

Ferric Iron Reduction by *Cryptococcus neoformans*

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The pathogenic yeast *Cryptococcus neoformans* must reduce Fe(III) to Fe(II) prior to uptake. We investigated mechanisms of reduction using the chromogenic ferrous chelator bathophenanthroline disulfonate. Iron-depleted cells reduced 57 nmol of Fe(III) per 10⁶ cells per h, while iron-replete cells reduced only 8 nmol of Fe(III). Exponential-phase cells reduced the most and stationary-phase cells reduced the least Fe(III), independent of iron status. Supernatants from iron-depleted cells reduced up to 2 nmol of Fe(III) per 10⁶ cells per h, while supernatants from iron-replete cells reduced 0.5 nmol of Fe(III), implying regulation of the secreted reductant(s). One such reductant is 3-hydroxyanthranilic acid (3HAA), which was found at concentrations up to 29 μM in iron-depleted cultures but <2 μM in cultures supplemented with iron. Moreover, when washed and resuspended in low iron medium, iron-depleted cells secreted 20.4 μM 3HAA, while iron-replete cells secreted only 4.5 μM 3HAA. Each mole of 3HAA reduced 3 mol of Fe(III), and increasing 3HAA concentrations correlated with increasing reducing activity of supernatants; however, 3HAA accounted for only half of the supernatant's reducing activity, indicating the presence of additional reductants. Finally, we found that melanized stationary-phase cells reduced 2 nmol of Fe(III) per 10⁶ cells per h—16 times the rate of nonmelanized cells—suggesting that this redox polymer participates in reduction of Fe(III).

Cryptococcus neoformans is a pathogenic fungus which causes brain infections in immunocompromised patients. There is often no possibility for reconstituting cellular immunity in such patients. However, animals utilize a variety of nonspecific defense mechanisms against microbial infections in addition to specific, memory-based cellular immunity. In particular, animals deny iron to parasitic microorganisms in a form of nutritional immunity (1, 26). This defense, too, is breached in immunocompromised patients who develop cryptococcosis. We study iron uptake in *C. neoformans* in the hope that manipulations of iron availability will lead to improved therapy or prophylaxis against fungal infections.

In order to obtain scarce iron from the host or from insoluble ferric oxides in the aerobic environment, many bacteria and filamentous fungi secrete chelators of Fe(III) (21). However, *C. neoformans* does not secrete such siderophores (14). Rather, this organism reduces ferric iron as an essential step prior to iron uptake (16, 24). Here we describe three distinct systems for extracellular ferric reduction in *C. neoformans*: a cell-bound ferric reductase; secreted reductants, including 3-hydroxyanthranilic acid (3HAA); and melanin.

MATERIALS AND METHODS

Yeast strains and culture conditions. We used strain B3501, MAT α , serotype D, originally from the culture collection at the National Institutes of Health (12). Cultures were stored on brain heart glucose agar slants (Scott Laboratories, Inc.) and liquid cultures were started in GYE (2% glucose–2% yeast extract broth; BBL, Becton Dickinson and Co.). Limited-iron medium (LIM) contained, per liter, 20 g of glucose, 5 g of asparagine, 400 mg of K₂HPO₄, 100 mg of MgSO₄ · 7H₂O, 50 mg of CaCl₂ · 2H₂O, 1 mg of thiamine, 57 μg of boric acid, 396 μg of CuSO₄ · 5H₂O, 72 μg of MnCl₂ · 4H₂O, 4.2 mg of ZnCl₂, and 37 μg of (NH₄)₆Mo₇O₂₄ · 4H₂O, buffered with 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)–NaOH to pH 6.0. Salts of polyvalent metals were dissolved in Chelex-100-treated water (Bio-Rad), and other components were purified over a Chelex-100 column; total residual iron in LIM was 200 nM as determined with ferrozine pills (Hach Chemical Co.). All components were filter sterilized. Glassware was soaked in Citranox acid detergent (Alconox Inc.) and rinsed with distilled

water prior to use with low-iron medium. For iron repletion, 15 μM Fe(III) hydroxy-EDTA [Fe(III)HEDTA; Dow Chemicals] was added. All liquid cultures were grown with agitation at 225 rpm at room temperature. Growth was monitored by turbidimetry at 700 nm. Culture supernatants were sterilized by filtration through a 0.2-μm-pore-size cellulose acetate membrane (Nalgene).

