Reductive and Non-reductive Mechanisms of Iron Assimilation by the Yeast *Saccharomyces cerevisiae*

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Iron reduction and uptake was studied in wild-type and haem-deficient strains of *Saccharomyces cerevisiae*. Haem-deficient strains lacked inducible ferri-reductase activity and were unable to take up iron from different ferric chelates such as Fe(III)–citrate or rhodotorulic acid. In contrast, ferrioxamine B was taken up actively by the mutants as well as by the wild-type strains. At a low extracellular concentration, uptake was insensitive to ferrozine and competitively inhibited by Ga(III)–desferrioxamine B. Extracellular reductive dissociation of the siderophore occurred at higher extracellular concentrations. Two mechanisms appear to contribute to the uptake of ferrioxamine B by *S. cerevisiae*: one with high affinity, by which the siderophore is internalized as such and another with lower affinity by which iron is dissociated from the ligand prior to uptake.

INTRODUCTION

Iron transport in micro-organisms has been intensively studied, and most of this research has focused on the high-affinity iron uptake systems mediated by siderophores (for reviews see Winkelmann & Huschka, 1987; Bagg & Neilands, 1987). Secretion of siderophores in response to iron-limitation occurs in most micro-organisms including bacteria and fungi, but there are no reports of a siderophore secreted by the yeast *Saccharomyces cerevisiae*. Moreover, the chemical assay for the detection of siderophores (Schwyn & Neilands, 1987) gave negative results with this yeast (Neilands *et al.*, 1987). Nevertheless, it is well known that the use of siderophores as iron sources is not restricted to the organisms that produce them. A variety of siderophores that occur in soils may supply iron to plants either directly (receptor-mediated transport) or indirectly, after extracellular dissociation of the chelate (Crowley *et al.*, 1987; Castignetti & Smarrelli, 1986).

In a previous paper (Lesuisse *et al.*, 1987), we showed that, in common with several dicotyledonous plants (Bienfait, 1985, 1987; Romheld, 1987), *S. cerevisiae* is able to reduce extracellular ferric chelates – including siderophores – by a membrane-bound redox system that is induced in iron-deficient conditions. We proposed that iron must be reduced to be taken up by the cells, where it is stored in vacuoles, as are other divalent cations (Raguzzi *et al.*, 1988).

In this paper, we have re-examined the physiological role of the ferri-reductase activity in iron uptake by *S. cerevisiae* and the iron uptake mechanisms of this yeast.

METHODS

Growth conditions. The haploid strains FL100, G122 and G204 of *Saccharomyces cerevisiae* (Urban-Grimal & Labbe-Bois, 1981) were grown in liquid nutrient medium in Erlenmeyer flasks with mechanical agitation at 30 °C. The composition of media was as follows (per litre of distilled water). (1) Iron-rich medium: 30 g glucose; 6.7 g yeast nitrogen base without amino acids (Difco); 1 g yeast extract; 30 mg ergosterol; 2 ml Tween 80; 180 µmol

Abbreviations: ALA, 5-aminolevulinic acid; FOB, ferrioxamine B; DFOB, desferrioxamine B.

iron (as ferric citrate or FOB); and either 10 mg histidine (for G204) or 10 mg uracil (for G122). (2) Iron-deficient medium: as (1) without added iron; residual iron was removed by the method described by Nicholas (1957) before addition of 30 mg ergosterol, 2 ml Tween 80 and 50 mg ferrozine. (3) Preculture medium: as (1) without added iron. When necessary (see Results) the media were supplemented with 50 mg ALA l⁻¹.

Culture vessels were washed with HCl/HNO₃ (3:1, v/v) and rinsed with distilled water. Sterilized medium was inoculated with 10⁶ cells ml⁻¹ from a 24 h preculture and cells were harvested after 15 h, in the late exponential growth phase. The cells were washed twice with distilled water and once with 50 mM-trisodium citrate buffer, pH 6.5, and used the same day for experiments.

