Redox Buffering by Melanin and Fe(II) in Cryptococcus neoformans

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Melanin is a fungal extracellular redox buffer which, in principle, can neutralize antimicrobial oxidants generated by immunologic effector cells, but its source of reducing equivalents is not known. We wondered whether Fe(II) generated by the external ferric reductase of fungi might have the physiologic function of reducing fungal melanin and thereby promoting pathogenesis. We observed that exposure of a melanin film electrode to reductants decreased the open-circuit potential (OCP) and reduced the area of a cyclic voltammetric reduction wave whereas exposure to oxidants produced the opposite effects. Exposure to 10, 100, 1,000 or 10,000 μM Fe(II) decreased the OCP of melanin by 0.015, 0.038, 0.100, and 0.120 V, respectively, relative to a silver-silver chloride standard, and decreased the area of the cyclic voltammetric reduction wave by 27, 35, 50, and 83%, respectively. Moreover, exposure to Fe(II) increased the buffering capacity by 44%, while exposure to millimolar dithionite did not increase the buffering capacity. The ratio of the amount of bound iron to the amount of the incremental increase in the following oxidation wave was approximately 1.0, suggesting that bound iron participates in buffering. Light absorption by melanin suspensions was decreased 14% by treatment with Fe(II), consistent with reduction of melanin. Light absorption by suspensions of melanized Cryptococcus neoformans was decreased 1.3% by treatment with Fe(II) (P < 0.05). Cultures of C. neoformans generated between 2 and 160 μM Fe(II) in culture supernatant, depending upon the strain and the conditions (the higher values were achieved by a constitutive ferric reductase mutant in high concentrations of Fe(III)). We infer that Fe(II) can reduce melanin under physiologic conditions; moreover, it binds to melanin and cooperatively increases redox buffering. The data support a model for physiologic redox cycling of fungal melanin, whereby electrons exported by the yeast to form extracellular Fe(II) maintain the reducing capacity of the extracellular redox buffer.

Leukocytes attack pathogens with a flux of secreted strong oxidants (1). Thus, any extracellular microbial product which neutralizes oxidants is likely to protect the pathogen and promote invasive disease. Melanin, an extracellular redox buffer composed of polymerized catechols (5, 14, 15, 21), is a virulence factor for certain pathogenic fungi (3, 11). Its ability to neutralize strong oxidants is supported by several types of evidence: mutants of Cryptococcus neoformans selected for sensitivity to oxidants exhibit albinism (8); nonmelanized wild-type cells or mutants selected for albinism exhibit sensitivity to oxidants (9, 18); and melanin neutralizes oxidants and protects the melanized fungi Cryptococcus neoformans, Wangiella dermatididis, and Alternaria alternata from killing by hypochlorite and by per-manganate (8, 9). Thus, melanin may function as chemical armor in invasive disease. We reasoned that maintenance of the reducing capacity of extracellular melanin may be an important aggressive function in these pathogens. In studies of iron assimilation, we have found that C. neoformans (10, 16), like Saccharomyces cerevisiae (12), reduces extracellular Fe(III) directly at its cell membrane at a rate seemingly greater than needed for nutrition. C. neoformans also secretes the reductants 3-hydroxyanthranilic acid (3HAA) and α-ketoglutarate (AKG) (16). In the present work, we tested whether Fe(II), 3HAA, or AKG is able to reduce melanin and whether C. neoformans is able to generate concentrations of Fe(II) sufficient to drive the reduction of melanin.

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the solubility of 3HAA. The melanin film was washed with deaerated distilled water and reimmersed in buffer, and the new OCP was determined. The film was then subjected to a second voltammetric cycle. All operations were performed under argon, and all solutions were deaerated. During conditioning, the solutions were stirred by bubbling argon; electrochemical measurements were made in unstimred solutions. After each experiment, the melanin film was scoured off the carbon electrode with alumina paste and water. Voltammetric waves were integrated with a planimeter; since the potential is linear with respect to time, the wave area could be used to cage the faradaic charge and chemical mole equivalents. We made the assumption that electrode charging currents would cancel algebraically when the wave sizes were compared.