For the assay of ferric reductase, stationary-phase cultures in GYE were harvested, washed in LIM, inoculated into 100 ml of LIM [with or without Fe(III)HEDTA] at a density of 10⁶ cells/ml, and grown overnight. The cells were harvested, washed, resuspended in fresh LIM [with or without Fe(III)HEDTA] at a density of 10⁶ cells/ml, and agitated at room temperature.

For induction of melanization, B3501 was grown to stationary phase in LIM containing 2 g of glucose per liter and 10 μM Fe(III)HEDTA. The culture was divided into two cultures, and dopamine hydrochloride (1 mM, final concentration) was added to one. The cultures were incubated until their optical densities at 400 nm (OD₄₀₀) differed by at least 2 U (15). The cell densities of the two cultures were identical as determined by counting in a hemocytometer and by dilution and plating of cells (data not shown).

Assays for reduced iron. Fe(II) was quantitated in the presence of the chromogen bathophenanthroline disulfonate [BPDS; $\epsilon_{\text{Fe(II)BPDS}} = 22,140 \text{ M}^{-1} \text{ cm}^{-1}$ at 535 nm (3)]. The Fe(III) reducing activity of a culture was defined as follows. Total Fe(II) was measured by mixing 1 ml of culture (cells plus supernatant) with BPDS and Fe(III)HEDTA (final concentration, 1 mM each) to give a total volume of 2 ml. Supernatant Fe(II) was measured by mixing 1 ml of filter-sterilized supernatant with BPDS and Fe(III)HEDTA. Free Fe(II) was measured by mixing 1 ml of sterile supernatant with BPDS and Chelex-treated water. All samples were incubated for 1 h in the dark, and cells were centrifuged from the light path prior to reading of the absorbance. Cell-bound reductase activity was defined as the difference between total reduced iron and supernatant reduced iron, and secreted reductant was defined as the difference between supernatant reduced iron and free Fe(II). Results were normalized to cell density: 2×10^7 cells/ml gives an OD₇₀₀ of 1.0 (based on CFU) (data not shown). To measure in vitro reduction of Fe(III) by 3HAA, Fe(III)HEDTA (100 μM), 3HAA (0 to 25 μM), and BPDS (500 μM) were incubated at room temperature in the dark in 2 ml of 100 mM MES–NaOH, pH 6.0. Absorbance at 535 nm was monitored.

The effect of melanin upon reduction of Fe(III) was studied in pairs of melanized and nonmelanized cultures generated as described above. The cultures were washed twice in LIM to remove dopamine, resuspended in fresh LIM, and assayed for ferric reduction as above. Since melanin contributes to the OD₇₀₀ of the melanized cells, the data presented were normalized to the OD₇₀₀ of the nonmelanized culture.

Identification and assay of 3HAA. Anthranilic acid and 3HAA were assayed in sterile cultural supernatant preserved with 1 mM ascorbate and ultrafiltered (molecular weight cutoff, 5,000). Samples (10 μl) were subjected to C₁₈ reversed-phase high-pressure liquid chromatography (HPLC) using a 5-μm Spherisorb ODS2 4.6– by 250-mm column (Phase Separations, Ltd.). The solvent was 0.02 M sodium citrate, pH 5.3, containing 2 mM ZnSO₄ (25) and 4% 2-propanol, pumped at 1.1 ml/min. An SPF-500C spectrofluorometer (Spectronics Instruments, Inc.) was used for detection, with excitation at 315 nm and emission at 418

