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# A Transplasma Membrane Redox System in *Phycomyces blakesleeanus*: Properties of a Ferricyanide Reductase Activity Regulated by Iron Level and Vitamin $K_3$

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Intact *Phycomyces blaskesleeanus* mycelia are capable of reducing extracellular ferricyanide and this transmembrane reduction is an enzymatic process, which is enhanced by the presence of 10 mM lactate. It is modulated in response to intracellular iron levels and negatively regulated by iron and copper. It is inhibited by NEM, *p*CMB, iodoacetate, $Zn^{2+}$, $Cd^{2+}$, dicumarol, and capsaicine analog, but not by cloroquine, and activated by $Ca^{2+}$, $Mg^{2+}$, $Na^{+}$, and $K^{+}$. Ferricyanide reduction was concomitant with proton release into the extracellular medium, both processes being greatly promoted by vitamin $K_3$ following hyperbolic saturation kinetics with regard to ferricyanide concentration. No stoichiometric proton release was observed with regard to ferricyanide reduction in the absence or the presence of vitamin $K_3$. Proton release coupled with ferricyanide reductase activity does not appear to be due to $H^{+}$-ATPase. The relevance of these findings to the relationship between the two processes is discussed.

**KEY WORDS:** *Phycomyces blakesleeanus*; plasma membrane redox system; ferricyanide reductase; iron regulation; proton transport; Vitamin $K_3$.

## INTRODUCTION

Transplasma membrane redox systems seem to be ubiquitous in both prokaryotic and eukaryotic cells. These systems are involved in proton pumping, membrane energization, ion channel regulation, nutrient uptake, signal transduction, and growth regulation (for a review, see Lüthje *et al*., 1997; Medina *et al*., 1997). Iron is an indispensable element for living organisms since it participates in a wide range of metabolic functions, but in excess it can be toxic (Halliwell and Cutteridge, 1990), so organisms must tightly regulate their intracellular concentration by controlling uptake and intracellular storage. In organisms that secrete siderophores, such as bacteria, fungi, and some plants, iron transport is achieved by the uptake of a ferrisiderophore complex (Emery, 1987). Another strategy adopted by some organisms involves the extracellular reduction of ferric iron, via membrane-bound reductive iron assimilatory systems, i.e., ferric reductases, and subsequently the specific uptake of ferrous ion. Evidence for membrane-bound reductive iron assimilatory systems has been found in a broad array of fungi (Hass, 2003), in the root cell plasma membrane of dicotyledonous plants (Bienfait, 1985; Rubinstein and Luster, 1993; Robinson *et al*., 1999) and in the plasma membrane of mammalian cells (May *et al*., 1995; Van Duijn *et al*., 1998). The reductive iron assimilatory system has been intensively studied in yeasts. *Saccharomyces cerevisiae* expresses several metalloreductases, including the ferric reductases Fre1p and Fre2p, which are responsible for total plasma-membrane-associated activity (Dancis *et al.*, 1990; Georgatsou and Alexandraki, 1994). The FRE1 gene is regulated by both iron deprivation and copper deprivation, whereas the FRE2 gene is solely regulated by iron deprivation (Georgatsou *et al*., 1997; Martins *et al*., 1998). Iron levels in growth medium have also been reported to regulate ferric reductase and ferrous transport

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in *Candida albicans* (Knight *et al*., 2002; Morrissey *et al*., 1996).

On the other hand, in all the cells tested, the functioning of the plasma membrane redox system is accompanied by proton release into the medium (Crane *et al*., 1991; De Grey, 2003; Döring and Lüthje, 1996; Rubinstein and Luster, 1993). This proton efflux results in changes in the internal and external pH, which may be related with important cellular events. It has recently been proposed that this process may be involved in the life-extending action of caloric restriction, since the transmembrane proton gradient generated can conserve the free energy of oxidation of NADH (De Grey, 2001). A currently emerging area of interest is the protective role of the plasma membrane redox system versus oxidative stress damage (Medina *et al.*, 1997; Morré *et al*., 2000). In general, the nature and components of the electron transport system and the mechanism by which proton release is activated are yet to be discovered.

Fungal cells, with the exception of the yeast *S. cerevisiae*, have hitherto escaped the interest of researchers of plasma membrane redox systems. Nevertheless, filamentous fungi offer the advantage of a eukaryotic cell type with a less complex structure and specialization than most plant cells and the ability to specifically adapt to a very broad range of extracellular conditions. The present paper reports the existence and in vivo characterization of a plasma membrane redox system in the zygomycete fungus *Phycomyces blakesleeanus*, a fungus that accumulates a large amount of the antioxidant $\beta$-carotene. The results shown here are evidence of the existence of a cell surface ferrireductase activity, probably implicated in the reduction of Fe(III) to Fe(II) as a first step in the passage of iron across the plasma membrane.

## MATERIALS AND METHODS

*P. blakesleeanus* (strain NRRL 1555, genotype (−), wild-type) was used in all the experiments for this study. It was grown in liquid minimal medium (SIV) (iron-sufficient medium) [Sutter, 1975]. In some experiments an "iron-defficient" or "copper-defficient" medium was used, similar to SIV except that iron (7.5 mg of $Fe(NO_3)_3 \cdot 9H_2O$) or copper (2.5 mg of $CuSO_4 \cdot 5H_2O$), respectively, was not added. Fresh spores (up to 7 days old) were used. Spores were heat-shocked by incubating an aqueous suspension containing $10^8$ spores/mL for 15 min at 48°C. Liquid minimal media (100 mL) in 250-mL Erlenmeyer flasks were inoculated with 1.0 mL of the spore suspension. Cultures were incubated in an RC-TK Incubator Shaker (Infors, HT) at 200 rpm and 22°C in darkness. Mycelia were obtained by filtration at different stages of growth. Growth of *P. blakesleeanus* was determined as a function of mycelial dry weight. Aliquots of culture were taken and passed through a glass wool filter. They were kept at 50°C until weight was constant.

Ferricyanide reductase activity was monitored by production of ferrocyanide when *P. blakesleeanus* intact mycelia were incubated with ferricyanide. Ferrocyanide was measured by a modified version of Avron and Shavit's procedure (1963). Mycelia were collected by filtration, washed twice with distilled water, and resuspended in 50 mM sodium citrate buffer, pH 6.5, with 5% (w/v) glucose, except where indicated, in the reaction mixture. Each reaction sample contained, in 1.0-mL final volume: 50 mM sodium citrate buffer, pH 6.5, with 5% (w/v) glucose; 1 mM potassium ferricyanide; and mycelial suspension (50–200 $\mu$L). Reaction was initiated by the addition of potassium ferricyanide in Eppendorf tubes. After the desired incubation time at 37°C in a shaking water bath, reaction was stopped by chilling the Eppendorf tubes on ice. The mycelia were immediately separated by microcentrifugation and 875-$\mu$L aliquot of supernatant was transferred into an Eppendorf tube to which were added, in order, 125 $\mu$L of 3 M sodium acetate, 125 $\mu$L of 0.2 M citric acid, 62.5 $\mu$L of 3.34 mg/mL of bathophenantrolinesulfonate (BPS), and 62.5 $\mu$L of 3.3 mM $FeCl_3$, prepared in 0.1 M acetic acid. Samples were well-mixed, and the absorbance at 535 nm was measured after allowing for color development (30 min), with a molar extinction coefficient of 12556 $M^{-1}$ $cm^{-1}$ for BPS complex. Background levels were established using two parallel samples that contained no mycelia or no potassium ferricyanide; these values were subtracted from others obtained from samples that contained cells and ferricyanide. All experiments were performed on at least three separate occasions, and data from representative individual experiments are shown. Ferrireductase measurements were also made in duplicate and determined average values with standard error are presented.