Iron compounds. The ferric complexes ⁵⁵Fe-citrate, ⁵⁵Fe(OH)₃, ⁵⁵Fe-FOB and ⁵⁵Fe-rhodotorulic acid were prepared for uptake experiments from ⁵⁵FeCl₃ (Amersham) in order to give about 78 MBq (mg Fe)⁻¹.

Iron uptake. The washed cells were suspended at 50 mg wet wt ml⁻¹ in 50 mM-citrate buffer, pH 6.5, or in 50 mM-sodium acetate buffer, pH 5.2. Radioactive iron was added after 30 min preincubation at 30 °C with 5% (w/v) glucose with slow magnetic stirring. Samples (1 ml) were withdrawn at intervals and diluted 1:1 (v/v) with unlabelled iron (3 mM) on ice. The samples were washed to remove iron non-specifically bound to the cell surface, as follows: twice with 2% (w/v) EDTA in 50 mM-citrate buffer (pH 6.5); twice with dithionite (100 mM); and once with distilled water. Radioactivity in the washed samples was measured after solubilization of the cells (15 h at 50 °C in 1 ml NCS tissue solubilizer; Amersham).

Iron reduction. Ferri-reductase activity of the washed whole cells was measured as previously described (Lesuisse *et al.*, 1987), with 180 µM-ferric citrate as substrate and 5% (w/v) glucose in 50 mM-citrate buffer, pH 6.5.

RESULTS AND DISCUSSION

Ferri-reductase activity of haem-deficient cells

Whole cells of strains G122 and G204 were tested for ferri-reductase activity. Both strains lack cytochromes, have no catalase activity and do not grow on glycerol (Urban-Grimal & Labbe-Bois, 1981). In addition to its possible deficiency in iron metabolism (Urban-Grimal & Labbe-Bois, 1981), G122 was described as being deficient in protoporphyrinogen oxidase activity (Camadro *et al.*, 1982). G204 is an ALA-synthase-deficient mutant which responds to exogenous ALA by recovering the wild phenotype (presence of a functional respiratory chain) of the parental strain, FL100 (Urban-Grimal & Labbe-Bois, 1981).

As previously described (Lesuisse *et al.*, 1987), iron-deficient conditions induced ferri-reductase activity in cells with the parental phenotype (FL100 and G204 supplemented with ALA (Table 1). Basal activity (in iron-rich conditions) and induced activity (in iron-deficient conditions) were both maximal in the late exponential growth phase (not shown). In contrast, haem-deficient cells (G122 and G204) exhibited only a very low, non-inducible level of ferri-reductase activity (Table 1). This residual activity remained unchanged during all stages of growth, either in iron-rich or in iron-deficient conditions (not shown).

From these results, we conclude that haem is involved in the development of ferri-reductase activity. The residual activity exhibited by the two haem-deficient mutant strains could be due to intracellular traces of haem not detectable by spectral analysis; indeed, it had been previously suggested that the haem mutations of these strains could be leaky to some extent (Lewis *et al.*,

Table 1. *Ferri-reductase activity of different strains of S. cerevisiae grown in iron-rich or iron-deficient media*

Iron reduction was measured as described in Methods. Results are means ± SD of three (a), five (b) or nine (c) experiments.

Strain	Ferri-reductase activity [nmol h ⁻¹ (mg wet wt) ⁻¹]	
	Iron-rich medium	Iron-deficient medium
FL100 ^a	3.4 ± 0.88	37.2 ± 7.8
G122 ^b	0.36 ± 0.2	0.54 ± 0.2
G204 ^c	0.39 ± 0.2	0.60 ± 0.3
G204 supplemented with ALA ^c	4.14 ± 1.3	40.9 ± 9.5

Fig. 1. Absorbance spectra of cells of strain G204 grown for 24 h in iron-rich medium (as ferric citrate or FOB) with or without ALA. (a) Visible spectrum of FOB (100 μM) in water; (b) cells grown with FOB but without ALA; (c) cells grown with FOB and with ALA; (d) cells grown with ferric citrate but without ALA; (e) cells grown with ferric citrate and with ALA. The reference cuvette contained water (a) or suspension of cells of G204 grown in iron-deficient conditions without ALA (b–e). Cells were suspended at 100 mg wet wt ml^{-1} in the sample and reference cuvettes and were previously washed once with dithionite (100 mM) and twice with EDTA (2%, w/v).

