The plasma membrane ferrireductase activity of *Saccharomyces cerevisiae* is partially controlled by cyclic AMP

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The plasma-membrane-bound ferrireductase activity of *ras1* and *ras2* mutants of *Saccharomyces cerevisiae* is not induced in response to iron limitation. This phenotype was suppressed by the *bcy1* mutation in *ras2* but not in *ras1* mutants. The cellular haem content of *ras1*-bearing strains decreased dramatically when cells were grown in semi-synthetic medium (low yeast extract content), which could account for their very low ferrireductase activity. The ferrireductase activity of *cdc25* and *cdc35* mutants dropped when the cells were shifted to a non-permissive temperature. This drop was prevented in the double mutant *cdc35 sra5* by adding cyclic AMP to the growth medium. We propose that ferrireductase activity is under the control of a cyclic AMP-dependent protein phosphorylation.

INTRODUCTION

Whole cells of *Saccharomyces cerevisiae* can reduce a variety of extracellular ferric chelates via a trans-plasma-membrane redox system which catalyses the transfer of electrons from cytosolic NAD(P)H to extracellular Fe(III). The reduced ferrous ions are then taken up by the cells [1,2]. The ferrireductase activity of cells grown in iron-sufficient conditions is low (basal activity), but is dramatically increased when the cells are grown in iron-deficient medium (induced activity). Haem-deficient cells exhibit a very low, non-inducible, ferrireductase activity (residual activity) and are therefore unable to take up iron efficiently [2].

Higher plants contain similar reductive mechanisms for iron assimilation [3], but in spite of a profuse literature, very little is known about the physiological and biochemical events involved in trans-plasma-membrane electron transfer in eukaryotic cells.

Cyclic AMP (cAMP) is essential for growth in *S. cerevisiae* and may act as a second messenger, regulating a variety of metabolic and physiological functions by activating protein kinase A [4], including plasma membrane H⁺-ATPase activity [5]. In this study we have used strains with disrupted *ras1* or *ras2* genes, which are both involved in the GTP-dependent activation of adenylate cyclase [6], and other mutants affected in the RAS/cAMP pathway, to show that cAMP may also be involved in activating the ferrireductase system of *S. cerevisiae*.

MATERIALS AND METHODS

Strains

The haploid wild-type parental strain *S. cerevisiae* Jc482 (α *leu2 ura3-52 his4*) [7] and several isogenic mutants affected in the cAMP pathway were used: Jc302-26B (α *leu2 ura3-52 his4 ras2-530*) [8]; Jc308 (α *leu2 ura3-52 his4 ras1-545*) [9]; Jc303-51 (α *leu2 ura3-52 his4 ras2-530 sra1-3/bcy1*); BH101-1A (*ura3-52 leu2 his4 ras1-545 ras2-530 sra1-3/bcy1*); BH102-7D (*ura3-52 leu2 his4 ras1-545 sra1-3/bcy1*); Jc482//*RAS2^{val19}*; BeG334 (*a cdc35-10 arg1*); Be344 (*a cdc35-10 cas1-16 arg1*) (these two strains are derived from Be333 and Be333-16 [10]); BH100-3D (*a sra5::URA3 ura3-52 leu2 his4 cdc35-10*).

Cultures and assay conditions

Unless indicated, the cells were precultured aerobically at 26 °C for 40 h in medium containing (in 1 litre of water): 20 g of glucose, 6.7 g of yeast nitrogen base (without amino acids), 1 g of yeast extract and 50 mg each of any amino acid required.

The cells were harvested, washed twice with 2% (w/v) EDTA (disodium salt) and twice with distilled water, and inoculated at 1 g (wet wt.)·litre⁻¹ in iron-rich and/or iron-deficient culture media, which were identical to the preculture medium except that (i) the iron-rich medium contained 180 μ M-Fe(III) citrate (1:20, mol/mol); and (ii) the iron-deficient medium was prepared by 5% (w/v) 8-hydroxyquinoline in chloroform (1:1, v/v) extraction as described by Nicholas [11] and supplemented with 50 mg of ferrozine [3-(2-pyridyl)-5,6-bis-(4-phenylsulphonic acid)-1,2,4-triazine]·litre⁻¹. In some experiments the culture medium was supplemented with 5 mM-cAMP previously sterilized by filtration.

