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IRON UPTAKE FROM FERRIOXAMINE AND FROM FERRIRHIZOFERRIN BY GERMINATING SPORES OF *RHIZOPUS MICROSPORUS**

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Abstract—Mucormycosis caused by the fungus *Rhizopus* has been documented in iron overloaded patients and more particularly in dialysis patients, both when treated with desferrioxamine B (DFO). This iron and aluminium chelator is thought to play a role in the pathogenesis of this infection. We therefore investigated *in vitro* the cellular pharmacology of iron chelated by DFO in the fungus *Rhizopus*. In a medium, designed for fungal cultivation, *Rhizopus microsporus var. rhizopodiformis* takes up iron from ferric–DFO complex (^{55}Fe.DFO) and from ^{55}Fe.rhizoferrin, the siderophore synthesized and secreted by *Rhizopus* [Drechsel *et al.*, *Biol. Metals* **4**: 238–243, 1991]. In both cases, iron accumulation is partially saturable with the duration of exposure and the chelator concentration. Fe.DFO binds to *Rhizopus*; iron becomes trapped and remains associated with the fungus, whereas the iron-depleted siderophore is released in the extracellular medium. In a medium designed for mammalian cell cultivation and in the absence of human serum, the fungal iron accumulation both from ^{55}Fe.DFO and from ^{55}Fe.rhizoferrin is proportional to the chelator concentration. Human serum at 40% does not influence the iron accumulation from Fe.DFO but it significantly affects that from Fe.rhizoferrin which, in the presence of serum, only occurs at concentration > 5 μM. This difference finds its explanation in the iron transfer observed between Fe.rhizoferrin and seric apotransferrin, the latter making the metal unavailable to *Rhizopus*. By contrast, no iron transfer takes place between Fe.DFO and apotransferrin, allowing fungal iron utilization from this complex, even at very low concentrations. The iron uptake, being inhibited by NaN_3 and KCN, is energy-dependent; being inhibited by bipyridyl, it requires prior reduction of ferric iron; being unaffected by the covalent linkage of Fe.DFO to albumin, it does not require the entry of Fe.DFO within the fungus. These *in vitro* results strongly suggest that, upon administration of DFO to iron overloaded or dialysis patients, the formed Fe.DFO is efficiently used as an iron source by *Rhizopus*, even in the presence of serum apotransferrin or rhizoferrin. The consequent promotion of the growth of *Rhizopus* helps explain the increased risk of mucormycosis in DFO-treated patients.

Key words: iron; *Rhizopus*; ferrioxamine; feroxamine; rhizoferrin; mucormycosis

Mucormycosis (also called zygomycosis) is an infection caused by one of the fungi belonging to the class *Zygomycetes*. The genus *Rhizopus* is the one most often involved in this infection [1]. One of us reported the results of an international registry of mucormycosis in dialysis patients [2] and found that 46 of the 59 patients with this infection were treated with the iron and aluminum chelator DFO‖ at the time the infection developed. The pathogenic role of this chelator was strengthened by studies on experimental zygomycosis, where simultaneous administration of DFO significantly decreased the survival of the infected animals [3]. Our group found that *Rhizopus* takes up iron from the Fe.DFO and that this iron uptake is accompanied by a fungal growth stimulation. It was therefore concluded that zygomycosis in DFO-treated dialysis patients is a siderophore-mediated infection [4]. The fact that dialysis patients, treated with DFO, accumulate Fe.DFO in their serum for prolonged periods, renders them particularly susceptible to this infectious complication [5].

In the present study, the mechanism of iron uptake by *Rhizopus* from Fe.DFO is further investigated. We used *in vitro* experimental conditions mimicking as closely as possible conditions encountered in human pathology. It is concluded that iron accumulation by *Rhizopus* from Fe.DFO is an energy-requiring process, which involves dissociation of iron from the Fe.DFO complex and which could require a reductive step.

MATERIALS AND METHODS

Strains and culture conditions. Two strains of *Rhizopus microsporus var. rhizopodiformis*, further referred to as *R. microsporus*, were studied. They belong to the class of *Zygomycetes*, order *Mucorales*,

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‖ Abbreviations: BDM; basal defined medium; DFO; desferrioxamine B; Fe.DFO, ferric–desferrioxamine complex.

genus *Rhizopus*. The first strain (B 51321) was isolated from a dialysis patient who died from mucormycosis while on DFO treatment and the second one (B58739) from nature [6]. They were routinely maintained on Sabouraud dextrose agar (Difco, Detroit, MI, U.S.A.) Sporangiospores (spores) were prepared as described by Boelaert *et al.*[4].

Chelators. DFO (Desferal^R, Ciba Geigy, Basel, Switzerland) was labeled with ^3H by reductive methylation of the amino group to form [^{3}H]Me-DFO, as described previously [7]. [^{14}C]DFO was labeled biosynthetically and had a very low specific radioactivity (0.086 μCi/mmol); it was kindly provided by Dr H. H. Peter, (Ciba Geigy, Basel, Switzerland). Fe.DFO was prepared, as described previously [7], by mixing stoichiometric amounts of either DFO with $^{55}FeCl_3$ (Amersham International, Amersham, U.K.) or [^{3}H]Me-DFO with $FeCl_3$. Rhizoferrin, the siderophore synthesized and secreted by *Rhizopus* [8] was kindly provided by Prof. G. Winkelmann (Institut für Mikrobiologie and Biotechnologie, Tübingen, Germany).