1985). In any case, the absence of significant inducible ferri-reductase activity in the haem-deficient strains made them a valuable tool for investigating the role of reduction in iron uptake.

Accumulation of iron during growth

The effect of ALA on the capacity of strain G204 to take up iron from $^{55}\text{Fe(III)}$ -citrate and from ^{55}Fe -FOB was examined. The absence of ALA in the culture medium significantly lowered the amount of iron accumulated from ferric citrate but not from FOB: after 24 h growth in iron-rich medium, G204 had accumulated 268 pmol iron (mg wet wt) $^{-1}$ when FOB (180 μM) was used as iron source and only 13 pmol iron (mg wet wt) $^{-1}$ when FOB was replaced by ferric citrate; the values were 175 and 256 pmol (mg wet wt) $^{-1}$ respectively when ALA was added to the culture medium (data from a single representative experiment). This indicates that reduction is not an obligatory step in iron uptake from FOB as previously suggested (Lesuisse *et al.*, 1987). Fig. 1 shows the absorbance spectra of the washed cells after growth on iron-rich medium (as FOB or ferric citrate). As shown by the high absorbance in the range 420–440 nm, significant amounts of FOB were accumulated without dissociation by haem-deficient cells; however, in the case of ALA-supplemented cells the ferri-siderophore was largely dissociated, probably by reduction.

Iron uptake by non-growing cells

To evaluate the relative contributions of the reductive and non-reductive mechanisms of iron assimilation, the rate of iron uptake was measured for different ferric chelates under different conditions. As shown in Table 2, iron was effectively taken up from ferric citrate, Fe(OH)_3 or from the siderophore rhodotorulic acid only by cells with induced ferri-reductase activity (ALA-supplemented), uptake being strongly inhibited by the iron(II)-trapping reagent ferrozine. In contrast, two distinct mechanisms contributed to the uptake of iron from FOB (Table 2): at a low extracellular concentration (7 μM), uptake appeared to be essentially non-reductive, as iron was accumulated not only by ALA-supplemented cells grown in iron-deficient conditions, but

Table 2. *Rate of iron uptake from different ferric chelates by cells of strain G204 grown in iron-rich medium (as FOB or ferric citrate) or in iron-deficient medium, with or without ALA*

Uptake experiments were done as described in Methods, except that with $\text{Fe}(\text{OH})_3$, citrate buffer was replaced with 50 mM-MES/KOH, pH 7. When present, ferrozine, Ga^{3+} as $\text{Ga}(\text{NO}_3)_3$ or $\text{Ga}(\text{III})\text{-DFOB}$ were added together with iron, after preincubation. The condition '+ O_2 ' refers to the bubbling of oxygen through the cell suspension during the entire experiment. Results are means of five (a) or three (b) experiments, or are from one experiment only (c). Values in parentheses are the percentage inhibition of uptake by 1 mM-ferrozine.

	Iron uptake rate [$\text{pmol h}^{-1} (\text{mg wet wt})^{-1}$] by resting cells previously grown in medium containing:					
	ALA + FOB	ALA + ferric citrate	ALA (iron-deficient conditions)	FOB	Ferric citrate	(Iron-deficient conditions)
Fe(III)-citrate (7 μM) ^a	9.8 (91)	11.3 (95)	59 (94)	1.2 (9)	1.2 (11)	0.2 (0.5)
Fe(III)-citrate (360 μM) ^c		162 (99)				
Fe(OH) ₃ (7 μM) ^c		0.8 (100)	6.4 (99)		0.2	0.7
Rhodotorulic acid (7 μM) ^b		0.94 (77)	2.4 (82)		0.07	0.16
FOB (7 μM) ^a	7.2 (12)	12.3 (12)	69.7 (27)	60 (0)	59.4 (0)	64 (0)
FOB (7 μM) + O_2 ^b			37.5 (0)			
FOB (7 μM) + Ga(III)-DFOB (50 μM) ^b		2.8	23.5		5.2	4
FOB (7 μM) + Ga^{3+} (50 μM) ^b		9.2 (40)	74 (53)		61.3 (0.2)	63.2 (0)
FOB (360 μM) ^b	41.7 (60)	62 (70)	480 (79)	113.5 (0)	127 (0)	121 (2)