Measurements of physiologic Fe(II) concentrations. The wild-type strain of C. neoformans used was B3501, originally from the National Institutes of Health culture collection. Strain 620, a UV-induced mutant with a constitutionally de-repressed plasma membrane ferric reductase, and strain 643, a mutant which lacks the ferric reductase, will be fully described in a future report. Cultures were grown to stationary phase in glucose (2%)-yeast extract (2%), and 0.3 ml was inoculated into shake flasks containing 145 ml of pH 4.0 low-iron medium containing (per liter) asparagine, 5 g; succinic acid, 2 g; K₂HPO₄, 0.4 g; CaCl₂ · 2H₂O, 50 mg; thiamine, 0.4 mg; and 10 ml (added after autoclaving) of a trace element solution containing (per liter) CuSO₄, 0.5 mg; ZnSO₄ · 7H₂O, 2.0 mg; MnSO₄, 3.5 mg; sodium molybdate, 150 mg; and boric acid, 5.7 mg. After overnight incubation at room temperature, the resulting exponential-phase culture was centrifugally washed once with the same medium, resuspended at an optical density of 0.05 at 700 nm in 100 ml of the same medium supplemented with various concentrations of Fe(III)hydroxyethylenediamine triacetate [Fe(II-1HEDTA), and agitated at room temperature for 140 h. At intervals, the optical densities of 4-ml samples were measured at 700 nm to indicate growth, the samples were centrifuged, and the supernatants were filtered through 0.22-μm-pore-size membrane filters. A 1-ml sample of culture supernatant was mixed with 0.5 ml of distilled water and 0.5 ml of 4 mM bathophenanthroline disulfonate (BPD8) in reduced light, and the absorbance at 535 nm was immediately determined.

Optical measurements. Suspensions of autoxidative melanin were prepared by bubbling sterile air through a sterile solution of 1 mM dopamine hydrochloride (adjusted to pH 8.0 with sodium hydroxide) at room temperature for 1 week, filtering the resulting precipitate, air drying it, and hand grinding it in a mortar. The resulting powder (particle size, up to 50 μm) was suspended in phosphate buffer at an optical density of 0.4 at 400 nm, using matched 12-mm round cuvettes in a Coleman Jr. II spectrophotometer (Bacharach, Inc.). Anaerobic stirring was performed by bubbling argon.

Melanized C. neoformans was prepared by inoculating a stationary-phase culture as above into 145 ml of a medium containing (per liter) asparagine, 1 g; MgSO₄ · 7H₂O, 0.5 g; KH₂PO₄, 3 g; thiamine, 1 mg; dopamine hydrochloride, 190 mg; and 1 ml of Fries trace elements solution containing (per liter) boric acid, 114 mg; CuSO₄ · 5 H₂O, 792 mg; MnCl₂ · 4 H₂O, 144 mg; ammonium molybdate, 74 mg; FeCl₃ · 6 H₂O, 1.96 g; ZnCl₂, 4.4 mg; NaCl, 200 g; and CaCl₂ · 2H₂O, 200 g. The culture was agitated at room temperature for 7 days, and the resulting melanized yeast cells were washed twice and resuspended in phosphate buffer so that the optical density at 400 nm (with reference to sterile buffer) was approximately 0.4. When iron was to be added, the cells were suspended in 70 mM Tris-HCl (pH 7.2) instead of phosphate buffer. The aerosols generated by bubbling argon were trapped in an auto claveable cotton fiber in a laminar-flow cabinet.

RESULTS

Generation of melanin film electrode. We have confirmed that electroactive melanin can be incrementally deposited onto a glassy carbon electrode by cyclic voltammetric oxidation of the melanin precursor, 5,6-dihydroxyindole, as described previously (6). The scan obtained during generation of the melanin film shows incremental growth of the redox waves (Fig. 1).