DFO was covalently linked to bovine serum albumin with glutaraldehyde, as described by Yokoyama *et al.*[9] and as modified by Koizumi *et al.* [10]. After extensive dialysis against PBS, $^{55}FeCl_3$ was added to complex DFO. E_{400} was measured in order to evaluate the quantity of Fe.DFO effectively linked to the albumin and protein was assayed according to Lowry *et al.* [11]. The molar ratio DFO/albumin was 1:10.

Iron and chelator uptake. For the study of fungal iron uptake, 10^6 spores were cultivated in 1.1 mL Wickerham's medium (Difco Laboratories, Madison, WI, U.S.A.) which contains 0.65% (w/v) bacto yeast nitrogen (Difco) 2% (w/v) dextrose in 7.5 mM Tris buffer, pH 7.5 and 0.54 μM iron, assayed as in [12]. Spores were incubated for different durations at 37° in the presence of radiolabeled chelators. At the end of incubation, 1 mL of the suspension was filtered under vacuum through 0.22 μm microfiltration membranes, after 3 washings with 5 mL of PBS, filters and spores were assayed for radioactivity, after dispersion with 5 mL Aqualuma cocktail (Lumac-Lsc, Olen, Belgium) in a Tri Carb 460CD scintillation counter (Packard Instruments, San Diego, CA, U.S.A.).

When both the iron uptake and the growth of *Rhizopus* were measured in the same experiment, 10^6 spores were cultivated for 24 hr at 37° in 2.1 mL BDM, a synthetic medium for mammalian cell cultivation [13], which contains 1.35 μM of ferric iron. This growth medium was supplemented or not with 40% human serum from healthy human volunteers. Iron uptake was determined as above, while cell growth was measured as described below.

For competition experiments, 10^6 spores were incubated for 3 hr at 37° in 1.1 mL Wickerham's medium containing 2.5 μM ^{55}Fe.DFO, Fe.[^{3}H]Me-DFO or ^{55}Fe.rhizoferrin in the presence or absence of different concentrations of Fe.DFO or Fe.rhizoferrin. At the end of the experiments, samples were processed as for the uptake experiments.

For washout experiments, 10^6 spores in 1.1 mL of Wickerham's medium were preincubated with ^{55}Fe.DFO, Fe[^{3}H]Me-DFO or ^{55}Fe.rhizoferrin. Spores were centrifuged for 15 min at 2000 *g* in PBS and harvested either in ferrichelator-free Wickerham's medium, or in 100 mM acetate buffer pH 3.6 in 150 mM NaCl or in 100 μM Fe.DFO in Wickerham's medium. At the end of incubation, spores were filtered by centrifugation for 10 min at 2000 *g* through Ultrafree-MC membranes (pore diameter of 0.22 μm; Millipore) and washed twice with 1 mL PBS. Both the filter and the filtrate were analysed for radioactivity, as above.

In vitro *growth.* Spores (10^6) were incubated in 2.1 mL of BDM with human serum and chelators. After 24 hr at 37°, fungal growth was assayed by turbidimetry at 400 nm as described previously [4].

Iron transfer. ^{55}Fe (30 nmol), chelated with either DFO or rhizoferrin, were mixed in stoichiometric amounts with rhizoferrin, DFO or human serum (iron content; 16.7 μM; transferrin; 2.40 mg/mL, i.e. 30 μM; TIBC, 45.6 μM; iron saturation of transferrin; 24%); mixtures were then separated by agarose gel electrophoresis (Ciba Corning; Palo Alto, CA, U.S.A.) run in 0.05 M veronal buffer, pH 8.6. After electrophoresis, the gel was dried; autoradiography was carried out with Hyperfilm^{TM}-β max (Amersham International).

Statistics. Mean values are given together with SEM. Statistical significance between study groups was determined by the one factor ANOVA test.

RESULTS

Iron accumulation

Spores of *R. microsporus*, isolated from a dialysis patient who died from mucormycosis, were incubated for different durations in Wickerham's medium at 37° in the presence of 5 μM ^{55}Fe.DFO or ^{55}Fe.rhizoferrin. The accumulation of radioiron by the spores from ^{55}Fe.DFO or ^{55}Fe.rhizoferrin occurs within the first hour (DFO, Fig. 1A) or within a few minutes (rhizoferrin, Fig. 1B). In the same experimental conditions, the accumulation rate of ^{3}H label from Fe.[^{3}H]Me-DFO is proportional to the duration of incubation and results, after 3 hr incubation, in only 40% of the accumulation level of ^{55}Fe ($P < 0.0001$; not shown). A strain of *R. microsporus* isolated from the environment behaves similarly, giving similar accumulation levels as the clinical strain for both ^{3}H and ^{55}Fe (not shown).