also by cells grown without ALA in iron-rich or in iron-deficient media. In all cases, the inhibitory effect of ferrozine was low (0 to 27%) and was totally suppressed if the suspension was saturated with oxygen during incubation with iron (Table 2). The contribution of the reductive mechanism to the uptake process was increased when the concentration of FOB was raised; at 360 μM -FOB, most of the iron was taken up by the reductive mechanism in ALA-supplemented cells, as shown by the strong inhibitory effect of ferrozine (Table 2). Extracellular dissociation of FOB prior to iron uptake by *S. cerevisiae* had been previously shown by the use of ^{59}Fe -[^{14}C]FOB (Lesuisse *et al.*, 1987).

It is interesting to note that the ferrozine-insensitive FOB transport system was induced only in iron-deficient conditions in wild-type cells but in all conditions in haem-deficient cells, even when 180 μM -FOB was added to the growth medium (Table 2). This observation and the fact that undissociated FOB was accumulated by mutant cells (Fig. 1) led us to hypothesize that the deficiency in ferri-reductase activity of whole cells is accompanied by a deficiency in intracellular ferri-reductase activity which may be required for the removal – and subsequent utilization – of iron from the internalized siderophore.

The mechanisms of iron uptake from FOB were further investigated by studying the effects of Ga(III)-DFOB and Ga^{3+} on uptake. Emery & Hoffer (1980) showed that gallium analogues of siderophores could be taken up by micro-organisms in an active transport process indistinguishable from that of ferri-siderophores. In addition, it has been demonstrated that Ga^{3+} can displace iron from siderophores under reducing conditions (Emery, 1986). This was confirmed for the reduction of FOB by *S. cerevisiae*: the rate of formation of the Fe(II)-(ferrozine)₃ complex from a cell suspension (10 mg wet wt ml^{-1}) incubated with 200 μM -FOB,

Fig. 2. Dependence on extracellular FOB concentration of the rate of iron uptake by cells of strain G204. Cells were grown in iron-deficient conditions with (a) or without (b) ALA. Uptake experiments were done in acetate buffer as described in Methods, without additions (○) or with the addition of 14 μM -Ga(III)-DFOB (●) or 14 μM -Ga(NO₃)₃ (■).

1 mM-ferrozine and 5% (w/v) glucose was increased by more than 50% by the addition of 500 μM -Ga(NO₃)₃.

Ga³⁺ should therefore stimulate iron uptake from FOB if an essentially reductive assimilation is involved, while Ga(III)-DFOB should inhibit uptake in the case of a specific siderophore-mediated process. Results in Table 2 show that Ga(III)-DFOB had a strong inhibitory effect on the uptake of FOB, this effect being more pronounced for haem-deficient cells. In contrast, Ga³⁺ had little or no effect.