Amount of interference by residual iron. Before studying the interaction of iron with melanin, it was necessary to examine the effect of free iron in the system. The Fe(II)-Fe(III) couple in Tris buffer gave a reduction wave at −0.30 V and an oxidation wave at +0.11 V, using the unmodified glassy carbon electrode (not shown). The entire oxidation wave and a shoulder of the reduction wave fell within the potential window of the melanin signals; thus, free iron had the potential to interfere with any study of the interaction of iron and melanin. For this reason, we performed electrochemical measurements before or after 5-min exposures of melanin to reagents. Following such “conditioning,” the melanin film electrode was washed with deaerated water and reimmersed in buffer. The presence of contaminating residual iron in the latter solution was assessed by scanning with an unmodified glassy carbon electrode. The concentration found was approximately 17 μM, enough to give an oxidation wave which was about 2% as large as that of a typical melanin film. We decided that this residual contamination with iron did not interfere with our study.

Effects of reagents on the oxidation-reduction state of melanin. The OCP of water-washed, freshly deposited melanin at room temperature was between 0.00 and +0.03 V. Conditioning with the reductant, 1 mM sodium dithionite, resulted in a new OCP of −0.14 V. The area of the resulting reduction wave was only 5% of that of the untreated film control (Fig. 2A). This finding and the negative OCP indicate that the melanin film had been chemically reduced by the dithionite. The 50% increased area of the following oxidation wave (compared to that of the untreated control) supports this conclusion by indicating bulk reduction of the melanin film by dithionite, but it also indicates that the reactions occurring during scanning are not complete. Conditioning with an oxidant (1 mM Fremy’s salt, potassium nitrosodisulfonate) resulted in a new OCP of +0.08 V. The area of the resulting reduction wave was 30% increased, and that of the following oxidation wave was 10% decreased, compared to the control scans indicating that the melanin film was oxidized (Fig. 2B). When 1 mM Fe(II)(NH₄)₂(SO₄)ₓ[Fe(II)] was tested, the new OCP was −0.07 to −0.10 V. The area of the reduction wave was 50% decreased and that of the following oxidation wave was 150% increased compared with control values (Fig. 2C), indicating that the melanin film had been reduced by the Fe(II), and the color of the film changed from olive green to blue. The apparent increase in electrochemical buffering was of great interest. We compared...
the Fe(II)-related augmentation of the following oxidation wave over that of the untreated control to the total iron extractable from the melanin film following conditioning in 1 mM Fe(II) and 3 mM Na3HEDTA, voltammetric scanning, and washing successively with deaerated water and deaerated buffer. Iron was extracted by scraping the melanin film from the glassy carbon electrode into a solution of Ferrozine (Hach Chemical Co.), which was used as the iron assay reagent. The ratio of extracted iron to faradaic increment was 0.77 ± 0.44 (mean and standard deviation of six determinations), close to 1.0, suggesting a stoichiometric relation between the bound iron and the electrochemical augmentation. Thus, 1 mM Fe(II) reduces melanin, binds to it, and appears to increase the redox buffering by the system.

We extended the measurement into more physiologic concentrations of Fe(II). At 100 μM, Fe(II) decreased the OCP of the melanin film electrode by −0.038 V (P < 0.01) and decreased the area of the first reduction wave by 0.11 nmol (P < 0.05), or 35%. At 10 μM, Fe(II) decreased the OCP of melanin by −0.015 V (P < 0.01) and decreased the area of the first reduction wave by 0.050 nmol (P < 0.01), or 27%.