The same experiments were performed with spores preincubated in ferrichelator-free medium for 3 or 6 hr at 37°. Preincubation accelerates the morphological transformation of the fungus: after 3 hr of preincubation, no germination of spores is detected by phase contrast microscopy, whereas after 6 hr, some mycelia are present (not illustrated). After 3 or 6 hr of preincubation, *Rhizopus* spores accumulate more ^{55}Fe than in the absence of preincubation ($P < 0.0001$, for the accumulation of both ferrichelators without preincubation vs after 6 hr of preincubation). Figure 1 suggests a biphasic process: the first phase is comparable to the accumulation of radioiron in the absence of preincubation and a second phase results in an increased accumulation of ^{55}Fe, largely proportional

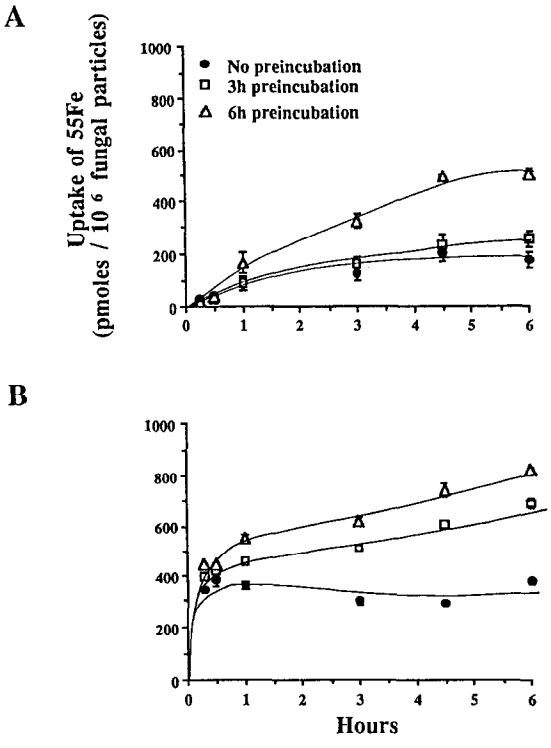

Fig. 1. Iron uptake from ^{55}Fe.DFO (A) or from ^{55}Fe.rhizoferrin (B) in function of the duration of incubation. *R. microsporus* spores were incubated in the presence of 5 μM ^{55}Fe.chelators with or without preincubation at 37° in Wickerham's medium (supplemented with Tris buffer). Mean results ± SEM of eight independent experiments are given.

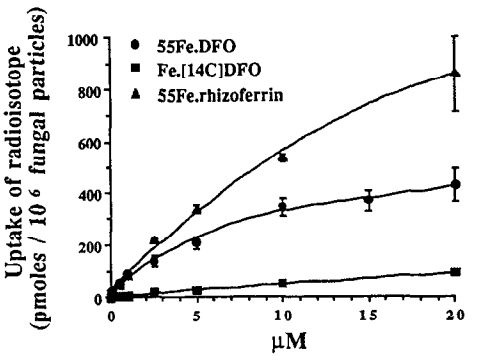

Fig. 2. Uptake of ^{55}Fe from radiolabeled Fe.DFO or Fe.rhizoferrin and uptake of ^{14}C-labeled DFO in function of chelator concentration. *R. microsporus* spores were incubated for 3 hr at 37° in the presence of different concentrations of ferrichelators. Mean results ± SEM of 12 independent experiments are given.

to the duration of incubation. This second phase continues up to at least 24 hr of incubation, at which time a complete germination of spores is observed by phase contrast microscopy (not illustrated).

Without preincubating the spores, iron accumulation from both Fe.DFO and Fe.rhizoferrin by germinating spores is not significantly affected by 10 mM of either NaN_3 or KCN; this indicates that, in these conditions, the phenomenon is energy-independent. By contrast, after 3 hr of preincubation at 37°, the iron accumulation from both ferrichelators is decreased by 46% (Fe.DFO + NaN_3), and 26% (Fe.DFO + KCN), as well as by 29% (Fe.rhizoferrin + NaN_3) and 21% (Fe.rhizoferrin + KCN). This indicates that, once the morphological transformation of *Rhizopus* spores is initiated, iron accumulation from these chelators is partially energy-dependent.

In another set of experiments, *Rhizopus* spores were preincubated for 24 hr at 37° in Wickerham's medium in the absence or presence of 5 μM DFO (to chelate the iron present in the cultivation medium). Spores were then isolated and reincubated for 3 hr with 5 μM ^{55}Fe.DFO. This preincubation in the presence of DFO does not significantly affect the subsequent iron accumulation ($P = 0.51$), which remains similar to the levels illustrated in Fig. 1A.