Cultures were grown in a reciprocal shaker at 26 °C or 36 °C. Cells were collected at intervals, washed as described above and assayed for their ferrireductase activity as previously described [2]. The total haem content of the cells was evaluated by the pyridine haemochrome method [12]. Low-temperature spectra of whole cells were recorded as described by Labbe & Chaix [13]. All results refer to wet wt. of yeast cells.

RESULTS

Various isogenic strains affected in the RAS/cAMP pathway were assayed for their ferrireductase activity when grown in iron-deficient and/or iron-rich conditions (Table 1). The strains with disrupted *ras1* or *ras2* genes had significantly lower ferrireductase activity than the wild-type counterpart when grown in iron-deficient conditions. The defect was particularly striking in the *ras1* mutant, which showed only residual activity under both conditions of growth (Table 1).

Mutations in the *BCY1/SRA1* gene coding for the regulatory subunit of the cAMP-dependent protein kinase can reverse the phenotypes of a disrupted *ras2* allele and the essential requirement of both *RAS* genes [14]. We therefore tested whether a *bcy1/sra1*-

Abbreviation used: cAMP, cyclic AMP.

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Table 1. Ferrireductase activity of yeast strains grown in iron-rich and iron-deficient media

The composition of the preculture and culture media was as described in the Materials and methods section; cell ferrireductase activity was determined after cell growth for 4 h in the appropriate culture medium.

Strain	Cell ferrireductase activity [nmol·h ⁻¹ ·(mg wet wt.) ⁻¹]	
	+ Fe	- Fe
Jc482 (wild-type)	9	85
Jc308 (<i>ras1</i>)	2	2.5
Jc302-26B (<i>ras2</i>)	2.5	29
BH102.7D (<i>ras1/bcy1</i>)	—	2
Jc303-5.1 (<i>ras2/bcy1</i>)	12	110
BH101.1A (<i>ras1/ras2/bcy1</i>)	—	9
Jc482// (<i>RAS2^{val19}</i>)	13	72

3 mutation could restore normal ferrireductase activity to *ras* mutants. The results (Table 1) show that ferrireductase activity could be restored for the *ras2* mutant, whereas the *ras1* and *ras1/ras2* mutants remained defective. The ferrireductase activity of strains with either *bcy1* or *RAS2^{val19}* mutations, which both constitutively activate the cAMP pathway, remained repressed under iron-rich growth conditions (Table 1).

The results obtained with *ras2* and *ras2/bcy1* mutants suggest that the increase in ferrireductase activity of the cells is under the direct or indirect control of a cAMP-dependent protein phosphorylation during iron deprivation. The observation that all of the *ras1*-bearing strains had only residual activity suggests that the RAS1 protein could be involved in the ferrireductase activity in a cAMP-independent manner, either directly or more probably via pleiotropic effects associated with the *ras1* mutation. This would be consistent with the hypothesis proposed by Toda *et al.* [15] that RAS protein could have more than a single action in *S. cerevisiae*.

The *ras1* mutant was strongly depigmented when cultured under our experimental conditions [synthetic medium supplemented with 0.1 % (w/v) yeast extract]. Since we had previously shown that haem-deficient mutants lack inducible ferrireductase activity [2], we tested the possibility that a defect in haem biosynthesis could be associated with the *ras1* mutation. As shown in Fig. 1 and Table 2, the *ras1*-bearing strains, unlike the *ras2* mutant, had a haem content and ferrireductase activity that depended strongly on the growth medium composition. The haem content of *ras1*⁻ cells grown in synthetic medium supple-

Fig. 1. Low-temperature spectra of whole cells in the exponential growth phase

The cells were precultured for 24 h (medium composition as described in the Materials and methods section) and then inoculated (at a final $A_{500} = 0.01$) in synthetic culture media supplemented with different amounts of yeast extract. The cells were harvested at $A_{500} = 4$. 1, Jc302-26B (*ras2*), 0.1 % (w/v) yeast extract; 2, Jc308 (*ras1*), 1 % (w/v) yeast extract; 3, Jc308 (*ras1*), 0.1 % (w/v) yeast extract, 5-aminolevulinic acid (50 mg·l⁻¹); 4, Jc308 (*ras1*), 0.1 % (w/v) yeast extract.