**Study of Fe(II)-melanin cooperativity.** The electrochemical activity of Fe(II) was compared to that of dithionite in greater detail, using 10 mM concentrations and extending the scanning so that second reduction waves could be observed. Following conditioning with 10 mM dithionite, the first reduction wave of melanin was almost abolished (Fig. 3B); the remainder of the scan showed residual effects of the prolonged reduction by dithionite (discussed above), namely, an oxidation wave larger than that of the control and a second following reduction wave smaller than that of the control. Thus, the melanin was strongly reduced and the cyclic scanning did not fully reverse the effect of the dithionite conditioning. A similar experiment was performed with Fe(II) as the reducing agent (Fig. 3A); a reduction was observed. Dithionite is a stronger reducing agent than Fe(II); this is reflected in the smaller reduction wave and lower OCP (−0.20 versus −0.12 V in the experiment shown) following dithionite than following Fe(II) treatment, indicating that the melanin film was reduced to a greater degree by dithionite. Interestingly, conditioning with Fe(II) increased both the following oxidation wave and the second following reduction wave over control values, while conditioning with dithionite increased only the following oxidation wave, the second following reduction wave having been decreased (summarized in Table 1). The augmentation by Fe(II) of both the following oxidation wave and the second following reduction wave over control values, while conditioning with dithionite increased only the following oxidation wave, the second following reduction wave having been decreased (summarized in Table 1). The augmentation by Fe(II) of both the following oxidation wave and the second following reduction wave cannot be explained as the result of a simple change in oxidation state but must indicate an increase in electrochemical buffering caused by binding of iron. Moreover, both the following oxidation wave and the second following reduction wave were larger after Fe(II) conditioning than after dithionite conditioning (Table 1). If this result were due simply to a more complete reduction, the oxidation wave (at least) should be larger after dithionite conditioning, since dithionite is the more powerful reductant. Clearly, exposure to Fe(II) has increased the amount of buffering by the melanin film. The electrochemical data in Table 1 are listed as chemical amounts, to facilitate comparison to the amounts of iron extractable from melanin films following each experiment. The amount of iron found after a 10 mM treatment, at 1.08 nmol, was more than the difference between integrated waves and therefore more than the amount predicted from the electrochemical result; at 10 mM, much of the Fe(II) appears to bind in a manner that is not electroactive. Thus, we observed binding of a stoichiometric quantity of Fe(II) only when 1 mM Fe(II)HEDTA was used for conditioning.
Effect of contaminating Fe(III). We thought it likely that Fe(II) is in equilibrium with Fe(III) in the extracellular space in vivo. Accordingly, we studied the electrochemical effects of conditioning with Fe(III) (chelated with HEDTA at treble the concentration of iron) and with various ratios of Fe(II) to Fe(III) (at HEDTA concentrations treble the sum of those of the iron species) in Tris. Fe(III) is a known oxidizing agent for melanin, and this was reflected by positive changes in the OCP and in the reduction wave area when melanin was conditioned with Fe(III) (Table 2). When Fe(II) and Fe(III) were both present, Fe(III) interfered only slightly with reduction and binding by Fe(II); even at a 1:10 ratio of Fe(II) to Fe(III), a net reduction occurred. Under the conditions used, then, Fe(III) is a relatively weak oxidizing agent. Letting “mel” symbolize a single aromatic catechol residue of melanin, at pH 7.2 and in the absence of oxygen, the equilibrium of the reaction $2\text{Fe(II)} + 2H^+ + \text{melanin(ox)} \rightleftharpoons 2\text{Fe(III)} + \text{melanin(red)}$ appears to be toward the right. Moreover, Fe(III) binds poorly to melanin compared with Fe(II) (Table 2). Thus, a 10-fold excess of Fe(III) did not prevent the reduction of melanin by Fe(II).

**Optical monitoring of the oxidation-reduction state of melanin.** We could further test the inference that melanin is reduced by Fe(II), since oxidants have been reported to darken melanin while reductants lighten it (14, 19). We confirmed this phenomenon for melanin in aqueous suspension and found that 10 mM Fe(II) decreased light absorption by aqueous melanin suspensions by 14% (Fig. 4); this effect is consistent with reduction of extracellular melanin by Fe(II). Under light microscopy, the melanin particles did not appear to have been aggregated by Fe(II). We next examined the effects of dithio-

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**TABLE 1. Comparison of conditioned melanin films to control films**