For some experiments, lyzed mycelia were collected as follows. Mycelia from cultures were collected by filtration and washed with 2 volumes of distilled water. Washed mycelia were suspended (1-g wet weight mycelia per 10 mL of buffer) in 50 mM Tris/HCl buffer, pH 7.6, containing 1 mM EDTA, and homogenized in a Braun MSK homogenizer-cell disrupter for two 35-s periods with a 15-s interval. Homogenized mycelia were filtered and lyzed mycelia resuspended in 50 mM sodium citrate buffer, pH 6.5, with 5% (w/v) glucose. All operations were performed at 4°C. The buffer was conditioned, for some indicated experiments, as follows. Mycelia collected by filtration and washed with 2 volumes of distilled water were

resuspended in 50 mM sodium citrate buffer, pH 6.5, with 5% (w/v) glucose and incubated in a shaking water bath for 30 min at 37°C. Mycelia were separated by filtration and conditioned buffer was used for ferrireductase determination. Protein concentration in conditioned buffer was determined by Bradford's method (1976). Bovine serum albumin fraction V was used as standard.

For some experiments, crude membrane fractions were obtained from *P. blakesleeanus* mycelia as described by Pollock *et al.* (1985).

Proton excretion by *P. blakesleeanus* mycelia was measured with a Metrohm combined LL micro pH glass electrode and a Metrohm 713 pH-Meter. The reaction mixture was stirred at room temperature (22–23°C). Untreated mycelia lowered pH of the medium from an initial pH of 6.46–6.43 within 10 min, after which pH decreased only very slowly or remained constant. Proton release produced by the addition of several compounds was measured using intact mycelia (39.3-mg dry weight) in 15 mL of 50 mM sodium citrate buffer, pH 6.5, and recorded with an Amersham Pharmacia recorder REC for 15 min. The lower limit of detectable pH change was 0.001. The number of protons released was calculated from the titration curve prepared with 0.1 M HCl.

## RESULTS

### Transmembrane Ferricyanide Reductase Activity of *P. blakesleeanus* Mycelium

*P. blakesleeanus* mycelia exhibited a reductase activity when grown in a liquid minimal medium (iron-sufficient medium) with 2% (w/v) glucose as a carbon source. This was studied using ferricyanide as a substrate to monitor $Fe^{+3}$ reduction, because both ferricyanide and its product ferrocyanide are membrane-impermeants, so this method provides a convenient measure for transplasma membrane ferricyanide reductase activity. Ferricyanide reduction required intact mycelia, as lyzed mycelia had less than 15% of reductase activity. To test whether some product secreted by intact (or damaged) mycelia was actually responsible for this activity, conditioned buffer at 37°C (as described in Materials and Methods) was added to the assay mixture, and it was found that ferricyanide reductase activity was less than 10%. The presence of mitochondria as a consequence of lyzed mycelia was ruled out when no inhibition was observed by mitochondrial inhibitor (Table I).

As shown in Fig. 1(A), a time-dependent reductase activity was observed when samples containing either 0.70 or 0.35 mg/mL of *P. blakesleeanus* mycelia were incubated with 1 mM ferricyanide at 37°C. The rate values for ferricyanide reduction were 4.02 ± 0.16 and 1.98 ± 0.16 nmol/min, respectively. Figure 1(B) shows the linear dependence of ferricyanide reductase activity with the quantity of mycelia and demonstrates that the ferricyanide reduction by *P. blakesleeanus* is mycelium-mediated. The ferricyanide reductase activity profile as a function of ferricyanide concentrations, which follows a hyperbolic saturation kinetics, is shown in Fig. 2. From the double reciprocal plot (inset of Fig. 2) a $K_m$ value of 0.08 ± 0.005 mM was calculated for ferricyanide and a $V_{max}$ value of 3.73 ± 0.05 nmol/min·mg mycelial dry weight. The pH optimum for ferricyanide reductase activity was determined by buffering the reaction mixture with 50 mM sodium citrate buffer (pH from 4.0 to 6.5), 50 mM potassium phosphate buffer (pH from 6.0 to 8.0), or HCl/Tris buffer (pH from 7.0 to 9.0). Maximum activity was detected about pH 8.0 (data not shown).

Table II illustrates the effect of incubation with glucose (5 mM) or lactate (10 mM) on ferricyanide reduction catalyzed by *P. blakesleeanus* intact mycelia. After 2 h of incubation, a similar decrease (about 50%) in ferricyanide reductase activity from mycelia with or without glucose was observed, most probably owing to depletion of NADH, the intracellular electron donor. However, the mycelia incubated with lactate were able to increase ferricyanide reduction, the highest reduction capacity (fourfold) being reached after 4 h. Regarding the effect of two glycolytic inhibitors, which are presumed to reduce the internal levels of NADH, sodium arsenate did not inhibit reduction activity whereas iodoacetate gave 35% inhibition (Table I). On the other hand, when plasma membrane fractions of *P. blakesleeanus* were assayed for ferricyanide reductase activity with ferricyanide as a ferric substrate and NADH or NADPH as the electron

**Table I.** Effect of Mitochondrial and Glycolytic Inhibitors on Transmembrane Ferricyanide Reduction by *P. blakesleeanus* Mycelia

| Assay conditions[a] | Activity (% of control) |
|---|---|
| Control | 100 |
| KCN (1 mM) | 114 |
| Sodium azide (10 mM) | 84 |
| Antimycine (10 mM) | 92 |
| Iodoacetate (5 mM) | 65 |
| Sodium arsenate (20 mM) | 120 |

*Note.* Reaction mixture contained *P. blakesleeanus* mycelia, 50 mM sodium citrate buffer with 5% (w/v) glucose (pH 6.5), 1.0 mM potassium ferricyanide, and the inhibitor at the indicated concentration.

[a] Mycelia were incubated with each inhibitor for 15 min at 37°C, and then the ferricyanide reductase activity was assayed as described in Materials and Methods. Data are given as means of three separate experiments.