Rhizopus spores were incubated at 37° with different concentrations of ^{55}Fe.DFO, Fe.[^{14}C]DFO or ^{55}Fe.rhizoferrin (Fig. 2). The iron accumulation from ^{55}Fe.DFO increases with the concentration of the ferrichelator and tends to a plateau level at concentrations ≥ 10 μM, suggesting a process that is at least partially saturable. Similar results are obtained in the presence of ^{55}Fe.rhizoferrin ($P < 0.0001$). With the latter ferrichelator, however, saturation is not reached at a concentration of 20 μM and the rate of iron accumulation is higher ($P < 0.0005$). The accumulation of ^{14}C (Fig. 2) or ^{3}H (not shown) by *R. microsporus* incubated in the presence of either biosynthetically labeled Fe.[^{14}C]-DFO or chemically derivatized Fe.[^{3}H]Me-DFO is proportional to the chelator concentration in the medium. However, the accumulation level of the chelator is significantly lower than that of radioiron ($P < 0.0001$).

Competition

In a further attempt to examine whether *Rhizopus* could express specific binding sites for the studied iron chelators, competition experiments were performed on germinating spores by incubation for 3 hr at 37° with 2.5 μM ^{55}Fe.DFO or ^{55}Fe.rhizoferrin in the presence of different concentrations of unlabeled ferrichelators. Unlabeled Fe.DFO and Fe.rhizoferrin inhibit almost completely radioiron accumulation from ^{55}Fe.DFO and ^{55}Fe.rhizoferrin, respectively, with half-inhibition at 3 and 6 μM (Fig. 3A and B). The inhibition of iron accumulation from ^{55}Fe.rhizoferrin by Fe.rhizoferrin starts at a concentration ≥ 2.5 μM. Fe.rhizoferrin inhibits radioiron accumulation from ^{55}Fe.DFO by 69% and Fe.DFO that from ^{55}Fe.rhizoferrin by 51%. These results suggest the binding of chelators to different sites of which one of them can fix the two chelators.

Washout

R. microsporus spores were preincubated for 1 hr at 37° with either ^{55}Fe.DFO, Fe.[^{3}H]Me-DFO or

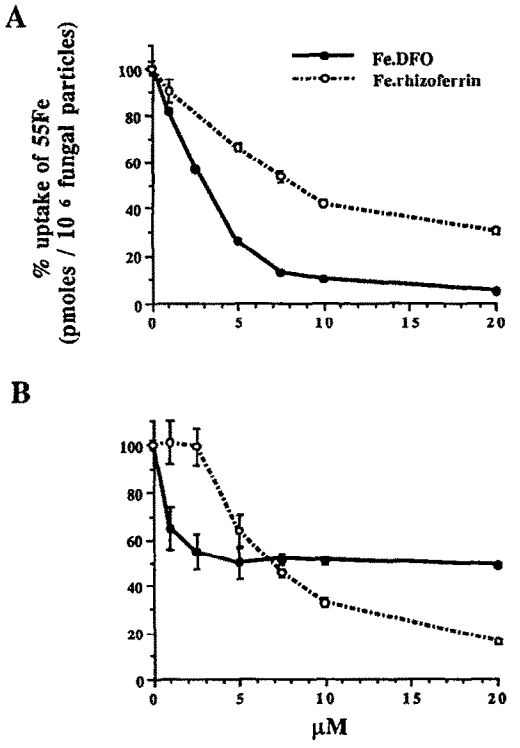

Fig. 3. Competition experiments. *R. microsporus* spores were incubated for 3 hr at 37° with 2.5 μM of either ^{55}Fe.DFO (A) or ^{55}Fe.rhizoferrin (B). Different concentrations of unlabeled Fe.DFO or Fe.rhizoferrin have been added and iron uptake was measured. Results were normalized, with the radioisotope uptake without additive considered to be 100%. Mean results ± SEM of eight independent measures are given.

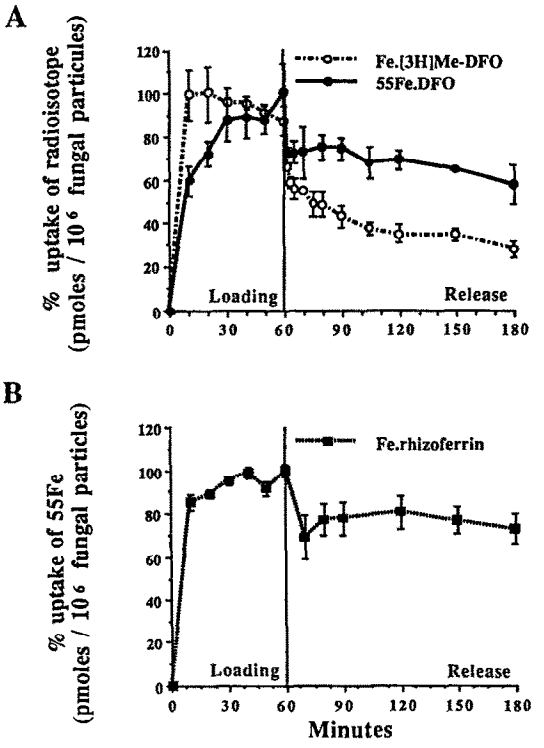

Fig. 4. Washout experiments. *R. microsporus* spores were first incubated with 5 μM ^{55}Fe.DFO, Fe.[^{3}H]Me-DFO (A) or ^{55}Fe.rhizoferrin (B)(*loading*). Spores loaded for 60 min were then washed and subsequently reincubated two more hours in a Fe.DFO- or Fe.rhizoferrin-free medium (*release*). Results were normalized, with the maximum uptake of radioisotope considered to be 100%. Mean results ± SEM of eight independent experiments are given.