mented with 1 % yeast extract did not differ significantly from that of the *ras2* mutant or the wild-type counterpart. The haem content of *ras1*⁻ cells fell significantly, but that of the *ras2* mutant did not, when the yeast extract concentration was lowered to 0.1 % or 0.05 % (Fig. 1 and Table 2). The fall was prevented by adding 5-aminolevulinic acid to the growth medium (Fig. 1). The *ras1*⁻ cells also recovered a basal ferrireductase activity in yeast-extract-rich medium, but this activity was absent under other growth conditions (Table 2). We conclude that the *ras1* mutation results in some defect in an early step of the haem biosynthesis pathway, which is not bypassed by the *bcy1* mutation. This defect remains masked when the cells are grown in complete medium, and probably accounts for the lack of ferrireductase activity of the *ras1*-bearing strains under our experimental conditions.

The role of cAMP in controlling the ferrireductase activity of iron-deprived cells was further investigated using the temperature-sensitive *cdc25* and *cdc35* mutants. Adenylate cyclase encoded by the *CDC35* gene is positively regulated by the *CDC25* gene product; a shift of *cdc25* and *cdc35* mutants to a non-permissive temperature results in a drastic decrease in their intracellular cAMP level [10,16]. As shown in Fig. 2, the ferrireductase activity of a *cdc35* mutant growing in iron-deficient conditions was strongly and rapidly inhibited when the cells were

Table 2. Ferrireductase activity and cellular haem content of yeast strains grown in synthetic medium supplemented with yeast extract

The cells were grown for 40 h in synthetic medium [YNB; 2 % (w/v) glucose] supplemented with histidine, leucine and uracil (50 mg·l⁻¹ each), plus 1 %, 0.1 % or 0.05 % (w/v) yeast extract. The haem content was then determined and the cells were re-inoculated at 1 g (wet wt.)·l⁻¹ in the same medium; they were allowed to grow for 5 h before measuring the ferrireductase activity.

Yeast extract (% (w/v) ...)	Cell ferrireductase activity [a, nmol·h ⁻¹ ·(mg wet wt.) ⁻¹] and haem content [b, nmol·(g wet wt.) ⁻¹]					
	1		0.1		0.05	
	a	b	a	b	a	b
Jc482 (wild type)	16	10	30	11	23	9
Jc302-26B (<i>ras2</i>)	16	6	24	10	20	7.5
Jc308 (<i>ras1</i>)	23	10	1	2	1	1.5
BH102.7D (<i>ras1/bcy1</i>)	38	7	0.6	2.5	0.6	1.2
BH101.1A (<i>ras1/ras2/bcy1</i>)	6	10	0.7	4	0.4	3

Fig. 2. Effect of temperature on the ferrireductase activity of a *cdc35* mutant

Cells of the strain BeG334 (*cdc35*; $\square$, $\blacksquare$, $\triangle$) and Jc482 (wild-type; $\square$, $\blacksquare$) were precultured and cultured as described in the Materials and methods section; cultures were carried out in iron-deficient medium at either 26 °C ($\square$, $\triangle$), or 36 °C ($\blacksquare$, $\bullet$), or were shifted from 26 °C to 36 °C at the indicated time ($\triangle$).

shifted from 26 °C to 36 °C. The same results were obtained with a *cdc25* mutant (results not shown).