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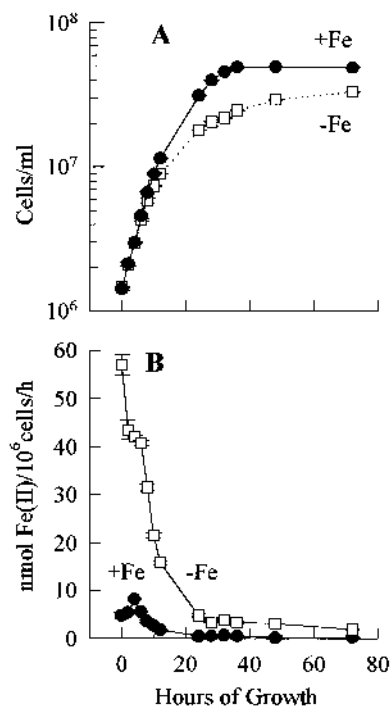


FIG. 1. Growth (A) and ferric reductase activity (B) of *C. neoformans* wild-type cells in LIM (-Fe) and in LIM plus 15 μ M Fe(III)HEDTA (+Fe). Each point is the mean \pm standard deviation from three samples.

nm. The fluorescence spectra of putative 3HAA and authentic 3HAA were compared in 10-mm quartz cuvettes by using 40 μ l of cultural supernatant mixed with 2.96 ml of chromatographic solvent (putative 3HAA) and 40 μ l of 25 μ M authentic 3HAA in LIM mixed with 2.96 ml of chromatographic solvent (authentic 3HAA).

The effect of exogenous iron on secretion of 3HAA was studied in two ways: first, by assay for 3HAA in supernatants of cultures grown with or without 15 μ M Fe(III)HEDTA; second, by transfer of iron-depleted or iron-replete cells to LIM and assay of supernatants after overnight incubation. In the latter experiment, cells were grown to stationary phase in LIM supplemented with 0, 15, or 40 μ M Fe(III)HEDTA. Portions (5 ml) of cells were washed twice in 20 ml of LIM, resuspended in 10 ml of LIM, and incubated with agitation. 3HAA was assayed in the resulting supernatants.

In order to generate supernatants with a range of reductant concentrations, B3501 was grown to stationary phase in GYE, harvested, washed, and resuspended at various densities in LIM. Supernatants were harvested from exponential- and stationary-phase cultures and assayed for iron reduction as described above. Aliquots were preserved with 1 mM ascorbate and frozen at -80°C prior to analysis for 3HAA by HPLC. Linear regression analysis was performed by the least-squares method (Sigmaplot; Jandel Scientific).

RESULTS

Cryptococcal cells must reduce extracellular Fe(III) to Fe(II) prior to uptake, as shown by the observation that addition of the chromogenic Fe(II) chelator BPDS to a culture colors the supernatant red and inhibits growth (16). In the following experiments, we assume that the rate of formation of the Fe(II)-BPDS complex [or Fe(II) produced] is equivalent to the rate of Fe(III) reduction.

For comparative purposes, we studied Fe(III) reduction in cultures that were iron depleted by growth in LIM and in cultures that were iron replete by growth in LIM supplemented with 15 μ M Fe(III)HEDTA. Under these conditions, the doubling time was ~ 3.75 h in exponential phase for both iron-depleted and iron-replete cultures, although the iron-supplemented culture grew to higher density (Fig. 1A).

Reduction of Fe(III) by cells. We determined the reducing activity of cryptococcal cells by measuring the activity of a

culture and subtracting the activity of its cell-free supernatant. Wild-type cells reduced Fe(III) at a maximal rate of 57 ± 2.24 nmol of Fe(II) produced per 10^6 cells per h when depleted of iron and at 8.24 ± 0.058 nmol per 10^6 cells per h when replete in iron (Fig. 1B). The activity was maximal in the exponential phase and minimal in stationary phase in both iron-depleted and iron-replete cultures. These features define a cell-associated ferric reductase, regulated by availability of iron and growth phase.