^{55}Fe.rhizoferrin (*loading*). At the end of the preincubation with Fe.DFO, germinating spores have accumulated 47-fold more ^{55}Fe than ^{3}H label and after washings, they were reincubated in a chelator-free medium (*release*). After an immediate release of 27% of the accumulated radioiron from ^{55}Fe.DFO, 73% of the metal remains associated with *Rhizopus* for at least 120 min. In contrast, 68% of the accumulated ^{3}H labeled material is washed out from the spores, with a half-life of 15 min (Fig. 4A). Analysis of the radioactive material released in the reincubation medium indicates that 91% is extractable by benzyl alcohol, suggesting that the ^{3}H is still associated with DFO or extractable metabolites (not shown). At 120 min, 112-fold more ^{55}Fe than ^{3}H label is associated with the spores. When the *loading* experiment is performed with ^{55}Fe.rhizoferrin (Fig. 4B), 74% of the iron remains associated with the spores, after a short phase of immediate release.

The association of radioiron with *R. microsporus* spores was further confirmed in another set of experiments. Spores, incubated for 3 hr with 10 μM ^{55}Fe.FDO, were washed for 10 min at 4° either in acetate buffer at pH 3.6 or in 100 μM Fe.DFO in Wickerham's medium. In both cases, less than 25% of the radioiron initially accumulated by the spores is released in the washout medium (Fig. 5).

Effect of human serum

In order to study the iron uptake in conditions closer to human pathology, *R. microsporus* spores were also cultivated in BDM, a synthetic medium designed for mammalian cell cultivation. Iron uptake was measured after 24 hr incubation at 37° in the presence or absence of human serum, in order to mimic the infection within different body compartments. In the absence of serum (Fig. 6A), the rates of iron uptake from ^{55}Fe.DFO or ^{55}Fe.rhizoferrin are not significantly different and are proportional to the ferrichelator concentrations. However, when 40% human serum is added to the cultivation medium, radioiron uptakes from ^{55}Fe.DFO and ^{55}Fe.rhizoferrin become very different (Fig. 6B). Iron uptake from ^{55}Fe.DFO is observed at the lowest Fe.DFO tested (10 nM) and it increases proportionally to its concentration. By contrast, no significant iron accumulation is observed from ^{55}Fe.rhizoferrin up to 5 μM; at higher ^{55}Fe.rhizoferrin concentrations, iron uptake increases as a function of its concentration.

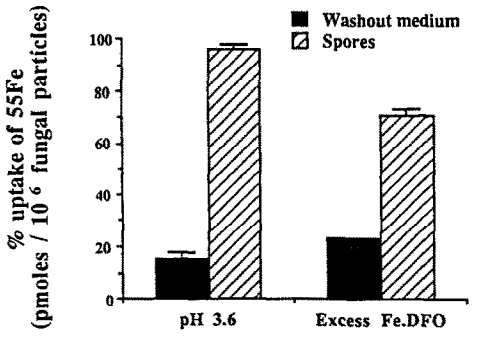

Fig. 5. Treatment experiments. *R. microsporus* spores were incubated for 3 hr at 37° with 10 μM ^{55}Fe.DFO in the presence of an acetic buffer (pH 3.6) or an excess of Fe.DFO (100 μM). Results were normalized, as described in the legend of Fig. 3. Mean results ± SEM of eight measures are given.

Iron transfer

Autoradiography of an agarose gel submitted to electrophoresis indicates that neither rhizoferrin nor apotransferrin nor human serum can take up radioiron from Fe.DFO (Fig. 7). By contrast, DFO complexes all the radioiron initially associated with Fe.rhizoferrin. In our experimental conditions, human apotransferrin takes up part of the iron from Fe.rhizoferrin; this transfer is only observed in the presence of serum.

Metabolic inhibitors and bipyridyl

The effect of two metabolic inhibitors (NaN_3 and KCN) was studied, both on the accumulation of ^{55}Fe from ^{55}Fe.DFO and on the growth of *Rhizopus* in BDM supplemented with 40% human serum (Fig. 8). NaN_3 almost completely abolishes iron uptake, while it inhibits growth by 88.5%. KCN also strongly decreases both iron uptake and growth in a concentration-dependent process, iron uptake being more inhibited than fungal growth.

Bipyridyl, a chelator of Fe^{2+}, also inhibits both the iron accumulation and the growth of the fungus induced by Fe.DFO (Fig. 9). The effect of bipyridyl is concentration dependent.

