Microbiology (2007), **153**, 3954–3962 **DOI** 10.1099/mic.0.2007/011049-0

Cryptococcus neoformans laccase catalyses melanin synthesis from both D- and L-DOPA

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The human fungal pathogen *Cryptococcus neoformans* produces melanin in the presence of various substrates, including the L enantiomer of 3,4-dihydroxyphenylalanine (DOPA). The enzyme laccase catalyses the formation of melanin by oxidizing L-DOPA, initiating a series of presumably spontaneous reactions that ultimately leads to the polymerization of the pigment in the yeast cell wall. There, melanin protects the cell from a multitude of environmental and host assaults. Thus, the ability of *C. neoformans* to produce pigments from a variety of available substrates is likely to confer a survival advantage. A number of *C. neoformans* isolates of different serotypes produced pigments from D-DOPA, the stereoisomer of L-DOPA. Acid-resistant particles were isolated from pigmented *C. neoformans* cells grown in the presence of D-DOPA. Biophysical characterization showed the particles had a stably detectable free-radical signal by EPR, and negative zeta potential, similar to L-DOPA-derived particles. No major differences were found between L- and D-DOPA ghosts in terms of binding to anti-melanin antibodies, or in overall architecture when imaged by electron microscopy. *C. neoformans* cells utilized L- and D-DOPA at a similar rate. Overall, our results indicate that *C. neoformans* shows little stereoselectivity for utilizing DOPA in melanin synthesis. The ability of *C. neoformans* to use both L and D enantiomers for melanization implies that this organism has access to a greater potential pool of substrates for melanin synthesis, and this could potentially be exploited in the design of therapeutic inhibitors of laccase.

Received 26 June 2007
Revised 26 July 2007
Accepted 27 July 2007

INTRODUCTION

Melanin pigments are used by micro-organisms for myriad purposes, including cell wall stability, protection against UV radiation, protection against heat and cold, resistance against heavy metals, and reduced susceptibility to amoeboid predators (Nosanchuk & Casadevall, 2003; Steenbergen *et al.*, 2001). These functions enhance survival in the environment. Among pathogenic fungi, melanin synthesis has been associated with virulence, probably due to increased resistance to host defences (Nosanchuk & Casadevall, 2003). For example, melanization contributes to resistance of *Paracoccidioides brasiliensis* and *Exophiala dermatitidis* to killing by phagocytic cells (da Silva *et al.*, 2006; Schnitzler *et al.*, 1999).

Melanins are a family of pigments with shared properties, including dark colour, insolubility, resistance to acid hydrolysis, and susceptibility to degradation by strong oxidizing agents (Nosanchuk & Casadevall, 2003; Riley, 1997). In addition, melanins have a stable free-radical population, which produces distinctive electron spin resonance signals due to the presence of unpaired electrons in the polymer (Enochs *et al.*, 1993). One type of melanin found in fungi is eumelanin, derived from tyrosine or the L enantiomer of 3,4-dihydroxyphenylalanine (DOPA). Oxidation of this precursor by phenoloxidase enzymes initiates the melanization pathway (Butler & Day, 1998).

Cryptococcus neoformans is a human pathogenic fungus found worldwide. It is commonly found in the environment, but generally causes disease in immunocompromised hosts, such as AIDS patients (Perfect & Casadevall, 2002). It has a polysaccharide capsule surrounding the cell wall that is a major virulence determinant (Casadevall *et al.*, 2003). Differences in the polysaccharide capsule result in altered recognition by antibodies, resulting in the grouping of *C. neoformans* varieties into five serotypes: A, B, C, D

Abbreviation: DOPA, 3,4-dihydroxyphenylalanine.

and AD. Some of the serotype differences reflect vast phylogenetic distances, such that they are considered different species (Kwon-Chung & Varma, 2006).

C. neoformans strains unable to melanize are significantly less virulent in mouse infection models (Salas *et al.*, 1996). Inhibition of melanization with glyphosate translates into therapeutic benefits in experimental animal infection (Nosanchuk *et al.*, 2001). Previous studies have shown a wide range of functions for melanin in *C. neoformans*, and these include both environmental and host resistance. For example, melanized *C. neoformans* cells are more resistant to the action of antimicrobial defensins (Doering *et al.*, 1999). In addition, melanized cells are more resistant to phagocytosis by macrophages (Wang *et al.*, 1995).

Unlike other melanotic microbes, *C. neoformans* melanizes only in the presence of exogenous substrates such as L-DOPA (Kwon-Chung *et al.*, 1983). Melanization in *C. neoformans* is catalysed by laccase, a cell-wall-associated diphenoloxidase that catalyses the oxidation of diphenolic compounds to their respective quinones (Williamson, 1994; Zhu *et al.*, 2001). This enzyme is relatively promiscuous, and can oxidize numerous catecholamine compounds (Polacheck *et al.*, 1982). *C. neoformans* presumably melanizes during mammalian infection by scavenging substrates from tissue that remain unidentified (Rosas *et al.*, 2000a). Recent reports have enumerated an expanded list of substrates that can be used for melanization *in vitro*, and these include several bacterial products (Frases *et al.*, 2006, 2007; Garcia-Rivera *et al.*, 2005).

Laccase is a potential target for the development of novel therapeutics to target melanotic microbes. Defining the requirements for laccase catalysis is important for identifying potential lead compounds. Here, we investigate the ability of this enzyme to synthesize melanin from D- and L-DOPA, and report the surprising finding that there is little or no enantiomeric preference for DOPA in pigment formation. The results have potential significance for understanding the origin of melanin precursors in the environment, and provide a finding that could be exploited in inhibitor design.