<table>
<thead>
<tr>
<th>Voltamgram segment</th>
<th>Reduction wave area (nmol) after treatment with:</th>
<th>10 mM dithionite</th>
<th>10 mM Fe(II)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 mM dithionite</td>
<td>Control</td>
<td>Expt – control</td>
</tr>
<tr>
<td>First reduction wave</td>
<td>0.026</td>
<td>0.585</td>
<td>−0.559</td>
</tr>
<tr>
<td>Oxidation wave</td>
<td>1.283</td>
<td>1.007</td>
<td>+0.276</td>
</tr>
<tr>
<td>Second reduction wave</td>
<td>0.820</td>
<td>1.040</td>
<td>−0.220</td>
</tr>
<tr>
<td>Change in OCP following conditioning</td>
<td>−0.22 V</td>
<td></td>
<td>−0.13 V</td>
</tr>
<tr>
<td>No. of trials</td>
<td>11</td>
<td></td>
<td>9</td>
</tr>
</tbody>
</table>

*The size differences between waves for iron-conditioned and DT-conditioned films are +0.228 nmol for the oxidation wave and +0.447 nmol for the second reduction wave.*
TABLE 2. Effect of the Fe(II)/Fe(III) ratio upon the oxidation-reduction state and electrochemical capacity of the melanin film

<table>
<thead>
<tr>
<th>Conditioning solutiona</th>
<th>Change in OCP (V)</th>
<th>Change in reduction wave area (nmol)b</th>
<th>Iron content of film (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10Fe(II)</td>
<td>−0.12</td>
<td>−0.30</td>
<td>0.389</td>
</tr>
<tr>
<td>1Fe(II)</td>
<td>−0.08</td>
<td>−0.20</td>
<td>0.225</td>
</tr>
<tr>
<td>10Fe(II)–1Fe(III)</td>
<td>−0.10</td>
<td>−0.26</td>
<td>0.389</td>
</tr>
<tr>
<td>1Fe(II)–1Fe(III)</td>
<td>−0.05</td>
<td>−0.07</td>
<td>0.329</td>
</tr>
<tr>
<td>1Fe(II)–10Fe(III)</td>
<td>−0.07</td>
<td>−0.18</td>
<td>0.090</td>
</tr>
<tr>
<td>1Fe(III)</td>
<td>0.00</td>
<td>+0.13</td>
<td>0.015</td>
</tr>
<tr>
<td>10Fe(III)</td>
<td>+0.07</td>
<td>+0.19</td>
<td>0.045</td>
</tr>
</tbody>
</table>

*a Arabic numerals indicate concentrations in millimoles.

*b Area of wave following conditioning minus area before conditioning.

dione (10 mM) and Fe(II)HEDTA (10 mM) upon live, melanized C. neoformans cells. The cells were clumped by free Fe(II) (ferrous ammonium sulfate) but not by Fe(II)HEDTA. Dithionite decreased light absorption by suspensions of melanized cells by 21% (optical density of untreated cells, 0.35 ± 0.04; optical density of conditioned cells, 0.27 ± 0.04 [mean and standard deviation of four trials]); Fe(II)HEDTA decreased light absorption by an average of 1.3% ± 1.7% in 11 experiments (P < 0.05, Student’s paired t test), consistent with reduction of melanin. Thus, optical lightening of melanized cells required high concentrations of Fe(II)HEDTA (10 mM) and was at the limit of detection. Fremy’s salt (10 mM) did not darken suspensions of C. neoformans.

Extracellular concentrations of Fe(II) achieved. We wondered whether C. neoformans was capable of generating quantities of Fe(II) sufficiently large to affect the oxidation-reduction state of melanin, as defined above. In physiologic experiments, suspensions of live C. neoformans cells incubated in 1 mM Fe(III)HEDTA in the presence of the Fe(II)-binding chromogen BPDS generated concentrations of Fe(II) greater than 100 μM (16). However, the presence of BPDS during the physiologic incubation raises the possibility that the equilibrium was shifted by trapping of Fe(II) by the dye. To avoid this potential artifact, we repeated the measurement in a system in which strain 620, constitutive for the ferric reductase, was grown with succinate (a nonreducing energy source) and various concentrations of Fe(III)HEDTA, and in which BPDS was added to filter-sterilized samples of culture supernatant at intervals to monitor the Fe(II) concentrations. The maximum concentration of Fe(II) achieved by the culture was 20 to 40% of the amount of Fe(III) supplied (Fig. 5) and up to 160 μM in the presence of 1 mM Fe(III)HEDTA. The wild-type strain, cultivated in 15 μM Fe(III)HEDTA, maintained 2 to 6 μM Fe(II) in extracellular fluid for 2 days, despite down regulation of its ferric reductase. Strain 643, lacking ferric reductase, generated a maximum of 1 μM Fe(II) under the same conditions.