Binding of DFO to albumin

DFO was covalently attached to serum albumin and loaded with ^{55}Fe before exposure to the spores. *R. microsporus* spores were incubated for 24 hr at 37° in BDM supplemented with 40% serum with this complex at a Fe.DFO concentration of 1 μM; the accumulation of radioiron reaches 1316.5 pmol/10^6 fungal particles, which corresponds to 94% of the level of iron acquisition from unconjugated ^{55}Fe.DFO (no significant difference). At the end of the experiment, about 65% of iron initially present in the medium has been accumulated.

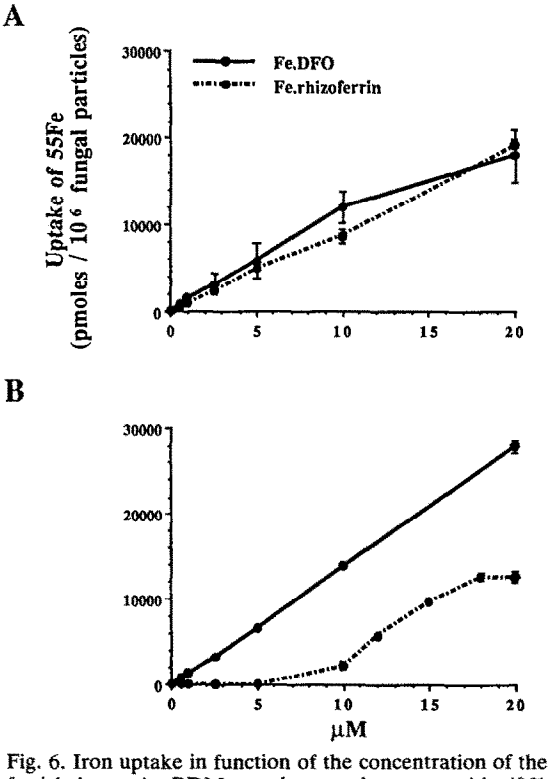

Fig. 6. Iron uptake in function of the concentration of the ferrichelators in BDM supplemented or not with 40% human serum. *R. microsporus* spores were incubated for 24 hr at 37° in BDM in the presence of different concentrations of ^{55}Fe.DFO or ^{55}Fe.rhizoferrin. BDM was supplemented (B) or not (A) with human serum. Mean results ± SEM of eight independent experiments are given.

DISCUSSION

Rhizopus spores accumulate iron from both ^{55}Fe.DFO and ^{55}Fe.rhizoferrin in a process almost immediate for Fe.rhizoferrin and completed in 3 hr for Fe.DFO (Fig. 1). The iron accumulation levels are comparable, although higher from Fe.rhizoferrin, the chelator synthesized by *Rhizopus* [8] than from the exogenous siderophore Fe.DFO. Metabolic inhibitors do not affect iron accumulation in spores, suggesting that this process is energy-independent and could correspond to the adsorption of the chelators on the spores. In spores initiating germination, the iron accumulation from both ferrichelators is increased, suggesting an additional process which is partially impaired by metabolic inhibitors.

The radioiron accumulation by spores from both ^{55}Fe.DFO and ^{55}Fe.rhizoferrin is partially saturable with the ferrichelator concentration but more metal is accumulated from Fe.rhizoferrin than from Fe.DFO (Fig. 2), suggesting the presence of more binding sites for Fe.rhizoferrin than for Fe.DFO, but a higher affinity for ferrioxamine than for rhizoferrin.

The accumulation by the spores of radiolabel from Fe.[^{14}C]DFO is proportional to its concentration and reaches much lower values than from ^{55}Fe. The different accumulation levels of the metal (^{55}Fe) and the ligand (^{14}C or ^{3}H) suggest that, upon binding of the complex to the spores, iron dissociates and becomes trapped, while the iron-free ligand is released back into the extracellular medium. The washout experiments confirm that the bulk of