Reduction of Fe(III) by cultural supernatants. In addition to reduction of Fe(III) directly, cells reduced Fe(III) indirectly in the presence of air by secretion of reductants. We measured the Fe(II) produced by cell-free supernatants and subtracted the contributions of free Fe(II). Rates were normalized to cell density prior to filtration. Supernatants from iron-depleted cultures generated up to 2.236 ± 0.023 nmol of Fe(II) per 10^6 cells per h, whereas supernatants from iron-replete cultures generated up to 0.523 ± 0.00473 nmol of Fe(II) per 10^6 cells per h (Fig. 2). The reducing activities peaked in early stationary phase, although this was partially due to the fact that the cells were washed free of accumulated supernatant when the cultures were started. The supernatants from both iron-depleted and iron-replete cultures had less than 1/10 of the activity of the corresponding cell-associated activities.

A candidate for the identity of the secreted reductant is 3HAA, which is secreted by *Saccharomyces cerevisiae* (19). We examined supernatants of iron-starved cultures of strain B3501 for the presence of 3HAA by fluorescence spectroscopy. The supernatants fluoresced at maximum excitation and emission wavelengths identical to those of authentic 3HAA (315 and 418 nm, respectively [Fig. 3]). Since anthranilic acid and 5-hydroxyanthranilic acid also fluoresce with peak excitations at 315 and 330 nm and peak emissions at 418 and 440 nm, respectively, we performed HPLC separations, using either C₈ reversed-phase (8) or C₁₈ reversed-phase adsorbants with fluorescence detection. A single fluorescent component cochromatographed with authentic 3HAA in the C₁₈ system (Fig. 4). No anthranilic acid or 5-hydroxyanthranilic acid was detected (lower limit of detection: 2 μ M [data not shown]). Putative 3HAA also cochromatographed with authentic 3HAA in the C₈ reversed-phase system (data not shown), but this system was less reliable in daily use. Because no interfering compounds were seen under these conditions, spectrofluorometric assays for 3HAA were subsequently performed without separations.

To measure the reducing power of 3HAA, we mixed various

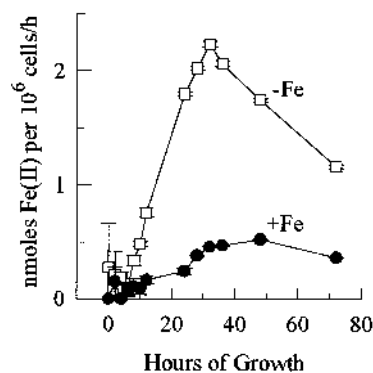


FIG. 2. Iron reduction by supernatants of wild-type cells grown in LIM (-Fe) and in LIM plus 15 μ M Fe(III)HEDTA (+Fe) from the cultures in Fig. 1. Each point is the mean \pm standard deviation from three samples.