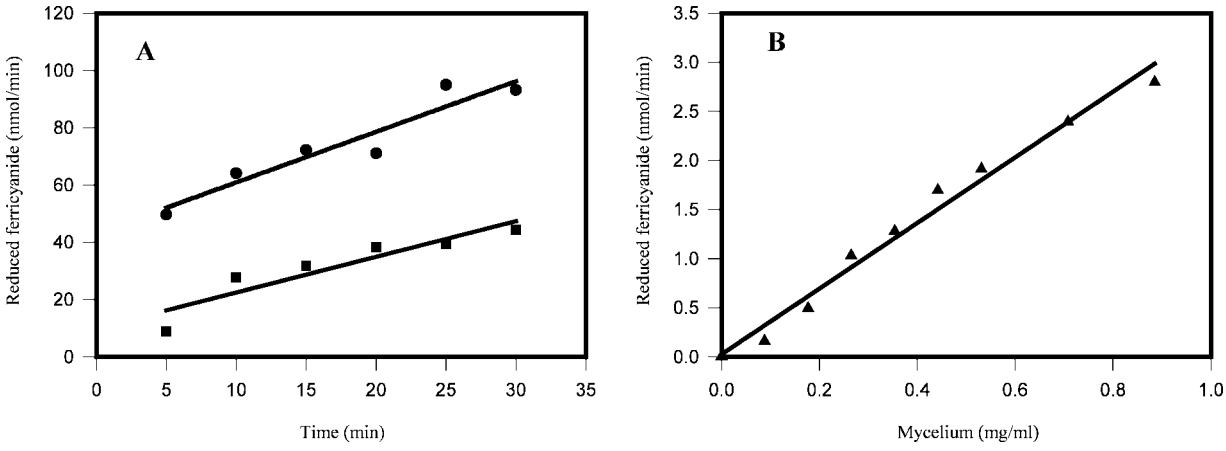


**Fig. 1.** Dependence of *P. blakesleeanus* mycelium transmembrane ferricyanide reductase activity with (A) time course and (B) mycelium amount (▲). Ferricyanide reductase activity was assayed with 1.0 mM potassium ferrycianide in 50 mM sodium citrate buffer containing 5% (w/v) glucose (pH 6.5) at 37°C, by using 0.7 mg/mL (●) or 0.35 mg/mL (■) of *P. blakesleeanus* mycelia grown in an iron-sufficient medium, as indicated in Materials and Methods.

donors, maximal reduction rate was obtained with NADH (apparent $V_{max}$ value of $1.72 \pm 0.23$ $\mu$mol/min·mg protein for NADH and $0.4 \pm 0.01$ $\mu$mol/min·mg protein for NADPH).

Specific ferricyanide reductase activity from *P. blakesleeanus* mycelia grown in a minimal liquid medium (iron-sufficient medium) increased during the exponential growth phase peaking at 24-h growth (Fig. 3), and remaining practically unchanged up to 54 h, when growth stopped. We investigated to ascertain whether *P. blakesleeanus* transmembrane ferrireductase activity was dependent on the iron levels in the growth medium. Thus, *P. blakesleeanus* mycelia were grown in an iron-deficient medium. As can be seen in Fig. 3, ferricyanide reductase activity was maximal at the onset of the exponential growth phase (16 h) in iron-deficient medium with a 20-fold increase in activity with respect to enzymatic activity from mycelia grown in iron-sufficient medium. From this growth time ferricyanide reductase activity decreased markedly to become about fourfold at 24-h growth, whereas similar lower activity levels in the stationary growth phase were detected in both media. No effect on dry weight was detected between the two growth conditions, in line with Hilgenberg *et al.*'s findings (1987). The increase in ferricyanide reductase activity observed from mycelia growing in iron-deficient medium was completely

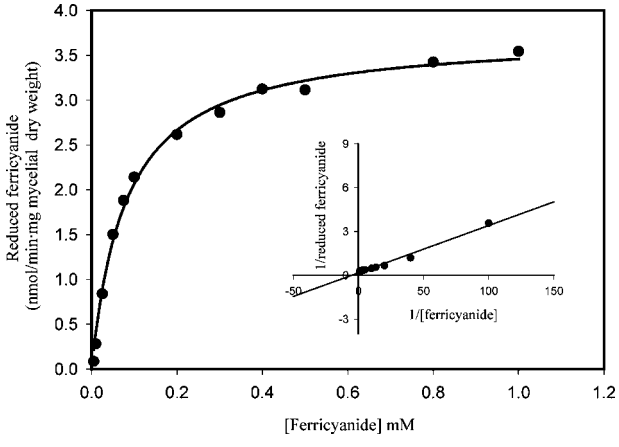


**Fig. 2.** Kinetics of *P. blakesleeanus* transmembrane ferricyanide reductase. The activity was assayed as indicated in Materials and Methods by using a concentration range of potassium ferricyanide from 0.005 to 1 mM in 50 mM sodium citrate buffer containing 5% (w/v) glucose (pH 6.5) at 37°C. Inset: reciprocal plot of data.

**Table II.** Effect of Lactate on *P. blakesleeanus* Transmembrane Ferricyanide Reductase Activity

| | Ferricyanide reductase activity[a] (nmol reduced ferricyanide/min·mg mycelial dry weight) | | |
|---|---|---|---|
| Incubation (h) | Control | +Glucose (5 mM) | +Lactate (10 mM) |
| 0 | $3.04 \pm 0.59$ | $2.88 \pm 0.37$ | $3.81 \pm 0.34$ |
| 2 | $1.80 \pm 0.13$ | $1.73 \pm 0.13$ | $4.05 \pm 0.92$ |
| 3 | $1.70 \pm 0.33$ | $1.79 \pm 0.20$ | $5.60 \pm 0.47$ |
| 4 | $1.74 \pm 0.23$ | $1.70 \pm 0.09$ | $6.40 \pm 0.56$ |

[a] In all the cases, ferricyanide was present at 1.0 mM concentration. *P. blakesleeanus* mycelia were incubated for the time indicated and then ferricyanide reductase activity was assayed as described in Materials and Methods. Means ± SD are from three separate experiments.

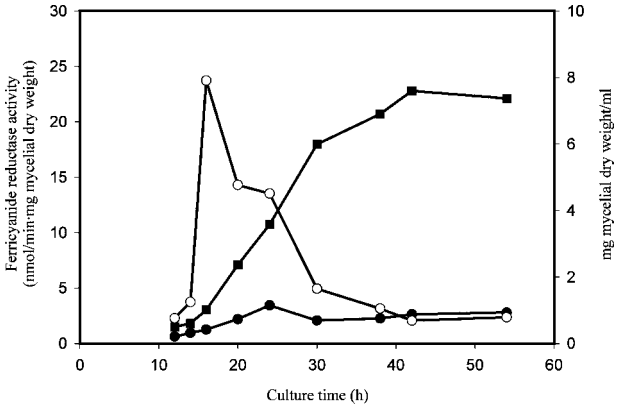


**Fig. 3.** Time course of *P. blakesleeanus* transmembrane ferricyanide reductase activity of mycelia grown in iron-sufficient medium (●) or iron-deficient medium (○). Mycelial dry weight (■). Growth conditions and ferricyanide reductase assay were determined and described in Materials and Methods.