The *SRA5* gene encodes the low- K_m cAMP phosphodiesterase [17]. Wilson & Tatchell [17] have reported that the *sra5* mutation could result in an increase in the intracellular cAMP concentration large enough to suppress the lethality of a double *ras1/ras2* mutant when exogenous cAMP is added to the medium. We have observed an identical suppressive effect of *sra5* and *cas1* [10] mutations on the *cdc35* growth defect at restrictive temperatures. We therefore assayed the ferrireductase activity of the double mutants *cdc35/sra5* and *cdc35/cas1* grown in iron-deficient conditions with or without added cAMP. Extracellular cAMP completely prevented the fall in ferrireductase activity when the double mutant *cdc35/sra5* was shifted to 36 °C (Table 3). cAMP also had a strong stimulatory effect at permissive temperature (Table 3), which was no longer observed in the iron-rich medium (results not shown). In contrast, extracellular cAMP had little or no effect on the ferrireductase activity of the *cdc35/cas1* mutant (Table 3).

DISCUSSION

The results reported here show that the plasma-membrane redox system of *S. cerevisiae* is partially controlled by the RAS/cAMP pathway: a cAMP-mediated activation of protein kinase A may be necessary to fully de-repress the ferrireductase activity of the cells, while a strong nutritional cAMP-independent control is probably exerted under repression conditions (high Fe^{3+} concentration). Haem-deficient mutants of *S. cerevisiae* have a very low residual ferrireductase activity [2], indicating that haem is also involved in regulating the enzyme activity, though no haem was found in purified plasma-membrane ferrireductase [18]. We now show that the cellular haem content of *ras1*-bearing strains grown in synthetic media sharply decreases, which could account for the loss of their ferrireductase activity.

The ferrireductase activity of resting cells is strongly stimulated by fermentable sugars within a lag period of 2–3 min [1]. This is consistent with the observation of Mbonyi *et al.* [19] that there is a transient increase in intracellular cAMP within 1–2 min of adding glucose to stationary-phase cells. Glucose also has a marked effect on the plasma-membrane H^+ -ATPase activity of *S. cerevisiae* [20], which is actually controlled by cAMP [5]. Lastly, Portillo & Mazon [21] showed that the H^+ -ATPase activity of a *cdc25* mutant could no longer be activated by glucose.

Our data indicate that cAMP acts as a positive effector of the ferrireductase activity of the cells, but we cannot conclude that an increase in intracellular cAMP concentration leads to modification(s) in the plasma-membrane redox system itself. cAMP could affect the ferrireductase activity by modifying the plasma-membrane H^+ -ATPase activity. A coupling between the reductase-dependent efflux of electrons and the ATPase-mediated efflux of protons is theoretically possible, since an increased electron flow from intracellular NAD(P)H through the plasma-membrane is expected to cause membrane depolarization and cytosolic acidification associated with oxidation of NAD(P)H. Moreover, there is a stoichiometrically related increase in the glucose-induced proton efflux during extracellular iron reduction by resting cells (E. Lesuisse & P. Labbe, unpublished work). However, plasma membranes isolated from iron-deficient cells have greater ferrireductase activity than those isolated from iron-rich cells [18], although the plasma-membrane ATPase activities are the same (E. Lesuisse, B. Horion, P. Labbe & F. Hilger, unpublished work). Also, the plasma-membrane H^+ -ATPase activity of the double mutant *cdc35/cas1* at 36 °C can be restored by exogenous cAMP addition [5], whereas the ferrireductase activity cannot. These data are in favour of direct activation of the plasma-membrane redox system via cAMP.

E. L. is a grantee of FEBS. We thank Dr. K. Tatchell for the yeast strains and Dr. O. Parkes for help in preparing the manuscript.

Table 3. Ferrireductase activity of *cdc35* mutants under various growth conditions

The cells were precultured for 40 h at 26 °C and then cultured for 5 h at 26 °C, or for 2 h at 26 °C and then shifted to 36 °C for a further 3 h. All cells were grown in iron-deficient media with or without added cAMP (5 mM).

Strain	Cell ferrireductase activity [nmol · h ⁻¹ · (mg wet wt.) ⁻¹]			
	26 °C	Shift (26 to 36 °C)	26 °C, cAMP	Shift (26 to 36 °C), cAMP
BeG334 (<i>cdc35</i>)	52	0.5	69	0.7
BH100.3D (<i>cdc35 sra5</i>)	53	4.5	122	61
Be344 (<i>cdc35 cas1</i>)	44	1.0	72	3.5

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Received 15 July 1991/18 September 1991; accepted 1 October 1991