Reaction of 3HAA and AKG with melanin. Because the reducing agent 3HAA is also secreted by C. neoformans, we assayed its reaction with melanin electrochemically. We detected a slight reduction of melanin only by pushing the solubility to 5 mM with 10% 2-propanol. Thus, a plot of first reduction wave area against concentration (1 to 5 mM) gave a statistically significant correlation but a plot of OCP against concentration gave a trend that was not significant (data not shown). When AKG was assayed, neither the OCP nor the reduction wave area was altered.

FIG. 4. Effect of various reagents upon light absorption by melanin. The optical density (OD400) of melanin was monitored under bubbled argon as it was exposed to reagents (10 mM) as indicated. mel, melanin.

DISCUSSION

A mechanistic explanation for the role of melanin in pathogenesis is based upon the ability of each monomeric residue of melanin to exist in either a reduced state, analogous to hydroquinone, or an oxidized state, analogous to quinone (14), and thereby to constitute a redox buffer; indeed, melanin has been shown to neutralize potentially microbicidal strong oxidants similar to those produced by leukocytes in response to infection (9, 23). However, melanin, even under the ambient atmosphere, exists mainly in the oxidized form (5). Thus, in an active infection, presumably involving waves of host-derived oxidative fluxes, a reductive role for melanin seems dependent upon the ability of the pathogen to maintain melanin in a substantially reduced state and upon a mechanism for transport of reducing equivalents to the exterior of the yeast. The observed reduction of Fe(III) to Fe(II) on the external surface of C. neoformans appears to provide one such electron transport mechanism. One might ask whether reduction of Fe(III) might have a deleterious effect upon the pathogen, based upon the propensity of Fe(II) to react with H₂O₂ and generate potentially harmful hydroxyl radicals; however, binding of Fe(II) by melanin suppresses this reaction (17), while the hydroxyl radical appears to be harmlessly absorbed by melanin (22). Inasmuch as secreted low-molecular-weight reductants can themselves neutralize extracellular oxidants without mediation by melanin, an important use of melanin may be its ability to concentrate and immobilize a quantity of reducing equivalents just outside the surface of the cell.

Transition metals are known to associate with melanin (20); indeed, in the presence of strong chelators of Fe(II) and Fe(III) and in the absence of initial Fe(II), melanin generates Fe(II) from Fe(III) (17), a reaction which we also have observed (16). However, the reverse reaction, reduction of melanin by Fe(II), has not previously been shown, and until very recently it has not been possible directly to monitor the oxidation state of melanin itself as it reacts with redox reagents. The physicochemical measurements which we made allow estimations of the extent of reaction of the catecholic groups of melanin with 1 mM Fe(II). Under the conditions where OCPs were measured, equilibrium is attained, and so the conditions of the melanin half-cell should be those predicted by the Nernst equation, E = E₀ + 0.059/n log (oxidized species)/(reduced species) (2), where n is the number of electrons in the half-cell reaction. Setting n = 2 for a hydroquinone-quinone oxidation-reduction potential mechanism.
reaction, the potential difference between two oxidation states is given as $E_1 - E_2 = 0.0295 \log \frac{(\text{ox}_1)/(\text{red}_1)}{(\text{ox}_2)/(\text{red}_2)}$. Since Fe(II) changed the OCP by $-0.10$ V, we estimate that the ratio $(\text{ox})/(\text{red})$ was decreased by antilog ($-0.10/0.0295$), or more than 1,000-fold. Moreover, since reduction of melanin occurs even when the initial concentration of Fe(III) is 10 times that of Fe(II), it appears that in the absence of air, the reaction equilibrium is toward the reduction of melanin and oxidation of iron.