METHODS

***C. neoformans* strains.** *C. neoformans* serotype D strain 24067, and serotype A strain H99, were obtained from the American Type Culture Collection. *C. neoformans* serotype D strain Cap67, containing a mutation in the *CAP59* gene, was provided by J. Kwon-Chung (Chang & Kwon-Chung, 1994). The congenic serotype D strains 2ETU (laccase deletion) and 2ETU-C (complemented strain) have been described (Salas *et al.*, 1996). The serotype A laccase deletion CHM3 was obtained from Dr Joseph Heitman (Duke University, Durham, NC, USA). This strain contains a partial deletion of the *LAC1* gene (Hicks *et al.*, 2004). The strains used in the rapid melanization assay were a combination of clinical, veterinary and environmental isolates. The *C. neoformans* var. *gattii* isolates (serotypes B and C) were obtained from Dr Thomas Mitchell (Duke University, Durham, NC, USA) and Dr June Kwon-Chung (National Institutes of Health, Bethesda, MD, USA). The *C. neoformans* var. *neoformans* (serotype A) isolates were obtained from New York City hospitals. The *C. neoformans* var. *neoformans* (serotype D) isolates were obtained from Dr Laurie Watt (bioMérieux, Marcy l'Etoile, France). Serotype AD isolates were obtained from Mary Brandt (Centers for Disease Control, Atlanta, GA, USA).

Melanization assays. For the *in vitro* melanization assay, *C. neoformans* cells were streaked on chemically defined minimal medium [15 mM glucose, 10 mM $MgSO_4$, 29.4 mM KH_2PO_4, 13 mM glycine, 3 μM thiamine, and 1.5 % (w/v) Bacto agar, pH 5.5], with and without 1 mM L- or D-DOPA (Sigma-Aldrich), and incubated in the dark for several days at 30 °C. Plates were examined daily to monitor growth and pigment production. The rapid melanization test was based on a previously described assay (Chaskes *et al.*, 1981). Each strain was streaked onto Sabouraud dextrose agar medium (Becton, Dickinson and Company), and incubated at 30 °C for several days. A toothpick was used to transfer a thick patch of cells to starvation medium [0.2 g l^{-1} K_2HPO_4, 0.1 g l^{-1} KH_2PO_4, and 1.5 % (w/v) Bacto agar, pH 7], and this was incubated at 30 °C for 2 days. Two drops of a solution of L- or D-DOPA (0.3 %, w/v) were pipetted directly onto the cells. Pigment formation was monitored visually over a time of 5–60 min. Initially, a pink pigment formed, usually within a few minutes. Over time, a brown pigment formed in many of the strains, usually within 1 h.

Preparation of melanin ghosts, and determination of yield. *C. neoformans* strain 24067 was cultured in chemically defined minimal medium with L- or D-DOPA (1 mM) at 30 °C in a shaking incubator in the dark for 7–14 days. Melanin ghosts were isolated by enzymic digestion of the cell wall, proteolysis, chloroform extraction, and boiling in concentrated HCl, as described previously (Rosas *et al.*, 2000b). Ghosts were viewed and imaged with an Olympus AX70 microscope. For pigment yield, *C. neoformans* cells were grown in chemically defined minimal medium with L- or D-DOPA for 1 week. Melanized cells were recovered, washed and dried. Dried cells were boiled in 6 M HCl for 1 h, washed and dialysed against dH_2O for several days. The resultant acid-resistant fraction was dried and weighed. The yield was calculated from the weight of the sample divided by the weight of the input DOPA.

EPR spectroscopy. One of the unique properties of melanin is the presence of unpaired electrons in the polymer from the dopaquinone moieties. These can be detected by EPR spectroscopy (Enochs *et al.*, 1993). Melanin ghosts or unmelanized cells (negative control) were suspended in water at a concentration between 4×10^7 and 5×10^7 cells ml^{-1}. Approximately 500 μl suspension was pipetted into 4 mm quartz EPR tubes (Wilmad LabGlass), and slowly frozen in liquid nitrogen. EPR spectra were obtained with a Varian E112X-Band model spectrometer. The parameters for EPR were as follows: modulation amplitude, 0.16 mT; centre field, 325 mT; sweep width, 8 mT; microwave frequency, 9.1 GHz; microwave power, 5.00 mW; and temperature, 77 K.

Zeta potential measurements. Zeta potential is an indicator of the surface charge (Richmond & Fisher, 1973). Since melanin is a negatively charged polymer, measurement of zeta potential is one way to determine whether various substrates are equally incorporated into melanin. The *C. neoformans* strain 24067, and the acapsular strain Cap67, were grown in chemically defined minimal medium with the respective pigment substrate for 10 days. The zeta potentials of 24067 and Cap67 strains were measured with a Zeta Potential Plus machine (Brookhaven Instruments). The zeta potential, or surface charge, of the particles was determined by applying an electric field to the particles in suspension, and determining the direction and velocity of the particle movement by measuring light scattering of a laser beam passed through the sample. Samples were prepared at a concentration

of 10^7 cells (or melanin ghosts) ml^{-1} in 10 mM KCl. For each experiment, each sample was measured three times, with 10 readings per measurement. Measurements more than three standard errors from the mean were thrown out. Values represent means of all readings.

Immunofluorescence of melanin ghosts. Approximately 5×10^6 melanin ghosts were washed in PBS (8 g l^{-1} NaCl, 0.2 g l^{-1} KCl, 0.2 g l^{-1} KH_2PO_4, and 1.2 g l^{-1} Na_2HPO_4, pH 7.4), and resuspended in 10 μg ml^{-1} antibody solution (ascites diluted in PBS), and incubated at 37 °C for 2 h. mAb 6D2 is a melanin-binding antibody that has been described previously (Rosas *et al.*, 2000a). The mAb 5C11 to mycobacterial polysaccharide was used as an isotype-matched negative control (Glatman-Freedman *et al.*, 1996). mAb binding was detected with a goat anti-mouse IgM conjugated to FITC (Southern Biotech). Ghosts were washed in PBS, and suspended in 100 μl 10 μg ml^{-1} secondary antibody, and incubated at room temperature in the dark for 1 h. Ghosts were washed in PBS, and resuspended in 20 μl mounting solution [0.1 M *N*-propyl gallate in 50 % (v/v) glycerol in 0.5 × PBS], and slides were prepared. Stained ghosts were viewed and imaged with an Olympus AX70 microscope equipped with an FITC filter.