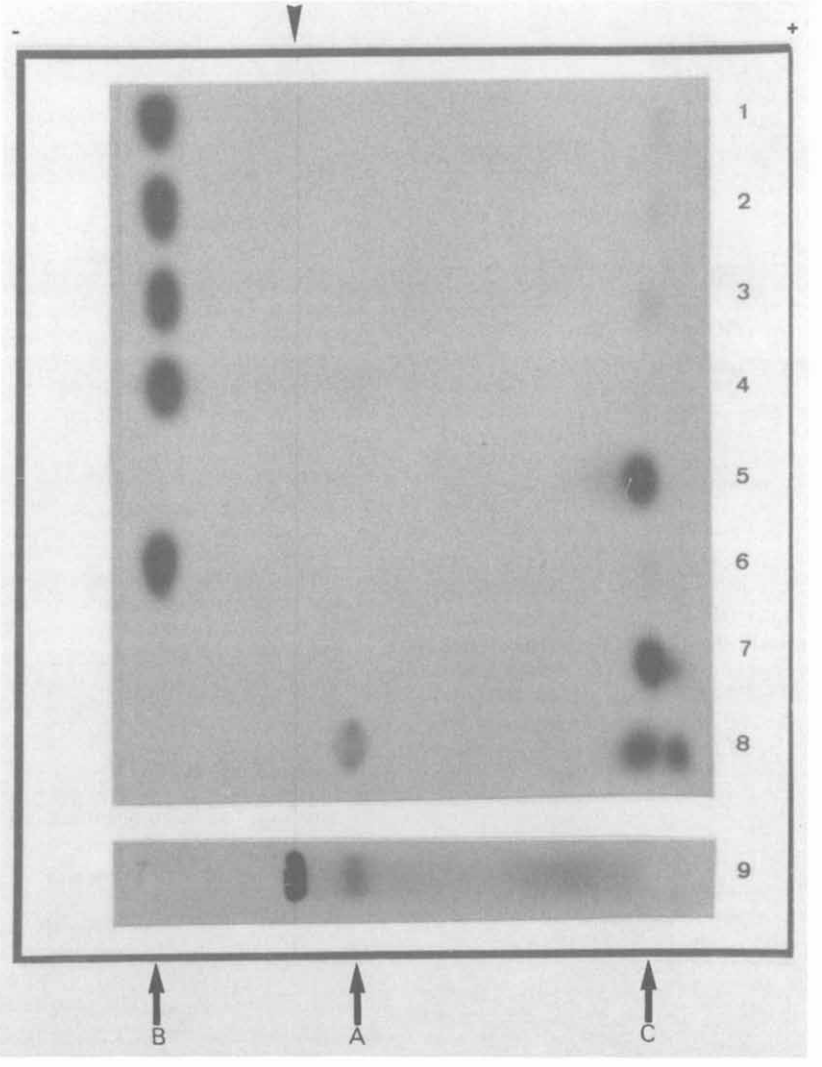

Fig. 7. Autoradiography of agarose gel. (Lane 1) ^{55}Fe.DFO (30 nmol); (Lane 2) ^{55}Fe.DFO + rhizoferrin (30 nmol); (Lane 3) ^{55}Fe.DFO + 18 nmol human apotransferrin; (Lane 4) ^{55}Fe.DFO + human serum corresponding to 13 nmol apotransferrin; (Lane 5) ^{55}Fe.rhizoferrin; (Lane 6) ^{55}Fe.rhizoferrin + DFO; (Lane 7) ^{55}Fe.rhizoferrin + human apotransferrin; (Lane 8) ^{55}Fe.rhizoferrin + human serum; (Lane 9) human transferrin.

radioiron accumulated by the spores remains associated with them upon different washings (Figs 4 and 5). The immediate release of 27% of the accumulated ^{55}Fe could correspond to the detachment of labeled material still associated with an exchangeable compartment. The release of 68% of the ^{3}H label from the spores during washout is in agreement with the concept that iron and the siderophore ligand dissociate. The observation that iron accumulation from Fe.DFO is unaffected by covalent binding of this complex to albumin, supports the hypothesis that Fe.DFO does not require to enter *Rhizopus* to deliver iron.

Competition experiments (Fig. 3) indicate that Fe.DFO and Fe.rhizoferrin almost completely inhibit the accumulation of radioiron from ^{55}Fe.DFO and from ^{55}Fe.rhizoferrin, respectively. We hypothesize that, at low concentrations, the Fe.rhizoferrin complex first saturates the binding sites; higher concentrations are required for competition with the accumulation of radioiron from ^{55}Fe.rhizoferrin. The iron accumulation from both ^{55}Fe.DFO and ^{55}Fe.rhizoferrin is only partially inhibited by Fe.rhizoferrin (69% inhibition) and Fe.DFO (51% inhibition), respectively, suggesting that spores display two distinct binding sites for Fe.DFO and Fe.rhizoferrin and that only one of them can be utilized by both chelators.

When incubated in conditions closer to human pathology, *Rhizopus* also accumulates iron from both ferrichelators (Fig. 6). Human serum has no significant effect on the radioiron uptake from ^{55}Fe.DFO. By contrast, it strongly affects the accumulation of iron from ^{55}Fe.rhizoferrin: at concentrations $\geq 5\,\mu$M, the iron accumulation is almost completely inhibited. Agarose gel elec-

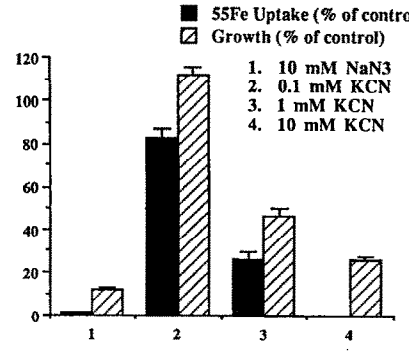

Fig. 8. Effect of metabolic inhibitors (NaN_3 and KCN) on iron uptake and growth of *R. microsporus*. Spores were incubated for 24 hr at 37° in BDM supplemented with 40% human serum, in the presence of 1 μM ^{55}Fe.DFO and metabolic inhibitors. Iron uptake from ^{55}Fe.DFO was measured and growth was evaluated by turbidimetry at 400 nm. Results were normalized: for iron uptake, as described in the legend of Fig. 3; growth in BDM with 40% serum and Fe.DFO was considered to be 100%. Mean results ± SEM of eight independent experiments are given.