inhibited by the presence of cycloheximide (100 $\mu$g/mL) (data not shown), indicating that protein synthesis is required to reach this increase in enzymatic activity. Inoculation of the mycelia exponentially grown (12-h-old) in iron-sufficient medium into fresh iron-deficient medium, as well as that of the mycelia exponentially grown in iron-deficient medium into fresh iron-sufficient medium, resulted in a ferricyanide reductase activity similar in magnitude to that registered when mycelia were grown in iron-sufficient medium. Moreover, inoculation of stationary phase mycelia grown in iron-sufficient medium into either iron-sufficient or iron-deficient medium resulted in an enzymatic activity similar to that registered in iron-sufficient medium (data not shown). Similar experiments were carried out with *P. blakesleeanus* spores obtained from solid iron-deficient medium, which were used to inoculate liquid iron-deficient and iron-sufficient media, respectively. Maximum mycelial ferricyanide reductase activity was obtained with 16-h-old mycelia grown in liquid iron-deficient medium. Similar results were obtained by using spores grown in a solid iron-sufficient medium. However, the maximum mycelial ferricyanide reductase activity obtained at 16 h was about half that obtained from spores grown under iron-depletion conditions. On the other hand, no substantive differences were observed from experiments carried out with mycelia obtained from iron-sufficient medium, regardless of whether spores were obtained from solid iron-deficient or iron-sufficient media. Thus, these results indicate that ferricyanide reductase activity of *P. blakesleeanus* mycelia increased in response to iron-limiting medium, and that there was no simple dose–response relationship between medium iron content and ferricyanide reductase activity, suggesting that it is somehow dependent on cellular iron level. In addition, these results also indicate that *P. blakesleeanus* ferricyanide reductase activity is not regulated by mycelium growth.

We have determined the kinetic parameters of *P. blakesleeanus* transmembrane ferricyanide reductase activity using 16-, 24-, and 42-h-old mycelia from both iron-sufficient and iron-deficient medium. The apparent kinetics parameters, $K_m$ and $V_{max}$, were unchanged for enzymatic activity from mycelia grown in iron-sufficient medium at any of the growth times assayed. However, both apparent $K_m$ and $V_{max}$ values determined for ferricyanide reductase activity of mycelia grown in iron-deficient medium decreased as growth time increased. Double reciprocal plots of velocity *versus* ferricyanide concentrations gave straight lines intersecting in the lower-left quadrant (Fig. 4), and this kinetic pattern could indicate the presence of a mixed-type effector (Segel, 1993), whose concentration changed with the growth time. If that effector were an inhibitor, its concentration should increase throughout growth time. On the other hand, if it were an activator, its concentration would decrease with growth time. A kinetic comparison of the ferricyanide reductase activity for plasma membrane fractions of *P. blakesleeanus* obtained from iron-deficient and iron-sufficient media showed no significant difference between apparent kinetic parameters (data not shown).

In addition, we tested the effect of low copper concentration media on *P. blakesleeanus* ferricyanide reductase

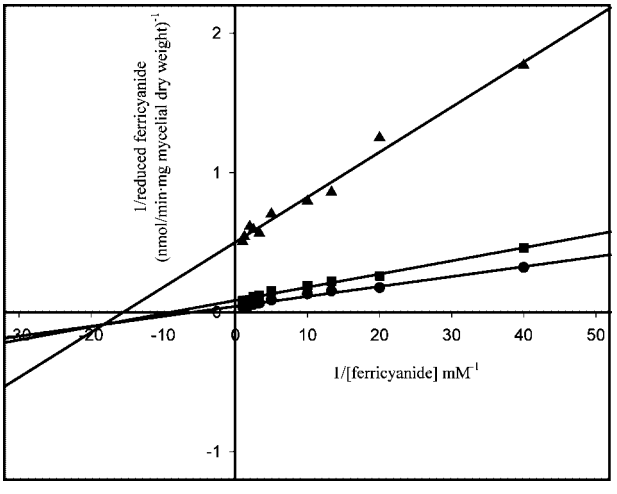


**Fig. 4.** Double reciprocal plots for the transmembrane ferricyanide reduction catalyzed by *P. blakesleeanus* mycelia obtained at 16- (●), 24 (■), and 42-h growth (▲) in iron-deficient medium. The assays were performed at 37°C in 50 mM sodium citrate buffer with 5% (w/v) glucose (pH 6.5) at the indicated ferricyanide concentrations (0.005–1 mM range).

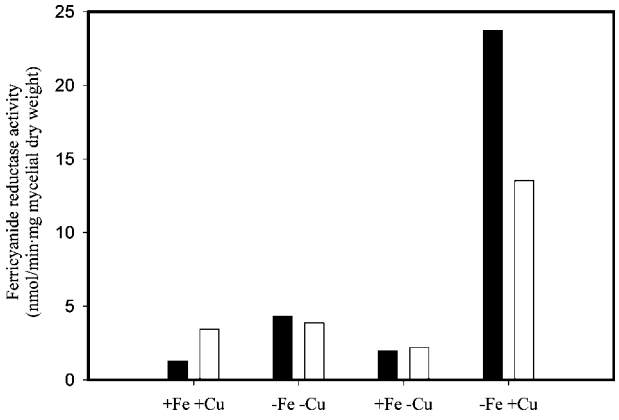


**Fig. 5.** Ferricyanide reductase activity of *P. blakesleeanus* mycelia grown in iron- and copper-sufficient medium (+Fe+Cu), iron- and copper-deficient medium (−Fe−Cu), iron-sufficient and copper-deficient medium (+Fe−Cu), or iron-deficient and copper-sufficient medium (−Fe+Cu). The assay was performed either with 16-h-old mycelia (■) or 24-h-old mycelia (□), in all cases as described in Materials and Methods.

activity from mycelia grown in both iron-sufficient and iron-deficient media (Fig. 5). The ferricyanide reductase activity was maximum for mycelia grown in iron-deficient media regardless of the copper supplementation or otherwise in the growth media. Also, enzymatic activity was higher for mycelia grown in copper-supplemented media regardless of iron depletion or otherwise. These results indicate that ferricyanide reduction activity increases in the presence of copper and rule out negative regulation by the metal.

The effect produced by several effectors on ferricyanide reductase activity of *P. blakesleeanus* mycelia obtained from growth in iron-sufficient and iron-deficient media, are summarized in Table III. $Ca^{2+}$, $Mg^{2+}$, $Na^{+}$, and $K^{+}$ stimulate enzymatic activity in both growth conditions. Stimulation of ferrireductase activity by salts (NaCl, KCl, $CaCl_2$, and $MgCl_2$) was also reported for carrot cells (Barr *et al.*, 1985) and *S. cerevisiae* (Crane *et al.*, 1982). The presence of 20 $\mu$M $Zn^{2+}$ in the reaction mixture produced a greater inhibition of reductase activity for mycelia from iron-sufficient medium than from iron-deficient medium. This inhibition by $Zn^{2+}$ appears to be a common feature of the plasma membrane redox system (Medina *et al.*, 1997). Recently, Santos *et al.* (2003) have suggested the existence in *S. cerevisiae* of an iron–zinc connection similar to the iron–copper connection. The effect of $Cu^{2+}$ cannot be determined, as this ion interferes with the colorimetric assay used for ferricyanide reduction. $Cd^{2+}$ (250 $\mu$M) produced a total inhibition of reductase activity in both cases, which required the continued presence of $Cd^{2+}$-mycelia pretreated with 250 $\mu$M $Cd^{2+}$ for 30 min and subsequently filtered and washed prior to the assay failed to show any effect. This argues against a nonspecific action of the divalent cation on protein sulfhydryl groups. A similar behavior was reported for extracellular ferrireductase from K562 cells (Inman *et al.*, 1994). The effect of inhibitors of plasma membrane electron transport, namely chloroquine, dicumarol, and capsaicin synthetic analog (*N*-vanillylnonanamide), was also tested.

