with 20 mg Fe/l	0.63 ± 0.11 (1)	7.10 ± 2.34 (11)	0.48 ± 0.10 (0.8)	0.05 ± 0.01 (0.1)

Mean ± deviations of two experiments

adaptation. Moreover, this supply of iron to the mitochondria is accompanied by the decrease of vacuolar iron concentration. From these data, it seems likely that vacuolar iron is mobilised for mitochondriogenesis.

If we consider the kinetics of intracellular iron distribution (fig.2), we can see that the presence of iron in the vacuolar compartment occurs very soon after its uptake by the cells. Moreover, the accumulation rate of iron in this compartment is maximal at the beginning of the incubation of the cells with iron, whereas it is very low in the mitochondrial and cytosolic fractions but increases after 30 min. This might suggest that the iron which is taken up by the cells could transit in the vacuoles before its redistribution in the other cellular compartments.

4. DISCUSSION

The subunit molecular mass of yeast ferritin was found to be rather small compared to that of other ferritins: 11 kDa compared with 20 kDa for mammalian ferritins [7] and 15 kDa to 17 kDa for

Table 3

Evolution of the intracellular iron distribution of cells grown on 5% glucose and adapted on 3% ethanol; evolution of the cytochrome oxidase activity

		Time after ethanol adaptation (h)			
		0	6	12	20
Total cellular iron (μg Fe·(g cells)$^{-1}$)		109.6 ± 19.8	94.7 ± 14.1	80.8 ± 13.6	44.8 ± 9.8
Iron content[a] (μg Fe·(mg protein)$^{-1}$)	protoplasts	0.58 ± 0.07	0.65 ± 0.08	0.55 ± 0.07	0.52 ± 0.06
	vacuoles	5.52 ± 1.14	4.40 ± 1.38	4.02 ± 1.52	1.79 ± 0.84
	mitochondria	0.67 ± 0.09	0.54 ± 0.05	0.35 ± 0.04	0.49 ± 0.02
	cytosol	0.042 ± 0.019	0.022 ± 0.003	0.026 ± 0.003	0.024 ± 0.002
Cytochrome oxidase specific activity (nmol·min^{-1}·(mg protein)$^{-1}$)	protoplasts	24.7 ± 3.5	28.3 ± 4.8	49.5 ± 5.3	102.6 ± 7.1
	mitochondria	10.6 ± 3.4	74.3 ± 10.6	222.9 ± 31.9	372.9 ±47.4

[a]These values are corrected in order to counterbalance the dilution of cellular iron due to cellular growth.
Mean ± deviations of two experiments

Table 4

Time after ethanol adaptation (h)	Cytochrome oxidase specific activity (nmol·min^{-1}·(mg protein)$^{-1}$)	Total iron in the mitochondrial fraction (μg·(mg protein)$^{-1}$)	Mitochondrial iron (μg·(mg protein)$^{-1}$)
0	10.6	0.67	0.02
6	74.3	0.54	0.11
12	222.9	0.35	0.21
20	372.9	0.49	0.49

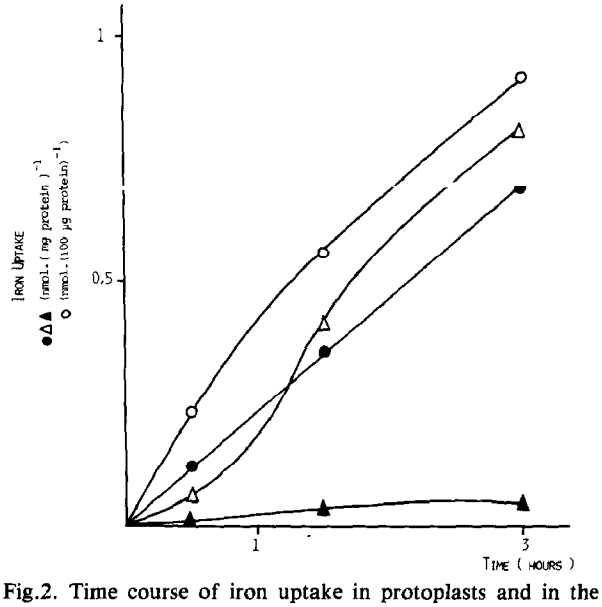

Fig.2. Time course of iron uptake in protoplasts and in the subcellular fractions: (●) protoplasts; (○) vacuoles; (△) mitochondria; (▲) cytosol.

bacterioferritins [4–6]. In contrast, a global molecular mass of 274 kDa is close to that found for the bacterioferritin of *E. coli* [5] which was 269 kDa. The ratio of the subunit to oligomer molecular masses of yeast ferritin suggests that it is made of 24 subunits. As in bacterioferritins, the presence of a haem group was found in our ferritin. This could suggest a closer structural resemblance with bacterioferritins than with other ferritins. A quite surprising fact for a protein which was thought to be a major iron storage compound is its low iron content. Moreover, this iron content is essentially independent of intracellular iron concentrations. This led us to suspect that another mechanism was implied in iron storage at least to protect the cells from excess iron when growing with high iron contents.

Our results show that the vacuole is the major iron storage compartment in the yeast cell: it is the only compartment to greatly increase its iron concentration when the cells are submitted to a high iron content in their environment. This vacuolar iron storage is theoretically possible taking into account the acidic pH of this compartment and the presence of polyphosphates [11] to which ferric iron can bind. We note here that the cytosolic iron content is relatively constant over a 20-fold increase of extracellular iron concentration and this agrees well with the invariability of ferritin iron content. However, it seems that the vacuole is not a passive storage form for excess iron in the cell

but that its iron can be utilised by the cell for iron requiring processes such as mitochondriogenesis. Other in vitro results show that vacuolar iron can be mobilised by the combined action of a FeII-chelating reagent and a reductant (not shown).

We conclude that there are two iron storage pools in yeast: a cytosolic one associated with a ferritin-like protein and a vacuolar one. The relationship between these two pools and their biological significance needs to be investigated.

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