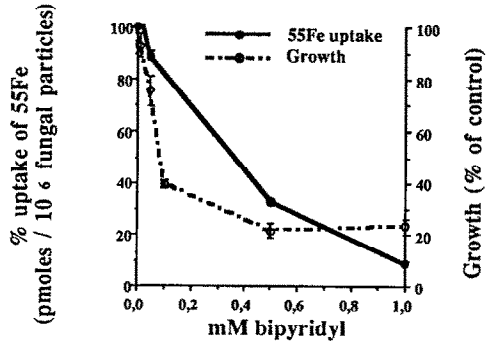

Fig. 9. Effects of bipyridyl on iron uptake from Fe.DFO and on growth of *R. microsporus*. Spores were incubated for 24 hr at 37° in BDM supplemented with 40% human serum, in the presence of 1 μM ^{55}Fe.DFO and different concentrations of bipyridyl. Iron uptake from ^{55}Fe.DFO was measured and growth was evaluated by turbidimetry at 400 nm. Results were normalized, as described for iron uptake in the legend of Fig. 3 and for growth in the legend of Fig. 6. Mean results ± SEM of eight independent experiments are given.

trophoresis (Fig. 7) indicates that apotransferrin chelates part of the iron initially complexed by rhizoferrin and that this transfer requires the presence of serum. Apotransferrin does not take up any iron from Fe.DFO, even in the presence of serum.

The nutritive medium (BDM) supplemented with 40% human serum contains 7.5 μM iron (6.68 μM provided by serum and 0.82 μM from BDM) and 12 μM transferrin (corresponding to a total iron binding capacity of 18.2 μM). Since iron is taken up from Fe.rhizoferrin by apotransferrin, iron uptake by *Rhizopus* should not occur at Fe.rhizoferrin concentration $\leq$ 18 μM. The experimental data (Fig. 6) indicate that iron uptake starts at concentrations $<$ 10 μM, what could be explained by a partial chelation of iron from Fe.rhizoferrin by human serum (Fig. 7).

Iron accumulation by *Rhizopus* is blocked both by the metabolic inhibitors NaN_3 or KCN (Fig. 8) and by the ferrous chelator bipyridyl (Fig. 9). In all cases, this decreased iron accumulation is accompanied by a decreased growth of *Rhizopus*. This indicates that the iron uptake by *Rhizopus* from Fe.DFO is an energy-dependent process which could involve a step of reduction. It also confirms that iron is required for fungal growth [4].

Several conclusions can be drawn from these observations. First, our results confirm the report by Thieken and Winkelmann [14] that *Rhizopus* takes up iron from its endogenous siderophore Fe.rhizoferrin in a receptor-mediated, energy-dependent process. Our experiments, carried out with different culture media and at a different pH (7.5 vs 6.0), are in agreement with these previous findings.

Second, *Rhizopus* also takes up iron from the exogenous siderophore Fe.DFO in a process that is largely similar. Iron uptake from Fe.DFO is energy-dependent and could require a reductive step. Competition experiments indicate, however, that this ferrichelator also interacts with binding sites different from those to which Fe.rhizoferrin attaches. The intact Fe.DFO complex does not enter the fungal cell but iron uptake is accompanied by the release of the DFO-ligand extract in the extracellular medium.

Third, when using *in vitro* conditions as close as possible to the clinical situation, we found that an important proportion of the iron bound to rhizoferrin is taken up by serum apotransferrin. The latter protein therefore makes iron unavailable to *Rhizopus*. By contrast, iron within Fe.DFO is not trapped by serum apotransferrin and it is easily utilized by *Rhizopus*.

Microorganisms have recently been classified according to their capacity to utilize or not iron from Fe.DFO [15]. However, the mechanism of iron transport from Fe.DFO is not uniform among the microbes which can utilize this iron source. The producer strain *Streptomyces pilosus* has been shown to take up the intact Fe.DFO complex [16]. In two yeasts, i.e. *Saccharomyces cerevisiae* and *Geotrichum candidum*, a similar uptake of the entire Fe.DFO complex coexists with a reductive pathway [17, 18]. In the present study, we demonstrate that iron accumulation from Fe.DFO by *Rhizopus* is not accompanied by the entry of the Fe.DFO complex. It seems that *Rhizopus* uses an iron reductive pathway with dissociation of iron from the ligand. The reduction of iron by a membrane reductase is a very common mechanism utilized by several microorganisms to acquire iron from different sources [19]. Further characterization of the reductase produced by *Rhizopus* was beyond the scope of the present study.

In a serum-containing medium, *Rhizopus* efficiently utilizes Fe.DFO, even at nanomolar concentration, as an iron source. Neither serum

transferrin, nor the endogenous siderophore, rhizoferrin, interfere with this process. These *in vitro* results help explain the mechanisms whereby the *in vivo* production of Fe.DFO during DFO-therapy can, in some patients, lead to iron accumulation by *Rhizopus*, to its growth stimulation, and ultimately to a siderophore-mediated infection [4].

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