Oxidation of melanins, and HPLC of oxidized melanins. The ghosts produced from *C. neoformans* cells grown in the presence of L- or D-DOPA were oxidized with acidic permanganate, as described previously (Garcia-Rivera *et al.*, 2005). The oxidation products were analysed by HPLC using an Amersham Biosciences ÄKTAbasic liquid chromatograph with a P-900 pump, a UV-900 detector, and a Hamilton PRP-1 C_{18} column with dimensions of 250 × 4.1 mm, and particle size of 7 μm. The mobile phase was 0.1 % (v/v) trifluoroacetic acid in water (solvent A), and 0.1 % (v/v) trifluoroacetic acid in acetonitrile (solvent B). At 1.0 ml min^{-1}, the elution gradient was as follows (min, %B): 0, 0; 1, 0; 12, 25; 14, 25; 16, 0. The UV detector was set at an absorbance of 280 nm.

Scanning electron microscopy. Melanin ghosts were prepared as described previously (Eisenman *et al.*, 2005). Imaging was performed with a JEOL JSM-6400 scanning electron microscope, using an accelerating voltage of 10 kV. Images were acquired with analySIS software (Soft Imaging System). To determine granule size, images were analysed using Adobe Photoshop software. The line tool was used to measure diagonally across each granule, and the size was determined based on the scale bar.

Transmission electron microscopy. Melanin ghosts were prepared as described previously (Eisenman *et al.*, 2005). Samples were viewed in a JEOL 1200EX transmission electron microscope at 80 kV. Photographs or negatives of the images were scanned and analysed in Adobe Photoshop. The line tool was used to measure across the width of the melanin layer, and the size was calculated based on magnification of the photograph or negative.

Laccase activity of cells. Cells of *C. neoformans* strain H99 or CHM3 were incubated at 30 °C, 150 r.p.m., in chemically defined medium. After 4 days, cells were transferred to starvation medium (0.2 g l^{-1} K_2HPO_4 and 0.1 g l^{-1} KH_2PO_4), at a concentration of 5×10^7 cells ml^{-1}, and incubated for an additional day. Cells were washed, collected by centrifugation, and washed in starvation medium. Cells were then resuspended in starvation medium at a concentration of 5×10^7 cells ml^{-1}. Assays were done in 96-well plate format. A 100 μl volume of cell suspension was added to 100 μl pre-warmed L- or D-DOPA substrate to final concentrations of 10, 5, 2.5, 1.25, 0.625, 0.313 or 0.156 mM and mixed by pipetting. Reaction mixtures were incubated at 30 °C for various times, from 5 to 60 min. The samples were subjected to centrifugation to remove the cells, and absorbance of the supernatant at 475 nm was measured immediately in a VERSAmax tunable microplate reader (Molecular Devices). To control for background oxidation of DOPA, absorbance values obtained for CHM3 cells were subtracted from those obtained for H99. The kinetics of substrate utilization were determined as follows. For each substrate concentration, A_{475} was plotted against time. The slopes of the resulting lines were considered to be the 'initial velocity' (V_i) for each substrate concentration. Overall utilization was determined by plotting V_i (units of A_{475} min^{-1}) against substrate concentration. Three samples for each substrate concentration and time point were measured, and the mean was plotted on the graphs.

Statistical analysis. The computer program JMPIN (SAS Institute) was used for statistical analysis. An analysis of variance was performed to determine whether data from multiple experiments could be pooled. Data were analysed for normal distribution by the Shapiro–Wilk *W* test. Non-parametric data were compared by Wilcoxon/Kruskal–Wallis tests. Parametric data were compared by the *t* test.

RESULTS

C. neoformans produces a dark pigment in the presence of D-DOPA

In vitro melanization assays were performed to determine whether *C. neoformans* produced pigment from D-DOPA. Cells were streaked onto medium containing either L- or D-DOPA, or with no substrate. Fungal cells turned dark brown/black within 4 days in agar containing either L- or D-DOPA (Fig. 1a). To determine whether pigment was produced from D-DOPA through the same pathway as L-DOPA, strains lacking laccase were tested. Laccase deletion strains of two different serotypes were tested: 2ETU (serotype D) and CHM3 (serotype A). No pigmentation was observed with D-DOPA in either of these strains, indicating that the laccase is required for pigment production from D-DOPA. The experiment was repeated three times with similar results. In addition to the above tests, a rapid melanization test was done with 23 *C. neoformans* strains. Four or five strains of each serotype were tested. Strains 2ETU and 2ETU-C served as negative and positive controls, respectively. For each strain, no major difference in the rate or intensity of colour change was observed between L- and D-DOPA. Nevertheless, differences were observed between strains, such that not all the strains produced pigment at the same rate, but each strain utilized L- and D-DOPA for pigment formation equally (Table 1). Two independent experiments with similar results were done for each strain, except for the serotype AD strains, which were tested once.

Melanin ghosts from D-DOPA

Previous studies have established that melanin produced from L-DOPA could be isolated from melanized *C. neoformans* cells by chemical and enzymic removal of other cellular material to recover acid-resistant particles called melanin ghosts (Rosas *et al.*, 2000b). Such particles retain the original cell shape. Melanized cells grown in the presence of D-DOPA were subjected to the same treatments, and the resulting particles appeared similar to