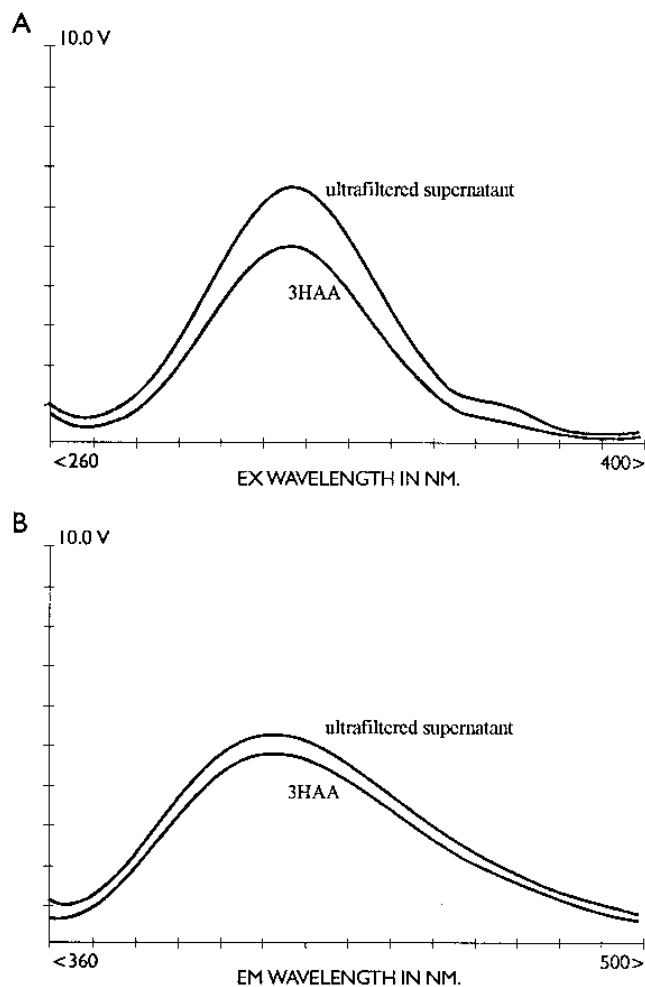


FIG. 3. Excitation (EX) and emission (EM) spectra of authentic 3HAA and putative, secreted 3HAA in 0.02 M sodium citrate (pH 5.3)–2 mM ZnSO₄–4% 2-propanol.

concentrations of 3HAA with Fe(III)HEDTA and BPDS and monitored the absorbance of the Fe(II)-BPDS complex over time (Fig. 5A). The reduction of Fe(III) was half-complete in 3 h. When the maximal concentrations of Fe(II) were replotted against the initial concentrations of 3HAA, each mole of 3HAA was seen to reduce 3 mol of Fe(III) (Fig. 5B).

We next examined regulation of 3HAA secretion by iron. Since Fe(III) is an oxidizer of 3HAA, it was not surprising that the concentrations of 3HAA in cultures supplemented with Fe(III)HEDTA were always less than 2 μ M (lower limit for detection, 0.4 μ M), while cultures not supplemented contained up to 29 μ M 3HAA. In order to prevent oxidation of 3HAA by free Fe, we washed and resuspended iron-depleted and iron-replete cells in LIM. After overnight incubation, cells originally grown in 0, 15, or 40 μ M Fe(III)HEDTA had secreted 20.4 ± 9.0 , 7.1 ± 1.0 , and 4.5 ± 1.7 μ M 3HAA, respectively. Since free iron was not detectable in the supernatants (<0.5 μ M), we conclude that secretion of 3HAA was regulated by the iron status of the cells.

In order to determine whether 3HAA could account for the reducing power of the supernatants, we obtained cultural supernatants with a variety of 3HAA concentrations and ferric reduction activities by growing cells in LIM without Fe(III)

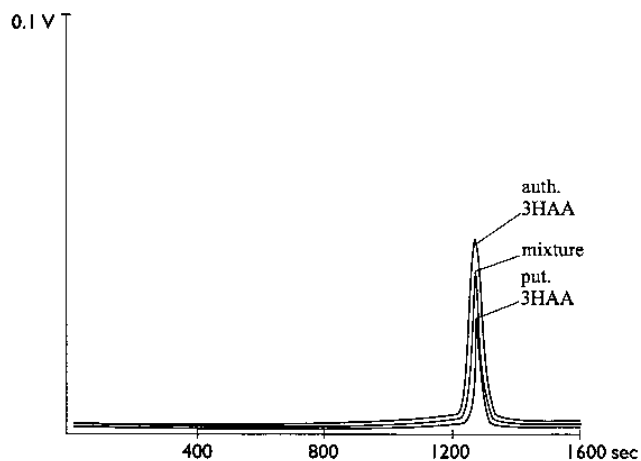


FIG. 4. HPLC separation of secreted 3HAA with fluorescence detection (excitation at 315 nm; detection at 418 nm). The chromatography solvent was 0.02 M sodium citrate (pH 5.3), 2 mM ZnSO₄, and 4% 2-propanol. Cultural supernatant contained 12.5 μ M putative (put.) 3HAA; the solution of authentic (auth.) 3HAA was 25 μ M. The mixture is equimolar in putative and authentic 3HAA.