**Table III.** Effect of Various Compounds on *P. blakesleeanus* Transmembrane Ferricyanide Reduction From Iron-Sufficient (+Fe 24-h Growth) and Iron-Deficient (−Fe 16-h Growth) Medium

| | Activity (%) (nmol/min·mg mycelial dry weight) | |
|---|---|---|
| Treatments | +Fe | −Fe |
| Control | 100 (3.43 ± 0.09) | 100 (23.72 ± 2.87) |
| $CaCl_2$ (1 mM) | 240 | 299 |
| $MgCl_2$ (1 mM) | 230 | 223 |
| NaCl (10 mM) | 154 | 220 |
| KCl (10 mM) | 165 | 244 |
| $CdSO_4$ (250 $\mu$M) | 0 | 0 |
| $CdSO_4$ (250 $\mu$M)[a] | 85 | 88 |
| $ZnCl_2$ (20 $\mu$M) | 25 | 70 |
| Chloroquine (20 $\mu$M)[b] | 95 | 90 |
| Dicumarol (10 $\mu$M)[b] | 70 | 44 |
| Capsaicine analog (10 $\mu$M)[b,c] | 80 | 45 |
| Putrescine (0.25 mM)[a,b] | 100 | ND |
| Chlorpheniramine (0.25 mM)[a,b] | 100 | ND |
| Dibutyril cAMP (50 $\mu$M)[a,b] | 100 | ND |
| Ascorbate (25 $\mu$M)[a,b] | 100 | ND |
| Dehydroascorbate (25 $\mu$M)[a,b] | 100 | ND |
| *p*CMB (20 $\mu$M)[b,d] | 74 | 78 |
| NEM (5 mM)[b] | 52 | 41 |
| NEM (5 mM)[a,b] | 4.4 | 6 |
| Vitamin $K_3$ (0.1 $\mu$M) | 139 | 50 |
| Vitamin $K_3$ (50 $\mu$M) | 570 | 50 |

*Note.* Reaction mixture contained *P. blakesleeanus* mycelia in 25 mM Tris-Mess buffer (pH 8.0), 1 mM potassium ferricyanide, and the effectors at the concentrations indicated. The assays were performed at 37°C as described in Materials and Methods.

[a] Mycelia were incubated with $CdSO_4$, Putrescine, Chlorpheniramine, or NEM for 30 min each at 37°C; with dibutyril cAMP, ascorbate, dehydroascorbate for 60 min each at 37°C, after which mycelia were filtered, washed with distilled water, and used to assay the ferricyanide reductase activity.

[b] The assay was carried out in 50 mM sodium citrate buffer with 5% (w/v) glucose (pH 6.5) with 1 mM ferricyanide.

[c] Capsaicin synthetic analog (*N*-vanillylnonanamide).

[d] Mycelia were incubated with *p*CMB for 15 min at 37°C, and then the ferricyanide reductase activity was assayed. Data are given as means of three separate experiments. ND: not determined.

Chloroquine had no effect on ferricyanide reductase activity whereas dicumarol and capsaicin synthetic analog inhibited reductase activity, with a higher inhibition on the enzymatic activity from mycelia grown in iron-deficient medium. Inhibition by chloroquine has been reported for animal plasma membrane AFR reductase (Villalba *et al*., 1995) and yeast cells (Santos-Ocaña *et al.*, 1995), whereas inhibition by dicumarol and capsaicin has been described for ferricyanide reductases (Döring *et al*., 1992; Santos-Ocaña *et al*., 1995; Villalba *et al*., 1993). *p*CMB and NEM caused a similar inhibition on ferricyanide reductase activity from *P. blakesleeanus* mycelia grown in both conditions, as with other ferrireductases (Inman *et al*., 1994; Van Duijn *et al*., 1998). Polyamines like putrescine or chlorpheniramine had no effect on ferricyanide reduction by *P. blakesleeanus* mycelia grown in either medium; in contrast, they significantly inhibited plasma membrane ferricyanide reductase from Erlich ascites tumor cells (Rodríguez-Caso *et al*., 1998). Dibutyril cAMP, which increases intracellular cAMP, has no detectable effect on *P. blakesleeanus* transmembrane ferricyanide reductase activity. Intracellular cAMP has, however, been reported to apparently control transplasma membrane electron transport in both *S. cerevisiae* (Lesuisse *et al*., 1991) and HL-60 cells (Rodríguez-Aguilera *et al*., 1993). On the other hand, while it has been reported that catalytic amounts of ascorbate or dehydroascorbate stimulate reduction of external ferricyanide from mammalian cells (Van Duijn *et al*., 1998), we detected no effect on transmembrane ferricyanide reductase from *P. blakesleeanus*.

Finally, the addition of vitamin $K_3$ to the reaction mixture greatly promoted ferricyanide reduction by *P. blakesleeanus* mycelia grown in iron-sufficient medium. Vitamin $K_3$ increased apparent $V_{max}$ value and decreased apparent $K_m$ value for ferricyanide (Table IV). On the other hand, ferricyanide reductase activity showed a saturable dependence on vitamin $K_3$ concentration, which had to be $1.23 \pm 0.6$ $\mu$M for 50% of apparent maximum velocity to be reached. However, vitamin $K_3$ inhibited this enzymatic activity of *P. blakesleeanus* mycelia grown in iron-deficient medium. Similar stimulation of ferrireductase activity by vitamin $K_3$ has been reported for yeast cells (Yamashoji and Kajimoto, 1986) and carrot suspension cultures (Barr *et al*., 1990). Contradictory results have also been reported—Vitamin $K_3$ depolarized the plasma membrane of *Zea mays* L. roots and stimulated ferricyanide reduction (Döring *et al*., 1992), whereas Bernstein *et al*. (1989) reported that the lipophilic $K_3$ depolarized the plasma membrane and inhibited the ferricyanide reduction in *Egeria densa* leaf cells.