Emery (1986) showed that citrate could inhibit the reductive exchange reaction between Ga³⁺ and FOB, and that acidic conditions were required for optimum reaction rate. Citrate buffer, pH 6.5, was therefore replaced by acetate buffer, pH 5.2, for studying the effects of Ga³⁺ and Ga(III)-DFOB on FOB uptake rate as a function of FOB concentration. In haem-deficient cells (Fig. 2a), uptake of FOB proceeded with apparent K_m and V_{max} values of about 5 μM and 30 $\text{pmol h}^{-1} (\text{mg wet wt})^{-1}$ respectively (V_{max} was 3 to 4-fold higher in citrate buffer, at pH 6.5; not shown). Inhibition by Ga(III)-DFOB was clearly competitive ($K_i = 10 \mu\text{M}$), while Ga³⁺ had no effect on the kinetic parameters. No simple Michaelis-Menten kinetics were obtained for the uptake of FOB by ALA-supplemented cells (Fig. 2b): below about 7 μM -FOB, the apparent K_m and V_{max} values were about 3 μM and 37 $\text{pmol h}^{-1} (\text{mg wet wt})^{-1}$ respectively, and Ga(III)-DFOB acted as a competitive inhibitor ($K_i = 4 \mu\text{M}$). As the FOB concentration was raised, deviations from Michaelis-Menten kinetics were observed, indicating that saturation could not be reached as rapidly as expected if a single high-affinity mechanism was involved. Ga³⁺ had a relatively low inhibitory effect, which was totally suppressed at FOB concentrations higher than 20 μM .

From the results in Table 2 and Fig. 2, we conclude that *S. cerevisiae* can produce a specific high-affinity transport system for FOB-type siderophores. In wild-type strains, the siderophore-mediated transport system co-exists with a reductive uptake mechanism of lower affinity and specificity (Lesuisse *et al.*, 1987), as shown by Emery (1987) for the fungus *Ustilago sphaerogena*.

Fig. 3. Schematic representation of the mechanisms involved in iron uptake by *S. cerevisiae*. S, inducible siderophore-mediated transport system; R, inducible ferri-reductase (X is the intracellular electron donor); C, divalent cation transport system.

Therefore, at low FOB concentrations, uptake occurs essentially by the high-affinity mechanism. Ga(III)-DFOB acts as a competitive inhibitor and Ga^{3+} inhibits the uptake by displacing iron from FOB; free ferrous ions are either re-oxidized or taken up by the cells via a low-affinity divalent cation uptake system (K_m around $270 \mu\text{M}$) (Lesuisse *et al.*, 1987). When the FOB concentration is raised, the reductive mechanism becomes quantitatively more important as the high-affinity system approaches saturation. A model of the mechanisms involved in our uptake assays is presented in Fig. 3.

The relative efficiency of the two transport systems under iron limitation was shown by experiments in which DFOB ($20 \mu\text{M}$) restored (by about 90%) the growth yield of cells of G204 supplemented with ALA in iron-poor medium (iron $< 0.1 \mu\text{M}$); desferri-rhodotorulic acid restored the growth yield by only 22%, whereas the yield was restored to 100% after addition of either $0.5 \mu\text{M}$ -FOB or $0.5 \mu\text{M}$ -ferri-rhodotorulic acid. This observation supports the view of a dual transport mechanism, one with high affinity for FOB-type siderophores and another with lower affinity for different ferric chelates including siderophores (reductive mechanism).

General conclusions

The system of iron acquisition by reductive and non-reductive mechanisms which is described here appears to be very similar to that described by Emery (1987) for *U. sphaerogena*; in this fungus, iron can be taken up from ferrichrome and ferrichrome A (both ligands being secreted by the cells under iron-deficient conditions) either by internalization of the chelate via a

highly specific transport system or indirectly, by means of a non-specific reductive system. The relative rate of iron uptake by the two systems depends, among other factors, on the nature of the siderophore, on its extracellular concentration and on the concentration of oxygen in the cell suspension (Emery, 1971, 1983, 1987; Ecker & Emery, 1983). The system described here for *S. cerevisiae* differs on essentially two points. (1) *S. cerevisiae* does not itself secrete any siderophore (Neilands *et al.*, 1987). The existence of a siderophore-mediated transport system in this fungus could therefore be considered as an 'opportunistic strategy', as found in certain plants (Crowley *et al.*, 1987). (2) The reductive system of *S. cerevisiae* is strongly induced in iron-deficient conditions, which is not so in *U. sphaerogena* to our knowledge. This could mean that the reductive mechanism of iron assimilation is physiologically required for iron supply to *S. cerevisiae* in iron-limited conditions, as in many plants (Bienfait, 1987).

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