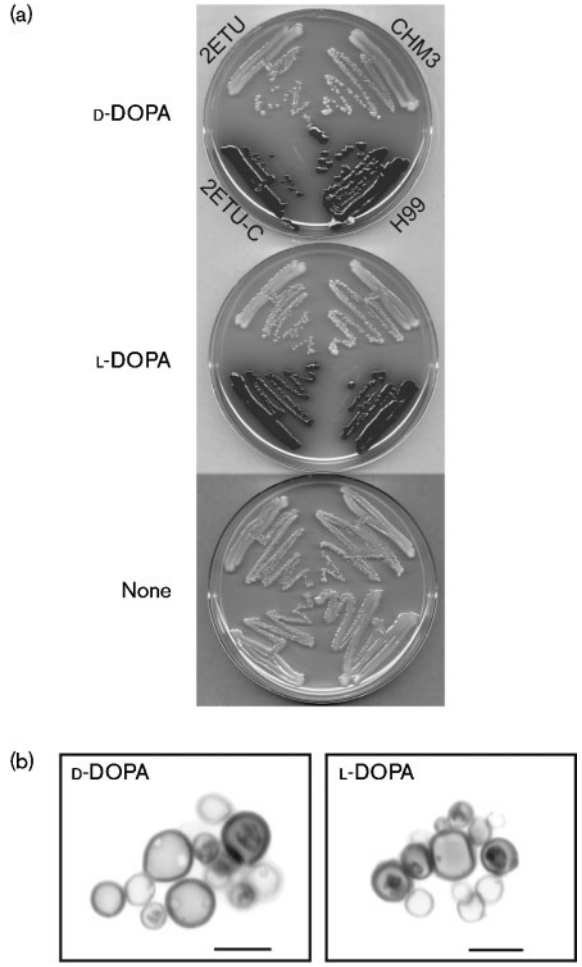

Fig. 1. *C. neoformans* produces pigment from D-DOPA. (a) Laccase deletion (2ETU and CHM3) and wild-type (2ETU-C and H99) *C. neoformans* cells were plated on medium containing L- or D-DOPA, and incubated for 4 days. (b) Melanin ghosts isolated from *C. neoformans* cells grown in the presence of L- or D-DOPA. Cells were subjected to enzymic and chemical degradation, as described in Methods. The resulting particles were imaged by light microscopy, without staining. Bars, 5 μm.

L-DOPA-derived ghosts when viewed by light microscopy (Fig. 1b). Melanin ghost preparation from D-DOPA was repeated twice. Next, we determined the yield of pigment produced from L- and D-DOPA. A greater yield of acid-resistant pigment was recovered from D-DOPA (85 %) compared with L-DOPA (63 %).

Biophysical properties of ghost particles derived from L- or D-DOPA

One of the hallmarks of melanins is the presence of a stable free-radical population that produces distinctive signals by EPR spectroscopy (Enochs *et al.*, 1993). Particles derived from L- and D-DOPA were tested for the presence of a stable free-radical signal by EPR. L- and D-DOPA produced a similar free-radical signal when the magnetic field was applied (Fig. 2). The negative control, non-melanized *C. neoformans* cells, did not produce a free radical signal. Two independent experiments were performed, and in both cases the signal from D-DOPA ghosts was similar to that of L-DOPA ghosts.

Next, the surface charge of the pigmented cells and ghosts was measured (Table 2). Previous studies have shown that melanin derived from *C. neoformans* has an overall negative charge (Nosanchuk & Casadevall, 1997). No significant differences were observed in the zeta potential for wild-type or acapsular cells grown with L- or D-DOPA. However, the acapsular cells were significantly less negative than the wild-type cells. Since zeta potential reflects surface charge, these data suggest that, for whole cells, the negative charge of the melanin is masked by the presence of the cell wall and capsule. Although a prior study has shown small differences in cell zeta potential with melanization, we note that in that case the cells were grown in rich medium that produces smaller capsules (Nosanchuk & Casadevall, 1997). For melanin ghosts, the L-DOPA ghosts were more negative than the D-DOPA-derived ghosts (-20.2 and -17.4 mV, respectively); however, this difference was not statistically significant at the 0.05 level ($P=0.07$). Two independent experiments were performed for whole cells, and three for melanin ghosts. The data presented are from one representative experiment.

Comparison of L- and D-DOPA melanin ghosts

mAb 6D2 is a melanin-binding IgM made against L-DOPA-derived melanin ghosts from *C. neoformans*. mAb 6D2 was used to probe for antigenic differences between L- and D-DOPA ghosts by immunofluorescence. mAb 6D2 bound to both L- and D-DOPA-derived melanin ghosts (Fig. 3a). No fluorescence was observed with the control IgM under identical conditions (data not shown). The experiment was repeated twice with similar results.

Transmission electron microscopy revealed that the overall appearances of the melanin cross-sections were similar, with layers of melanin visible, as previously observed (Fig. 3b) (Eisenman *et al.*, 2005). The thickness of the melanin was measured for each substrate (Fig. 3c). There was considerable variation in the thicknesses measured within each substrate group. Analysis of the data distribution revealed two peaks of thicknesses for both L- and D-DOPA. Most of the ghosts had a melanin thickness between 50 and 200 nm; however, a few had thicker melanin, between 200 and 400 nm. For the group with the thinner melanin thickness, the thickness of the D-DOPA melanin was greater than the L-DOPA melanin (means of 125 ± 49 and 96 ± 36 nm, respectively; $P=0.009$). The group with thicker melanin had a trend toward greater thickness with D-DOPA compared with L-DOPA, but this was not statistically significant (295 ± 58 and 274 ± 73 nm, respectively; $P=0.15$). Forty-three L-DOPA and 48 D-DOPA ghosts were measured. The experiment was

Table 1. Rapid melanization test

Colour changes from pink to brown were monitored and recorded for cells incubated in the presence of L- or D-DOPA: P, pink; S, salmon; RB, red/brown; B, brown; W, no change/white. Colour intensity is indicated by the number of plus symbols (+).