## Proton Release Associated With Transplasma Membrane Electron Transport

Twenty-four-hour-old *P. blakesleeanus* mycelia grown in iron-sufficient medium released protons in 50 mM sodium citrate buffer, pH 6.5, as shown in Fig. 6, which increased with ferricyanide concentration (Fig. 6(A)), showing a saturable hyperbolic kinetics. Under these conditions, proton release was greatly stimulated by the addition of vitamin $K_3$. Proton release by *P. blakesleeanus* mycelia induced by ferricyanide also showed a saturable hyperbolic dependence on vitamin $K_3$ concentration (Fig. 6(B)). In the absence of ferricyanide, vitamin $K_3$ did not cause proton release. To determine the proton excretion rate of the plasma membrane redox system, the background rate of protons excreted by the fungus under control conditions (without any addition) was subtracted from the rate for any of the other conditions. With regard to proton release induced by ferricyanide,

**Table IV.** Effect of Ferricyanide and Vitamin $K_3$ Concentration on the Apparent Kinetic Parameters of Ferricyanide Reductase Activity and Proton Release From *P. blakesleeanus* Mycelia; and the Ratio of Proton Release to Ferricyanide Reduction at pH 6.5

| | Ferricyanide reduction | | Proton release | | |
|---|---|---|---|---|---|
| Conditions | $K_m$ [a] | $V_{max}$ [b,c] | $K_m$ [a] | $V_{max}$ [b,c] | $H^+/e^-$ ratio |
| Variable ferricyanide concentration | | | | | |
| Vitamin $K_3 = 0$ | $0.08 \pm 0.005$ | $3.73 \pm 0.05$ | $4.35 \pm 1.64$ | $23.50 \pm 3.53$ | 6.3 |
| Vitamin $K_3 = 20$ $\mu$M | $0.03 \pm 0.01$ | $11.20 \pm 0.30$ | $0.12 \pm 0.01$ | $80.40 \pm 1.35$ | 7.3 |
| Variable vitamin $K_3$ concentration | | | | | |
| Ferricyanide = 1 mM | $1.23\ 10^{-3} \pm 0.60\ 10^{-3}$ | $7.95 \pm 0.67$ | $0.079 \pm 0.066$ | $376.8 \pm 11.6$ | 47.4 |

*Note.* Mycelia were 24-h-old grown in iron sufficient-medium.
[a] (mM).
[b] (nmol/min mg mycelial dry weight).
[c] The values of $V_{max}$ refer to maximum proton excretion rate obtained by subtracting the background proton excretion (19.90 nmol $H^+$/min mg mycelial dry weight).

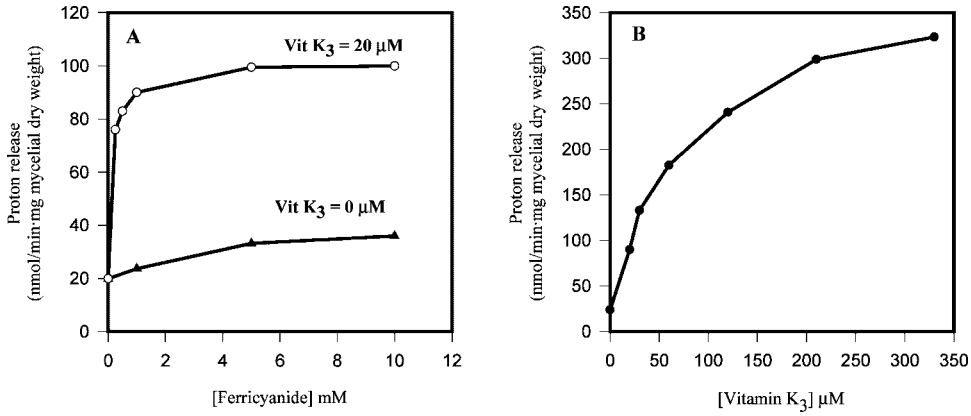


**Fig. 6.** (A) Effect of vitamin $K_3$ on proton release induced by ferricyanide reduction: vitamin $K_3$ absence (▲), vitamin $K_3$ (20 $\mu$M) presence (○). (B) Proton release dependence with vitamin $K_3$ concentration in the presence of 1 mM ferricyanide. The rate of proton release was determined using 15 mL of 50 mM sodium citrate buffer, pH 6.5, with *P. blakesleeanus* intact mycelia (39.3-mg dry weight) grown in iron-sufficient medium, as described in Materials and Methods.

the addition of 20 $\mu$M vitamin $K_3$ increased the apparent $V_{max}$ value for proton excretion, whereas the apparent $K_m$ value for ferricyanide decreased (Table IV). At 1 mM ferricyanide, the maximum proton excretion induced by vitamin $K_3$ was 376.80 ± 11.60 nmoles $H^+$/min mg mycelial dry weight, and the vitamin $K_3$ concentration required to produce 50% of this maximum value was 79.00 ± 6.64 $\mu$M. As can be seen in Table IV, the proton release and ferricyanide reduction, in the absence of vitamin $K_3$ proceeded nonstoichiometrically at a $H^+/e^-$ ratio around 6, whereas in the presence of vitamin $K_3$ this ratio increased from around 7 to 47. The effect of ferricyanide and vitamin $K_3$ was not parallel. As for ferricyanide reduction, the maximum transport of $e^-$ was reached as from 1 mM ferricyanide, whereas $H^+$ excretion was maximum for ferricyanide concentrations over 10 mM; thus the $H^+/e^-$ ratio increased with ferricyanide concentration to a maximum value of 7. This ratio also increased with vitamin $K_3$ concentration to a maximum value of 47.

Addition of 50 mM glucose enhanced proton release (about 145% activation) in the absence of ferricyanide. Proton release induced by glucose has been observed in suspensions of yeast cells (Serrano, 1980; Sigler *et al.*, 1981), suggesting that proton release requires metabolic energy. Table V shows that proton release induced by glucose from *P. blakesleeanus* mycelium is totally inhibited by diethylstilbestrol, which is an inhibitor of plasma membrane ATPase (Serrano, 1980) but not by dicumarol. Furthermore, diethylstilbestrol (DES) did not affect proton release induced by ferricyanide in the presence or the absence of vitamin $K_3$, but dicumarol caused inhibition of

**Table V.** Effect of Diethylstilbestrol (DES), Dicumarol, and Glucose on Proton Release

| Treatments | nmol $H^+$/min·mg mycelial dry weight | | |
|---|---|---|---|
| | Control | +DES (65 $\mu$g/mL) | +Dicumarol (100 $\mu$M) |
| None | 19.90 ± 2.34 | ND | 19.50 ± 2.52 |
| FeCNK (1 mM) | 23.80 ± 3.67 | 21.12 ± 2.95 | 19.90 ± 2.34 |
| FeCNK (1 mM) + Vitamin $K_3$ (20 $\mu$M) | 89.82 ± 5.59 | 87.41 ± 3.27 | 27.68 ± 3.33 |
| Glucose (50 mM) | 48.81 ± 4.22 | 1.84 ± 0.13 | 42.60 ± 3.82 |
| Glucose (50 mM) + FeCNK (1 mM) | 54.23 ± 3.76 | ND | ND |
| Glucose (50 mM) + Vitamin $K_3$ (20 $\mu$M) | 48.80 ± 4.85 | ND | ND |
| Glucose (50 mM) + Vitamin $K_3$ (20 $\mu$M) + FeCNK (1 mM) | 152.91 ± 4.65 | ND | ND |

*Note.* The rate of proton release was determined using 15 mL of 50 mM sodium citrate buffer (pH 6.5) with intact mycelia (39.3-mg dry weight) from *P. blakesleeanus* iron-sufficient medium, as described in Materials and Methods. Mean values ± SD are from three separate experiments. ND: not determined.