HEDTA for various periods of time. 3HAA concentrations ranged from 0.4 to 29 μ M, depending on phase of growth and degree of iron depletion. The concentrations of 3HAA increased as the reducing activities of the supernatants increased but could account for only 50% of the Fe(III) reduced (Fig. 6). Below the level of about 20 μ M Fe(III) reduced per h, 3HAA

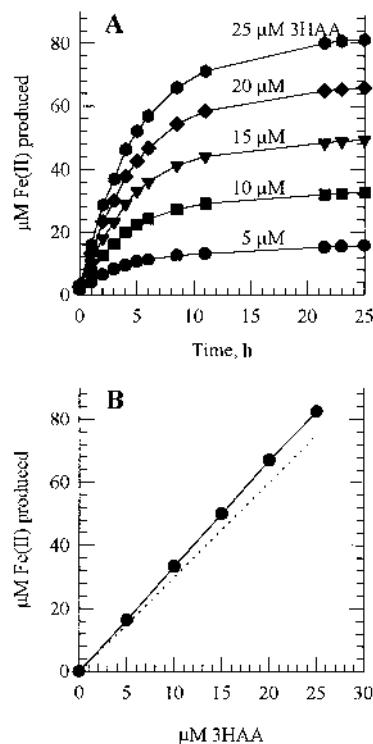


FIG. 5. Reduction of Fe(III) by 3HAA in vitro in 100 mM MES-NaOH, pH 6.0. (A) Time course of reduction of Fe(III) by various concentrations of 3HAA. (B) Plot of maximal Fe(III) reduction as a function of 3HAA concentration. The dotted line represents theoretical reduction of 3 mol of Fe(III) per mol of 3HAA.

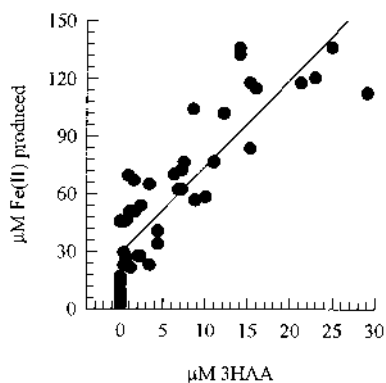


FIG. 6. Correlation of Fe(III) reduction activity and 3HAA concentration in supernatants of cultures grown in LIM without Fe(III)HEDTA. Solid line, linear regression analysis of all datum points.

was not detected. This level of activity may be due to an additional unidentified reductant or to loss of 3HAA by autooxidation.

Reduction of Fe(III) by melanin. *C. neoformans* synthesizes external melanin when grown in the presence of substrate catechols. Because pure suspensions of melanin reduce Fe(III) to Fe(II) in vitro (9, 11, 22), we tested this reaction in vivo by incubating melanized and nonmelanized cells with Fe(III)HEDTA and BPDS. Whereas the nonmelanized culture produced Fe(II) at a rate of 0.128 ± 0.0123 nmol per 10^6 cells per h, the melanized culture produced Fe(II) at a rate of 2.10 ± 0.0216 nmol per 10^6 cells per h, a 16-fold difference.

DISCUSSION

Although *C. neoformans* is a basidiomycete and therefore not closely related to the ascomycete *S. cerevisiae*, mechanisms of iron acquisition are similar in the two fungi. First, neither species secretes siderophores, although each may contain a receptor for exogenous ferric deferoxamine (14, 17). Second, iron uptake in both is subject to inhibition by an extracellular ferrous iron chelator and to stimulation by exogenous reductants (16, 18, 24), indicating that reduction of ferric iron normally precedes uptake.

The first mechanism of iron reduction in both organisms is by a plasma membrane ferric reductase. The ferric reductases of *S. cerevisiae* have been subjected to intense physiologic and genetic study (4, 5, 10), and we believe that we have detected a similar ferric reductase in *C. neoformans*. Both organisms exhibit similar maximal ferric reductase activities when depleted of iron—57 and 20 nmol of Fe(III) per 10^6 cells per h by *C. neoformans* and *S. cerevisiae*, respectively (4). Expression is downregulated by 15 to 20 μ M iron in both organisms. Finally, the ferric reductases are downregulated in the stationary phase of growth.