Serotype	Strain	0.3 % L-DOPA		0.3 % D-DOPA	
		5 min	60 min	5 min	60 min
A	36	S+++	B++	S+++	B++
	53	S+++	B++	S+++	B++
	69	S++	B++	S++	B++
	75	S+	B+	S+	B+
B	101.97	P+	B++	P+	B+++
	102.97	P+	RB++++	P+	RB++++
	123.97	RB+	RB+++	RB+	RB+++
	NIH 198	W	W	W	W
	NIH 444	RB++	RB+++	RB++	RB+++
C	106.97	W	S+	W	S+
	107.97	B+	B++	B+	B++
	1343	W	B+	W	B+
	NIH 34	W	W	W	W
	NIH 191	P++	RB++++	P++	RB++++
D	BMX303695	S+	P++	S+	P++
	BMX303696	S+	P++	S+	P++
	BMX303697	S+	P++	S+	P++
	BMX303698	S++	P++	S++	P++
	JEC 21	P++	B+++	P++	B+++
	2ETU	W	W	W	W
	2ETU-C	P+	P++	P+	P++
AD	92-224	S+	B+	S+	B+
	92-244	S+	B+++	S+	B+
	92-668	P+	B+++	P+	B+++
	92-885	P++	B+++	P++	B+++

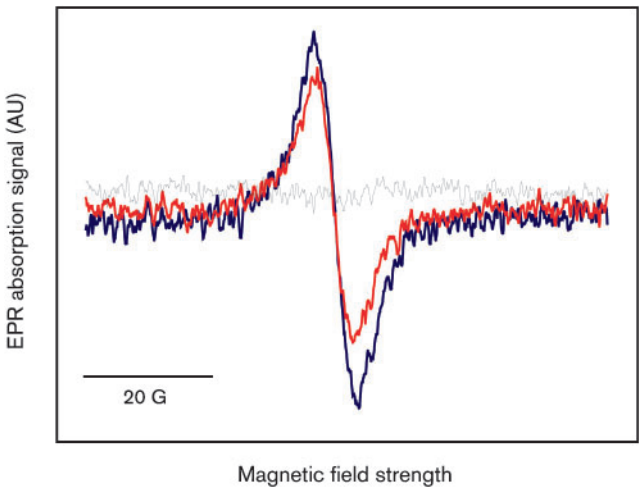

Fig. 2. L-DOPA (blue line) and D-DOPA (red line) ghosts have a stable free-radical signal. EPR was used to detect the presence of unpaired electrons in melanin ghosts as described in Methods. AU, Arbitrary units; black line, negative control.

repeated twice, and each time there was a trend toward increased thickness with D-DOPA ghosts.

Scanning electron microscopy revealed that D-DOPA-derived melanin had a similar granular appearance to that of L-DOPA-derived melanin (Fig. 3d). The size of granules on ghosts from each substrate was measured to determine if there were any differences in the melanin. Ten ghosts for each substrate were analysed, and 9–10 granules per ghost were measured. The measurements for D-DOPA granules

Table 2. Zeta potential of melanized cells and melanin ghosts

Cell type	Zeta potential (mV)		
	L-DOPA	D-DOPA	Minimal medium
Melanin ghosts	−20.22	−17.36	NA
Cap67 cells	−6.92	−5.76	−6.61
24067 cells	−33.83	−32.66	−35.01

NA, Not applicable.