**Table VI.** Effect of Vitamin $K_3$ on Ferricyanide Reductase Activity and Proton Release from *P. blakesleeanus* Mycelia; and the Ratio of Proton Release to Ferricyanide Reduction at pH 6.5

| Conditions | +Fe (24 h) | | | −Fe (16 h) | | |
|---|---|---|---|---|---|---|
| | Ferricyanide reduction | Proton[a] release | $H^+/e^-$ ratio | Ferricyanide reduction | Proton[a] release | $H^+/e^-$ ratio |
| Ferricyanide (1 mM) | 3.73 ± 0.09 | 3.90 ± 0.12 | 1.0 | 23.72 ± 2.87 | 51.57 ± 4.23 | 2.0 |
| Ferricyanide (1 mM) + Vitamin $K_3$ (20 $\mu$M) | 11.20 ± 1.33 | 60.50 ± 4.86 | 5.4 | 10.45 ± 1.30 | 54.23 ± 3.76 | 5.0 |

*Note.* Mycelia were from 24-h-old grown in iron-sufficient medium [+Fe (24 h)] or 16-h-old grown iron-deficient medium [−Fe (16 h)]. Mean values ± SD are from three separate experiments. Ferricyanide reduction and proton release are expressed as nmol/min mg mycelial dry weight.

[a] Proton release values were obtained by subtracting the background proton excretion: 19.90 nmol $H^+$/min mg mycelial dry weight for +Fe (24 h) and 7.6 nmol $H^+$/min mg mycelial dry weight for −Fe (16 h).

proton release induced by ferricyanide in both conditions (Table V). These results indicated that the basal rate of protons excreted by the fungus with or without glucose may be attributable to plasma membrane ATPase. Similar independence of the plasma membrane redox system of the ATPase pumping the protons has been reported for proton release coupled with ferricyanide reduction by yeast cells (Yamashoji and Kajimoto, 1986).

Additionally, we determined the influence of ferricyanide and vitamin $K_3$ on $H^+$ efflux when *P. blakesleeanus* mycelia were grown in iron-deficient medium. Table VI shows the comparison of the effects produced by ferricyanide and vitamin $K_3$ on ferricyanide reduction and proton release when *P. blakesleeanus* mycelia were grown in iron-sufficient and iron-deficient medium. Iron depletion noticeably activates both ferricyanide reduction and proton release in the absence of vitamin $K_3$, although proton release velocity was twice as high as ferricyanide reduction velocity, so the $H^+/e^-$ ratio increased to 2. However, the presence of 20 $\mu$M vitamin $K_3$ caused no effect on proton release and inhibited (about 50%) ferricyanide reduction, showing a $H^+/e^-$ ratio about 5. Under iron-sufficient conditions, the presence of 20 $\mu$M vitamin $K_3$ activated both ferricyanide reduction and proton release, proton release velocity being five times that of ferricyanide reduction velocity. Thus, although vitamin $K_3$ did not alter the $H^+/e^-$ ratio, this was reached by a differential effect on ferricyanide reduction and proton release. These results reinforced the hypothesis that the processes of ferricyanide reduction by plasma membrane ferrireductase and proton release by mycelia of *P. blakesleeanus* are coupled but independently regulated.

## DISCUSSION

Transmembrane redox activities have been found in eukaryotic cells. We have characterized the ability of intact mycelia from the zygomycete fungus *P. blakesleeanus* to catalyze extracellular ferricyanide reduction. Our results support the conclusion that this primary effect is due to a plasma membrane ferrireductase which uses internal NADH as its electron donor. Conditions that affect the integrity of mycelia hindered the ferricyanide reductase activity, most probably owing to the consumption of NADH, the potential source of the reducing equivalents for the system. The lack of significant reductase activity using conditioned buffer rules out the possibility that some product secreted by the mycelia might cause the residual activity. It has been reported that the ascorbate released by erythrocytes could reduce extracellular iron (Orringer and Roer, 1979). Moreover, the presence of mitochondria released from lyzed mycelia was excluded because the mitochondrial inhibitors had no effect. Ferricyanide reduction by *P. blakesleeanus* mycelia showed a linear relationship with the mycelial mass. All the foregoing, together with the fact that both ferricyanide and ferrocyanide are membrane impermeants, allows us to assume that ferricyanide reduction occurs extracellularly in association with plasma membrane ferrireductase activity.

Ferricyanide reductase activity in intact mycelia of *P. blakesleeanus* showed a hyperbolic saturation kinetics for ferricyanide at pH 6.5, with an optimum pH of about 8.0, as reported for *Phanerochaete chrysosporium* (Stahl and Aust, 1995). A similar $K_m$ value for ferricyanide has been described for ferricyanide reductase for intact Ehrlich ascites tumor cells (Medina *et al.*, 1988) and *Zea mays* root cells (Qiu *et al.*, 1985), whereas a lower $K_m$ value was found for K562 cells (Inman *et al.*, 1994). NADH has been reported to be the natural electron donor for the transplasma membrane redox system in different eukaryotic cells (Barr *et al.*, 1985; Crane *et al.*, 1985; Sun *et al.*, 1986), including yeast cells (Crane *et al.*, 1982; Yamashoji and Kajimoto, 1986) and white rot fungus *P. chrysosporium* (Stahl and Aust, 1995). Our results indicate that NADH may be used as a source of the intracellular reducing equivalents for ferricyanide reduction

by *P. blakesleeanus* mycelia. Incubation with lactate, an immediate source of cytoplasmic NADH, made it possible to maintain a higher ferricyanide reductase activity in comparison with that obtained with or without glucose in similar conditions. Therefore, glucose under these conditions was not a suitable source of available cytosolic NADH, because the glucose consumed may be used to supply ATP for proton release controlled by ATPase of plasma membrane (Marcos *et al.*, 1998). We cannot exclude the possibility that lactate formed may be ejected into the external medium, but not in sufficient quantities to provide the required NADH. However, when lactate was added to the incubation medium, it was incorporated into *P. blakesleeanus* mycelia and directly transformed into pyruvate, serving as an immediate source of NADH. Notably, lactate dehydrogenase in *P. blakesleeanus* is a regulatory enzyme controlled by the energy charge (Busto *et al.*, 1983; Soler *et al.*, 1982). On the other hand, we think that the slight inhibition by iodoactate is caused by its action as a sulphydryl groups reagent rather than as a glycolytic inhibitor. The inhibition by *p*CMB and NEM of transmembrane ferricyanide reduction in intact mycelia, as well as the inhibition produced by *p*CMB, NEM, and iodoacetate of ferricyanide reduction using plasma membrane fractions of *P. blakesleeanus* (data not shown), supports this hypothesis. In addition it indicates the involvement of a sensitive protein sulfydryl group(s) involved in the process of plasma membrane electron transport in *P. blakesleeanus*.