The second mechanism for iron reduction involves secretion of a reductant. Here we have presented evidence that 3HAA is a secreted ferric reductant in *C. neoformans*. It has been reported that *S. cerevisiae* secretes 3HAA and that the secretion is increased in iron-replete cells (19). In *C. neoformans*, we detected up to 29 μ M 3HAA in cultures growing without iron but <2 μ M in cultures grown with iron. Moreover, iron-depleted cells secreted fivefold more 3HAA than iron-replete cells when incubated overnight in LIM. Thus, 3HAA secretion appears to be regulated by iron in *C. neoformans*, a result which appears to contradict that reported for *S. cerevisiae*. Although the organic product of the reaction between 3HAA

and Fe(III) has not been identified, the stoichiometry of the reaction—3 mol of Fe(III) reduced per mol of 3HAA oxidized—suggests oxidative dimerization of 3HAA (2, 7). Because the levels of 3HAA detected were sufficient to account for only half of the ferric reducing activity of the supernatant, we conclude that additional secreted reductants remain to be identified.

In confirmation of earlier results (14), we detected α -ketoglutarate in concentrations between 10 and 1400 μ M in cultural supernatants, using an organic acid column in HPLC. However, we have not been able to find a role for α -ketoglutarate in iron uptake. Its secretion is not regulated by exogenous iron, and although α -keto acids reduce Fe(III) by oxidative decarboxylation in the presence of strong acid (20), the rate of the reaction of α -ketoglutarate under physiologic conditions is not sufficient to account for the activity of the secreted ferric reductant (data not shown). In *Proteus* bacteria, certain α -keto acids (but not α -ketoglutarate) function as siderophores (6), but *C. neoformans* does not produce functional siderophores of any type (14).

Melanin formed by *C. neoformans* may also reduce iron. Consistent with observations of Fe(III) reduction by melanin in vitro (11, 22), our experiments provide evidence that the reaction occurs in vivo. The reaction seemingly depends upon the presence of soluble Fe(III) and the occurrence of a proportion of melanin residues in the reduced (hydroquinonic) state. While synthetic 3,4-dihydroxy-DL-phenylalanine melanin has been reported to exist predominantly in the oxidized form (11), we have suggested that living organisms actively reduce their melanin (13). In the experiments described in this paper, the formation of Fe(II) and oxidized melanin was thermodynamically favored by the presence of BPDS, an avid and specific chelator of Fe(II). However, the reaction might also be driven by physiologic removal of Fe(II) into the yeast cell.

In the presence of excess Fe(II) and HEDTA [which chelates both Fe(II) and Fe(III) without preventing redox reactions] the equilibrium between melanin and iron favors Fe(II) oxidation and melanin reduction (13). This appears true even in the presence of a 10-fold ratio of Fe(III) to Fe(II). Thus, the position of the equilibrium between reduced iron and reduced melanin appears to be a function of the prevailing chemical and physiologic conditions.

In contrast to the case in saprophytic *S. cerevisiae*, the process of iron assimilation in pathogenic *C. neoformans* goes beyond simple nutrition and participates in pathogenesis. Thus, environmental iron regulates production of the capsular polysaccharide, an established virulence factor (23), while reduction of extracellular iron appears to mediate control of the oxidation state of melanin (13), a redox buffer also important in virulence (15). Finally, we note that exported reductants related to iron assimilation are potentially deployed to intercept and neutralize incoming oxidants secreted by immunologic cells.

In summary, *C. neoformans* has at least three mechanisms for the reduction of ferric iron. Compared quantitatively, the cellular reductase has the greatest capacity to reduce iron (57 nmol/ 10^6 cells/h), followed by reductants (2.24 nmol/ 10^6 cells/h) and then melanin (2.10 nmol/ 10^6 cells/h). However, the three systems peak at different phases of growth: the first during early exponential phase, the second during late exponential phase, and the third in late stationary phase. Of these three systems, only melanin has thus far been demonstrated in *C. neoformans* cells infecting its host. These seemingly redundant systems may allow regulation of iron reduction in response to many different environmental conditions.

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