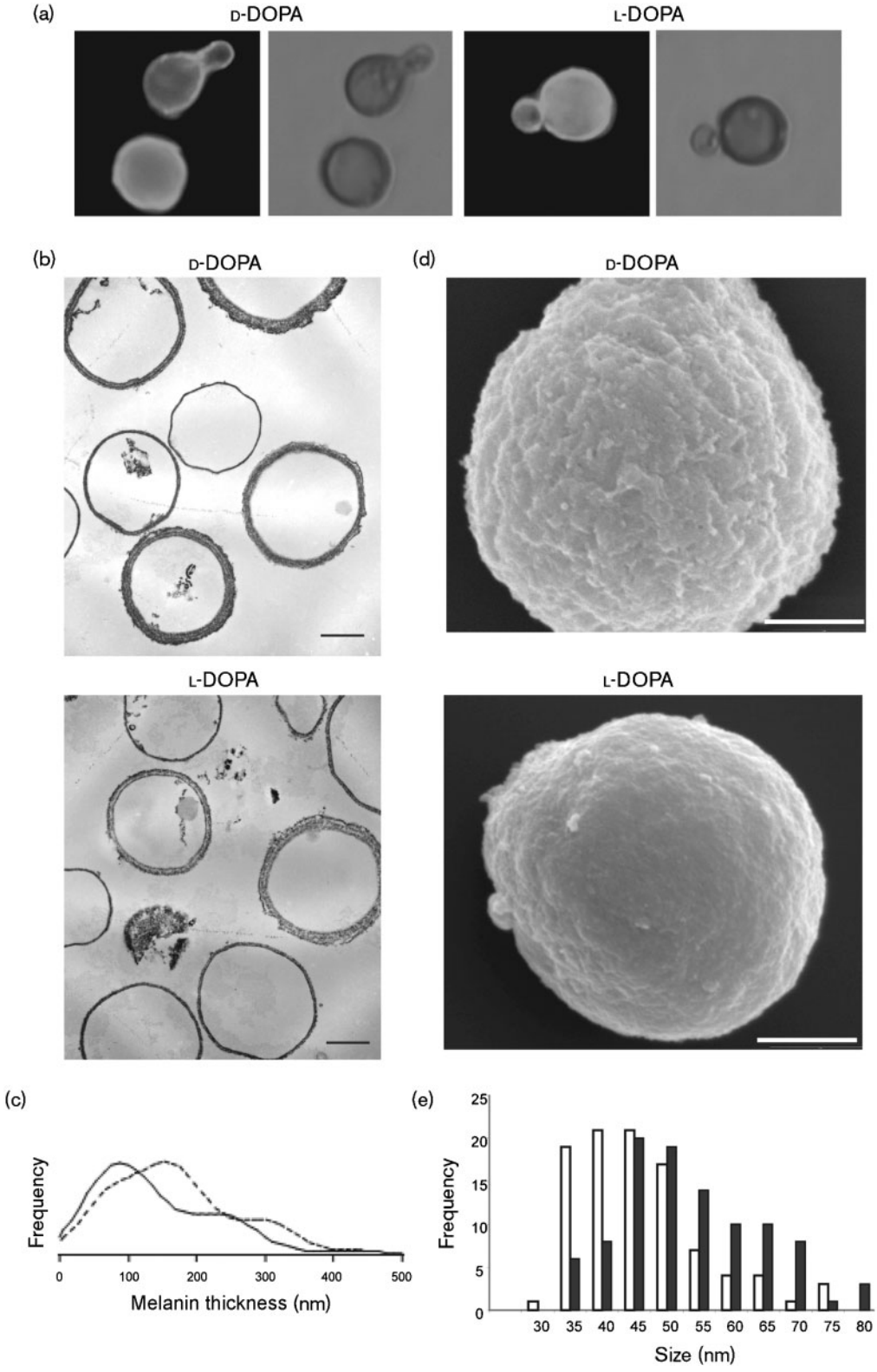

Fig. 3. Microscopy of L- and D-DOPA ghosts. (a) Melanin ghosts were incubated with anti-melanin antibodies and imaged by immunofluorescence as described in Methods. (b) Transmission electron microscopy images of representative L- and D-DOPA-derived melanin ghosts. Bars, 1 μm. (c) Distribution of melanin thickness for L-DOPA (solid line) and D-DOPA (dashed line) melanin ghosts. (d) Scanning electron microscopy images of representative L- and D-DOPA melanin ghosts. Bars, 1 μm. (e) Distribution of granule size for L-DOPA (white bars) and D-DOPA (black bars) melanin ghosts.

were uniform between ghosts, with a mean size of 57 nm. In contrast, the granules of the L-DOPA-derived ghosts were more variable between ghosts. The mean size was somewhat smaller at 49 nm. The average difference was due to the fact that the L-DOPA granules had a range of sizes from small to large, while the D-DOPA granules were larger and more uniform (Fig. 3e). Ten granules on each of ten different ghost images were analysed for each substrate. Two independent experiments were done, and each time the D-DOPA ghosts revealed larger granules.

Particles from pigmented cells generated with the various substrates were oxidized with acidic permanganate, and the products were separated by HPLC. Chromatograms showed peaks present in the L-DOPA-derived melanin at 8 and 12 min that were absent in the background solution. For the D-DOPA melanin, the treatment produced a similar pattern of peaks (data not shown). These data suggest that the composition of L- and D-DOPA melanin was similar.

Kinetics of substrate utilization

Since the *LAC1* gene is required for *in vitro* melanization with D-DOPA, we tested whether there were differences in the ability of laccase to use L- and D-DOPA. A whole-cell assay was performed to study the kinetics of substrate utilization by *C. neoformans*. A pink colour was apparent in the supernatant, probably due to the accumulation of dopachrome, an intermediate in the melanin synthesis pathway. Substrate utilization was assessed by monitoring the production of dopachrome by measuring absorbance at 475 nm. Overall, the kinetics of dopachrome formation were similar for L- and D-DOPA (Fig. 4). Three independent experiments were done, and in each experiment the results for L- and D-DOPA were similar. Data from one representative experiment are presented.

DISCUSSION

Melanization is critical to the virulence of many pathogenic fungi, including *C. neoformans*. A key to understanding melanization is to determine the relative efficiency of the substrates used in melanin synthesis. *In vitro*, *C. neoformans* must be provided with an exogenous source of substrate for melanization to occur. Often, catecholamines, such as L-DOPA, are used. Based on this evidence, and the fact that *C. neoformans* shows tropism for the central nervous system, it is thought that the fungus uses host neurotransmitters to produce melanin *in vivo* (Polacheck *et al.*, 1990). Recently, growing numbers of substrates for melanization in *C. neoformans* have been reported. These include several catecholamines, such as methyl-DOPA and norepinephrine (Garcia-Rivera *et al.*, 2005). In addition, *C. neoformans* can use substrates produced by bacteria for melanization (Frases *et al.*, 2006, 2007).

We have explored the question of substrate specificity further by testing the stereospecificity of the substrate requirements for DOPA. Stereospecificity is an attribute of most biomolecules and enzymes. Indeed, most enzymes are stereospecific in terms of substrate specificity, as well as reaction mechanism (Caldwell, 1995; Lamzin *et al.*, 1995; Rose, 1972). The results showed that *C. neoformans* can use both D- and L-DOPA for melanization. Remarkably, we found no major difference in melanization or the physical properties of melanin produced from L- and D-DOPA. The fact that very little difference was found between L- and D-DOPA-derived melanin may reflect the fact that the chiral carbon of DOPA is lost in the final melanin polymer, as suggested by most models of eumelanin synthesis. The only difference was an apparent larger granule size and melanin thickness, and increased yield, with D-DOPA. Although the mechanism for this effect is not understood, it could reflect local differences in the way the enantiomers melanize, possibly as a result of steric interactions with cell-wall polysaccharides, which are chiral molecules. This would suggest that melanin polymerization occurs with the cell wall acting as a template or scaffold for melanization.

The literature provides precedents for utilization of both DOPA enantiomers by diphenoloxidases or monophenol-oxidases, such as tyrosinase. *Mycobacterium leprae* can oxidize both L- and D-DOPA (Prabhakaran *et al.*, 1972). Mushroom tyrosinase can oxidize both L- and D-DOPA, albeit with a higher K_m for D-DOPA, a finding that presumably reflects chiral-side-chain effects on enzyme activity (Espin *et al.*, 1998). Similarly, polyphenoloxidase activity from the avocado plant (*Persea americana*) has similar reaction kinetics for the oxidation of L- and D-isomers of DOPA, and for L- and D-tyrosine (Kahn & Pomerantz, 1980). Polacheck *et al.* (1982) showed that laccase from *C. neoformans* cells has similar specific activity for L- and D-DOPA in a spectrophotometric assay, although they did not investigate the properties of the melanin product. In addition to D-DOPA, certain *C. neoformans* varieties can use D-tryptophan to produce non-melanin

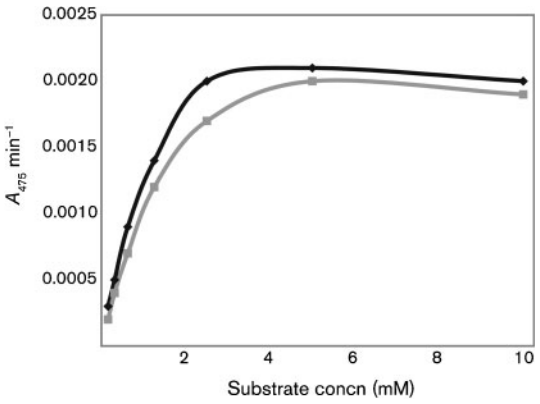

Fig. 4. *C. neoformans* utilizes L-DOPA (black) and D-DOPA (grey) with similar kinetics. Cells were incubated with various concentrations of substrate, and pigment production was monitored over time, as described in Methods.

pigments (Chaskes & Casadevall, 2006). In contrast to these bacterial, fungal and plant sources, tyrosinase from mammalian cells has a preference for the L isomers of DOPA and tyrosine (Pomerantz, 1963). Thus, our results are in agreement with previous studies, but also contribute significant new information by demonstrating that this lack of stereoselectivity observed in enzymic assays results in similar final melanin products. In fact, an exhaustive analysis of L- and D-DOPA-derived *C. neoformans* revealed no significant physical and chemical differences, except differences in granule size, melanin thickness and yield.