*P. blakesleeanus* transmembrane ferricyanide reductase activity was always present, but it increased remarkably under extracellular iron-limited conditions, which are often associated with specific iron-acquisition mechanisms. Iron-repressible cell surface ferric reductases, which potentially facilitate iron uptake, have been reported for *S. cerevisiae* (Lesuisse *et al*., 1987), *Schizosaccharomyces pombe* (Roman *et al*., 1993), *C. albicans* (Morrissey *et al*., 1996), *Cryptococcus neoformans* (Nyhus *et al.*, 1997), and *Histoplasma capsulatum* (Timmerman and Woods, 1999). In contrast with ferric reductase activity in *S. cerevisiae* (Eide *et al*., 1992) and *C. albicans* (Morrissey *et al*., 1996), in *P. blakesleeanus* it does not depend on the growth phase of the culture. However, our results also suggest that the regulation of *P. blakesleeanus* ferricyanide reductase is dependent, in some way, on the cellular iron content. Growth of *P. blakesleeanus* mycelia on iron-sufficient medium may result in an accumulation of intracellular iron, probably as ferritin, which could be used when mycelia are transferred to an iron-deficient medium. *P. blakesleeanus* mycelia store iron as ferritin (up to 1 mg of iron per gram of wet weight when they are grown on iron-rich medium) and previous results suggest that the level of cytoplasmic iron, derived either from ferritin or from an extracellular source, is carefully regulated, but the nature of this regulation is not known yet. The selective incorporation of ferritin into *P. blakesleeanus* spores has been reported, and in germinating spores, ferritin releases iron into a soluble pool (Cerdá-Olmedo and Lipson, 1987). As yet, our results do not allow us to confirm an induction of *P. blakesleeanus* transmembrane ferricyanide reductase activity under iron-limited conditions; alternatively it could be a constitutive reductase, which is activated or amplified under these iron-depletion conditions. A similar enhancement of iron-reducing activity (turbo-system) has been reported in the roots of many dicotyledons and some monocotyledons under iron deficiency (Bienfait, 1985; Döring and Lüthje, 1996).

Our results also suggest a possible connection between iron and copper metabolism in *P. blakesleeanus*, as has been described in *S. cerevisiae* (Georgatsou *et al*., 1995; Hassett and Kosman, 1995), *C. albicans* (Morrissey *et al*., 1996), and plants (Evans *et al*., 1992; Welch *et al*., 1993). However, maximum ferricyanide reductase activity was observed in *P. blakesleeanus* mycelia grown under iron-deficient and copper-sufficient conditions, which rules out the negative regulation by copper of *P. blakesleeanus* plasma membrane ferricyanide reductase activity. In contrast, the activity of both Fre1p from *S. cerevisiae* (Georgatsou *et al*., 1995) and ferric reductase from *C. albicans* (Morrissey *et al*., 1996) were induced by copper or iron depletion.

The parallel activator effect of magnesium and calcium ions appears to preclude a relationship to the calcium-stimulated proton transport system (Hinnen and Racker, 1979; Stroobant and Scarborough, 1979). On the other hand, inhibition by $Zn^{2+}$ is an interesting feature common to the plasma membrane redox system of different cells, and it has been interpreted as the involvement of histidine residues in the electron transfer to ferricyanide in plasma membranes of Ehrlich cells (Medina *et al*., 1994). The effects of the inhibitors, chloroquine, dicumarol, and the capsaicin synthetic analog are consistent with electron transport across the *P. blakesleeanus* plasma membrane. In addition, the differential sensitivity of ferricyanide reduction to chloroquine, dicumarol, and capsaicin analog reinforces the participation of a ferricyanide reductase in the *P. blakesleeanus* plasma membrane. In previous in vivo studies, we have also detected AFR reductase activity in *P. blakesleeanus* plasma membrane, which was strongly inhibited by chloroquine but not by dicumarol.

Reduction of ferricyanide by *P. blakesleeanus* mycelia is accompanied by the release of protons. This effect has been observed in mammalian, plant, and

microorganism cells (Crane *et al*., 1991; Döring and Lüthje, 1996; De Grey, 2003). Transfer of electrons across the plasma membrane may be accompanied by proton transference if protonated electron carriers are oxidized at the external surface by nonprotonated acceptors (e.g., quinones or flavins oxidized or cytochromes), or if the redox reactions drive or activate a proton pump. However, the relationship between proton secretion and ferricyanide reduction remains to be elucidated in most cases. The differential inhibition on proton release produced by diethylstilbestrol and dicumarol rules out the possibility of proton release coupled to ferricyanide reduction being due to plasma membrane ATPase, as has been suggested for yeast cells (Yamashoji and Kajimoto, 1986).

Both ferricyanide reduction and proton release followed a hyperbolic saturation kinetics for ferricyanide concentrations in the absence or the presence of vitamin $K_3$, and in both cases the transplasma membrane electron transport was accompanied by a nonstoichiometric proton release. In the absence of vitamin $K_3$, the $H^+/e^-$ ratio was about 6–7, which reached a maximum value of 47 in the presence of vitamin $K_3$ under our experimental conditions. Superstoichiometric proton efflux associated with transplasma membrane electron transport has been reported in mammals (Crane *et al*., 1991; De Grey, 2003) and plants (Crane *et al*., 1991; Rubinstein and Luster, 1993), and the evidence indicates that it could be based on activation of a channel for proton movement or proton pumping. Our results appear to rule out $H^+$-ATPase activation as the major factor responsible for proton release associated to ferricyanide reduction and stimulated by vitamin $K_3$. The stimulation effect produced by vitamin $K_3$ was not the same on ferricyanide reduction as on proton release and could be considered as a classic activator effect on some component(s) of both processes. However, a contribution to proton transfer by protonated electron carriers in the membrane cannot be ruled out. *P. blakesleeanus* is described as containing a ubiquinone with nine isopentenyl residues (Cerdá-Olmedo and Lipson, 1987), but we have no evidence for the K-type vitamin in the plasma membrane of this fungus. Added vitamin $K_3$ could integrate into the plasma membrane owing to its high hydrophobicity (Lüthje and Böttger, 1995). Vitamin $K_3$, like other quinones, could act as a mobile electron carrier within the lipid phase of the membrane and could be reduced by enzymes present in it, e.g., NAD(P) quinone oxidoreductase (Döring and Lüthje, 1996). Inhibition of *P. blakesleeanus* mycelium proton release by dicumarol is consistent with vitamin $K_3$ action. On the other hand, the different effect produced by vitamin $K_3$ on ferricyanide reduction and proton release under iron-deficient conditions when compared with the effect obtained under iron-sufficient conditions, is in agreement with the hypothesis that the processes of ferricyanide reduction and proton release in *P. blakesleeanus* are coupled but differently regulated. We are now researching other compounds and redox activities present in *P. blakesleeanus* plasma membrane to determine whether they are shared or each system operates independently, together with their roles in proton release. The characterization and molecular identification of the components of redox activity in *P. blakesleeanus* plasma membrane could certainly contribute to determining whether some of these redox activities are part of a signal transduction chain.

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