We have demonstrated that Fe(II) will detectably reduce a melanin film at concentrations as low as 10 $\mu$M. Since the electrochemical reference point (from which the reductive scan is begun) is near the midpoint of the complete reduction wave, it seems likely that 10 $\mu$M Fe(II) could reduce a completely oxidized sample of melanin almost by half. Can C. neoformans generate the necessary concentrations of Fe(II)? A constitutive ferric reductase strain is capable of generating more than 100 $\mu$M Fe(II); the wild type, cultivated in 15 $\mu$M Fe(III), generates 2 to 6 $\mu$M Fe(II), probably sufficient to bring melanin from the completely oxidized state to a partly reduced state, thereby regenerating the redox buffer. Thus, the physiologic system appears to achieve the thermodynamic requirement for partial reduction of melanin. Whether this process occurs under more complicated conditions, such as infections, would depend upon expression of the ferric reductase, secretion of reductants, and local iron concentrations in that situation; this information is not yet available.

3HAA, secreted by C. neoformans, appears to be an efficient reducing agent for Fe(III) (16), and on thermodynamic grounds, one might expect 3HAA to reduce melanin even more vigorously than does Fe(II); however, it should be remembered that the presence of BPDS, the chromogenic Fe(II) chelator, would promote the reduction of Fe(III) in vitro [as might physiologic uptake of Fe(II), in vivo]. It was therefore not totally surprising that 3HAA reacted poorly with melanin, with the rate of the reaction of 5 mM 3HAA being roughly comparable to that of 10 $\mu$M Fe(II). The reducing agent AKG did not reduce melanin at all. Thus, Fe(II) appears to be approximately 1,000-fold more reactive than 3HAA. Extracellular concentrations of 3HAA range from 20 $\mu$M [without exogenous Fe(III)] to 7 $\mu$M [in 15 $\mu$M Fe(III)]; if 3HAA participates in the reduction of melanin, the mediation of Fe(II) might be required.

Since the redox metabolism of melanin may be important in physiology and pathogenesis, it is desirable to be able to monitor the oxidation state of melanin over time. The electrochemical method introduced here is useful because of its fundamental nature; however, it appears to be limited to modeling in artificial preparations. We have performed in vitro redox titrations of melanized cells (8, 9), but that method seems too slow and clumsy for physiologic studies. The signal of the semiquinonic, unpaired electrons of melanin has been monitored semiquantitatively in vivo by electron spin resonance (22), but that technique alone does not seem to allow insight into changes of oxidation state. When electron spin resonance is combined with electrochemistry, changes in the oxidation state can be inferred from buildups and decays of the semiquinone species,
which is of intermediate oxidation state (13). That technique might allow electrochemical studies equivalent to those in the present work, provided that iron in solution did not interfere with the signal of the dissolved melanin.

In the present work, in addition to electrochemistry, we examined the use of optical absorbance to monitor the oxidation state of suspended melanin particles and suspended melanized cells. It has been shown that over a broad range of visible wavelengths, optical absorbance by dissolved melanin increases when the melanin is oxidized and decreases when the melanin is reduced (14, 19). We observed similar changes for melanin suspended in buffer. Thus, the inferences drawn by using electrochemical techniques were supported by this very different, very convenient, but very empirical method: suspensions of synthetic melanin seemed susceptible either to oxidation or to reduction by the reagents used. Inasmuch as we have repeatedly observed a slight lightening of melanized cell suspensions upon the addition of Fe(II)HEDTA, we have obtained evidence that Fe(II) reduces biomelanin in vivo. Although the optical technique is very insensitive, the concentration dependence of the lightening reaction does agree with the electrochemical results, in that only Fe(II) in the millimolar range appeared to reduce most of the melanin. Thus, it was perhaps to be expected that comparable concentrations would be required to induce a detectable optical change in melanin. A final problem is the lesser lightening of cells treated with Fe(II) compared with that of cells treated with dithionite; it is clear that the interaction of Fe(II) with live cells is more complex than its interaction with pure melanin. However, taken together with the more rigorous electrochemical results, these results constitute circumstantial evidence for physiologic reduction of melanin by Fe(II) and suggest that biosynthetic melanin in C. neoformans exists in a state which is susceptible to dissolved reductants.

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