Since melanization is critical to virulence, laccase is a potential drug target in *C. neoformans*. Inhibition of melanization with glyphosate improves experimental cryptococcal infection in mice. Hence, information regarding substrate specificity of the enzyme could be useful in inhibitor design. Lack of substrate stereospecificity may imply that not all of the groups of a chiral compound interact with the substrate-binding pocket. This may be the case since oxidation of the hydroxyl groups in L-DOPA occurs far from the chiral carbon in the molecule (Williamson, 1997). In addition, since other studies have suggested that mammalian enzymes are stereoselective, it may be possible to design enzyme inhibitors specific for the fungal enzymes. For example, fungal laccases may be inactivated by D-substrate suicide inhibitors that are not metabolized by the human tyrosinase.

Overall, the results significantly expand the potential repertoire of substrates utilized by *C. neoformans* in melanization, by eliminating the constraint of stereoselectivity. However, we acknowledge that our study has not excluded the possibility that D-DOPA is converted to L-DOPA by *C. neoformans* before melanization. However, the size difference in the granules formed from D- and L-DOPA would argue against a mechanism whereby D-DOPA was first converted to L-DOPA, such that the actual laccase substrate is L-DOPA. The fact that this organism can metabolize racemic mixtures of melanin substrates suggests that it could use both biotic and abiotic sources of substrates. Since *C. neoformans* is a wood-associated fungus, and laccases are thought to also function in deactivating reactive compounds formed in wood degradation, this property may allow it to deactivate both L- and D-phenolic compounds. The lack of stereoselectivity in substrate utilization for melanization highlights the importance of this process to survival of the fungus in the environment. The fact that *C. neoformans* can use many different compounds, and manifests no enantiomer preference in melanin synthesis, suggests that there is an evolutionary advantage in the ability to use an array of available compounds to synthesize this protective pigment.

ACKNOWLEDGEMENTS

H. C. E. is supported by the Ruth L. Kirschstein National Research Service Award AI063866. A. C. is supported by the National Institutes of Health awards AI033142, AI033774 and HL059842-08.

REFERENCES

Butler, M. J. & Day, A. W. (1998). Fungal melanins: a review. *Can J Microbiol* **44**, 1115–1136.

Caldwell, J. (1995). Stereochemical determinants of the nature and consequences of drug metabolism. *J Chromatogr A* **694**, 39–48.

Casadevall, A., Steenbergen, J. N. & Nosanchuk, J. D. (2003). 'Ready made' virulence and 'dual use' virulence factors in pathogenic environmental fungi – the *Cryptococcus neoformans* paradigm. *Curr Opin Microbiol* **6**, 332–337.

Chang, Y. C. & Kwon-Chung, K. J. (1994). Complementation of a capsule-deficient mutation of *Cryptococcus neoformans* restores its virulence. *Mol Cell Biol* **14**, 4912–4919.

Chaskes, S. & Casadevall, A. (2006). Biochemical differentiation of *Cryptococcus neoformans* var. *gattii* from *Cryptococcus neoformans* var. on D-tryptophan D-proline agar. In *American Society for Microbiology 106th General Meeting*. Orlando, FL.

Chaskes, S., Edberg, S. C. & Singer, J. M. (1981). A DL-DOPA drop test for the identification of *Cryptococcus neoformans*. *Mycopathologia* **74**, 143–148.

da Silva, M. B., Marques, A. F., Nosanchuk, J. D., Casadevall, A., Travassos, L. R. & Taborda, C. P. (2006). Melanin in the dimorphic fungal pathogen *Paracoccidioides brasiliensis*: effects on phagocytosis, intracellular resistance and drug susceptibility. *Microbes Infect* **8**, 197–205.

Doering, T. L., Nosanchuk, J. D., Roberts, W. K. & Casadevall, A. (1999). Melanin as a potential cryptococcal defence against microbicidal proteins. *Med Mycol* **37**, 175–181.

Eisenman, H. C., Nosanchuk, J. D., Webber, J. B., Emerson, R. J., Camesano, T. A. & Casadevall, A. (2005). Microstructure of cell wall-associated melanin in the human pathogenic fungus *Cryptococcus neoformans*. *Biochemistry* **44**, 3683–3693.

Enochs, W. S., Nilges, M. J. & Swartz, H. M. (1993). A standardized test for the identification and characterization of melanins using electron paramagnetic resonance (EPR) spectroscopy. *Pigment Cell Res* **6**, 91–99.

Espin, J. C., Garcia-Ruiz, P. A., Tudela, J. & Garcia-Canovas, F. (1998). Study of stereospecificity in mushroom tyrosinase. *Biochem J* **331**, 547–551.

Frases, S., Chaskes, S., Dadachova, E. & Casadevall, A. (2006). Induction by *Klebsiella aerogenes* of a melanin-like pigment in *Cryptococcus neoformans*. *Appl Environ Microbiol* **72**, 1542–1550.

Frases, S., Salazar, A., Dadachova, E. & Casadevall, A. (2007). *Cryptococcus neoformans* can utilize the bacterial melanin precursor homogentisic acid for fungal melanogenesis. *Appl Environ Microbiol* **73**, 615–621.

Garcia-Rivera, J., Eisenman, H. C., Nosanchuk, J. D., Aisen, P., Zaragoza, O., Moadel, T., Dadachova, E. & Casadevall, A. (2005). Comparative analysis of *Cryptococcus neoformans* acid-resistant particles generated from pigmented cells grown in different laccase substrates. *Fungal Genet Biol* **42**, 989–998.

Glatman-Freedman, A., Martin, J. M., Riska, P. F., Bloom, B. R. & Casadevall, A. (1996). Monoclonal antibodies to surface antigens of *Mycobacterium tuberculosis* and their use in a modified enzyme-linked immunosorbent spot assay for detection of mycobacteria. *J Clin Microbiol* **34**, 2795–2802.

Hicks, J. K., D'Souza, C. A., Cox, G. M. & Heitman, J. (2004). Cyclic AMP-dependent protein kinase catalytic subunits have divergent roles in virulence factor production in two varieties of the fungal pathogen *Cryptococcus neoformans*. *Eukaryot Cell* **3**, 14–26.

Kahn, V. & Pomerantz, S. H. (1980). Monophenolase activity of avocado polyphenol oxidase. *Phytochemistry* **19**, 379–385.

Kwon-Chung, K. J. & Varma, A. (2006). Do major species concepts support one, two or more species within *Cryptococcus neoformans*? *FEMS Yeast Res* **6**, 574–587.

Kwon-Chung, K. J., Tom, W. K. & Costa, J. L. (1983). Utilization of indole compounds by *Cryptococcus neoformans* to produce a melanin-like pigment. *J Clin Microbiol* **18**, 1419–1421.

Lamzin, V. S., Dauter, Z. & Wilson, K. S. (1995). How nature deals with stereoisomers. *Curr Opin Struct Biol* **5**, 830–836.

Nosanchuk, J. D. & Casadevall, A. (1997). Cellular charge of *Cryptococcus neoformans*: contributions from the capsular polysaccharide, melanin, and monoclonal antibody binding. *Infect Immun* **65**, 1836–1841.

Nosanchuk, J. D. & Casadevall, A. (2003). The contribution of melanin to microbial pathogenesis. *Cell Microbiol* **5**, 203–223.

Nosanchuk, J. D., Ovalle, R. & Casadevall, A. (2001). Glyphosate inhibits melanization of *Cryptococcus neoformans* and prolongs survival of mice after systemic infection. *J Infect Dis* **183**, 1093–1099.

Perfect, J. R. & Casadevall, A. (2002). Cryptococcosis. *Infect Dis Clin North Am* **16**, 837–874 (v–vi).

Polacheck, I., Hearing, V. J. & Kwon-Chung, K. J. (1982). Biochemical studies of phenoloxidase and utilization of catecholamines in *Cryptococcus neoformans*. *J Bacteriol* **150**, 1212–1220.

Polacheck, I., Platt, Y. & Aronovitch, J. (1990). Catecholamines and virulence of *Cryptococcus neoformans*. *Infect Immun* **58**, 2919–2922.

Pomerantz, S. H. (1963). Separation, purification, and properties of two tyrosinases from hamster melanoma. *J Biol Chem* **238**, 2351–2357.

Prabhakaran, K., Harris, E. B. & Kirchheimer, W. F. (1972). The nature of the phenolase enzyme in *Mycobacterium leprae*: structure–activity relationships of substrates and comparison with other copper proteins and enzymes. *Microbios* **5**, 273–281.

Richmond, D. V. & Fisher, D. J. (1973). The electrophoretic mobility of micro-organisms. *Adv Microb Physiol* **9**, 1–29.

Riley, P. A. (1997). Melanin. *Int J Biochem Cell Biol* **29**, 1235–1239.

Rosas, A. L., Nosanchuk, J. D., Feldmesser, M., Cox, G. M., McDade, H. C. & Casadevall, A. (2000a). Synthesis of polymerized melanin by *Cryptococcus neoformans* in infected rodents. *Infect Immun* **68**, 2845–2853.

Rosas, A. L., Nosanchuk, J. D., Gomez, B. L., Edens, W. A., Henson, J. M. & Casadevall, A. (2000b). Isolation and serological analyses of fungal melanins. *J Immunol Methods* **244**, 69–80.

Rose, I. A. (1972). Enzyme reaction stereospecificity: a critical review. *CRC Crit Rev Biochem* **1**, 33–57.

Salas, S. D., Bennett, J. E., Kwon-Chung, K. J., Perfect, J. R. & Williamson, P. R. (1996). Effect of the laccase gene *CNLAC1*, on virulence of *Cryptococcus neoformans*. *J Exp Med* **184**, 377–386.

Schnitzler, N., Peltroche-Llacsahuanga, H., Bestier, N., Zundorf, J., Lutticken, R. & Haase, G. (1999). Effect of melanin and carotenoids of *Exophiala* (*Wangiella*) *dermatitidis* on phagocytosis, oxidative burst, and killing by human neutrophils. *Infect Immun* **67**, 94–101.

Steenbergen, J. N., Shuman, H. A. & Casadevall, A. (2001). *Cryptococcus neoformans* interactions with amoebae suggest an explanation for its virulence and intracellular pathogenic strategy in macrophages. *Proc Natl Acad Sci U S A* **98**, 15245–15250.

Wang, Y., Aisen, P. & Casadevall, A. (1995). *Cryptococcus neoformans* melanin and virulence: mechanism of action. *Infect Immun* **63**, 3131–3136.

Williamson, P. R. (1994). Biochemical and molecular characterization of the diphenol oxidase of *Cryptococcus neoformans*: identification as a laccase. *J Bacteriol* **176**, 656–664.

Williamson, P. R. (1997). Laccase and melanin in the pathogenesis of *Cryptococcus neoformans*. *Front Biosci* **2**, e99–e107.

Zhu, X., Gibbons, J., Garcia-Rivera, J., Casadevall, A. & Williamson, P. R. (2001). Laccase of *Cryptococcus neoformans* is a cell wall-associated virulence factor. *Infect Immun* **69**, 5589–5596.

Edited by: D. Sanglard