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Using Solid-state NMR to Monitor the Molecular Consequences of *Cryptococcus neoformans* Melanization with Different Catecholamine Precursors

Subhasish Chatterjee¹, Rafael Prados-Rosales², Susana Frases^{2,§}, Boris Itin³, Arturo Casadevall², and Ruth E. Stark^{1,*}

¹Department of Chemistry, City College of New York, Graduate Center and Institute for Macromolecular Assemblies, City University of New York, New York, NY 10031, USA

²Department of Microbiology and Immunology, Albert Einstein College of Medicine, Yeshiva University, Bronx, New York 10461, USA

³New York Structural Biology Center, New York, NY 10027

Abstract

Melanins are a class of natural pigments associated with a wide range of biological functions, including microbial virulence, energy transduction, and protection against solar radiation. Because of their insolubility and structural heterogeneity, solid-state nuclear magnetic resonance (NMR) spectroscopy provides an unprecedented means to define the molecular architecture of these enigmatic pigments. The requirement of obligatory catecholamines for melanization of the pathogenic fungus *Cryptococcus neoformans* also offers unique opportunities for investigating melanin development. In the current study, pigments produced with L-dopa, methyl-L-dopa, epinephrine, and norepinephrine precursors are compared structurally using ¹³C and ¹H magic-angle spinning (MAS) NMR. Striking structural differences were observed for both aromatic and aliphatic molecular constituents of the mature fungal pigment assemblies, thus making it possible to redefine the molecular prerequisites for formation of the aromatic domains of insoluble indole-based biopolymers, to rationalize their distinctive physical characteristics, and to delineate the role of cellular constituents in assembly of the melanized macromolecules with polysaccharides and fatty acyl chain-containing moieties. By achieving an augmented understanding of the mechanisms of *C. neoformans* melanin biosynthesis and cellular assembly, such studies can guide future drug discovery efforts related to melanin-associated virulence, resistance to tumor therapy, and production of melanin mimetics under cell-free conditions.

*Corresponding Author Author to whom correspondence should be addressed: Ruth E. Stark. stark@sci.ccny.cuny.edu. Phone: +1-212-650-8916. Fax: +1-212-650-8719. CUNY Institute for Macromolecular Assemblies, The City College of New York, Department of Chemistry MR-1208B, 160 Convent Avenue, New York, NY 10031-9101.

§Present address: Laboratório de ultraestrutura celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, BRAZIL

Supplemental Information

These materials may be available free of charge via the Internet at <http://pubs.acs.org> 189 and 225 MHz solid-state CPMAS ¹³C NMR spectra of *C. neoformans* melanins.

150 MHz CPMAS solid-state ¹³C spectra of *C. neoformans* melanins produced with [U-¹³C₆]-glucose and natural abundance norepinephrine and epinephrine.

150 MHz CPMAS solid-state ¹³C spectra of *C. neoformans* melanin produced with natural abundance L-dopa and [U-¹³C₆]-glucose, acquired with time delays of 0–40 μs before ¹H decoupling.

150 MHz DPMAS solid-state ¹³C spectra of *C. neoformans* melanins produced with L-dopa and methyl-L-dopa.

600 MHz solid-state ¹H spectra of melanins produced with [U-¹³C₆]-glucose, and natural abundance L-dopa, norepinephrine, and epinephrine.

Keywords

Fungal Melanin; Solid-state NMR; Catecholamine; *Cryptococcus neoformans*; CPMAS; Eumelanin

Melanins, a family of ubiquitous natural pigments, are associated with biological functions that include energy transduction, protection against sunlight, radical scavenging, drug resistance, and microbial virulence.⁽¹⁻⁷⁾ These materials are categorized broadly in two groups depending on their chemical composition:^(3, 7) eumelanins (dark brown to black), and pheomelanins (reddish-brown). The pigments present in the human skin, in melanoma tumors, in melanized neurons (*Substantia nigra*), and in some fungal species are all eumelanins.^(6, 8, 9) In various opportunistic pathogenic fungi including *Cryptococcus neoformans*, melanin formation is associated with virulence, thus posing problems for individuals with compromised immune systems.^(10, 11) Because of that association as well as reduced fungal cell susceptibility to polyene- and echinocandin-type drugs, melanins are a natural target for drug discovery.^(3, 9, 10) Finally, these pigments are thought to contribute to the resistance of human melanoma cells to therapeutic radiation and chemotherapy.⁽⁹⁾ The similarities between fungal (*Cryptococcal*) and mammalian melanins have been exploited to develop monoclonal antibodies for the treatment of melanoma.⁽¹²⁾ Although melanins are present in all biological kingdoms, the molecular and supramolecular structures of these amorphous biomacromolecules have remained inadequately understood because of their heterogeneous, insoluble, hydrophobic, and charged polymeric physicochemical characteristics.^(5, 6) Chemical degradation studies have led to the proposal that these pigments are phenol- or indole-based polymers.⁽⁵⁾ Conversely, several “bottom up” approaches to the biopolymer structure have used chemical synthesis of heterocyclic building blocks leading to the formation of melanin mimics.⁽¹³⁻¹⁶⁾ Nonetheless, molecular-level structural investigations of intact melanin with established solution-state spectroscopic and optical techniques are precluded by the insolubility of the brown or black pigments.^(5, 6) Analogously, their amorphous physical characteristics do not allow the use of traditional crystallographic methods to elucidate their molecular structure. Although a potent tool for atomic-level structural investigation of melanin has been found in solid-state NMR,^(5, 6, 8) investigating the molecular structure and development of the mature melanin remains an extraordinarily challenging task.^(5, 6, 17, 18) Moreover, the relationship between pathways for natural pigment formation and ultimate molecular architecture remains unclear.

Structural models proposed for synthetic eumelanins have included both an extended heteropolymer⁽¹⁷⁾ with extensive cross-linking^(19, 20) and a stack of oligomers consisting of four to seven monomers;^(18, 19, 21) the latter model is supported by X-ray diffraction^(22, 23) and atomic force microscopy.^(21, 24) Nevertheless, synthetic melanin systems may be imperfect models for the natural biopolymers, differing with respect to their intrinsic structural details.^(7, 13, 24) The molecular basis for the unique paramagnetism^(8, 17) and optical absorptivity^(7, 25, 26) of natural eumelanins has so far remained undiscovered. Our understanding of the pathways by which natural melanins are formed is limited by the paucity of structural information on well-defined intact pigments and their possible covalent binding to neighboring proteins, lipids, and carbohydrates.^(5, 6)

Notably, the present study endeavors to build an understanding of melanin biosynthesis based on well regulated *in vitro* fungal melanogenesis. The pathogenic fungus *C. neoformans* produces melanin pigments only in the presence of exogenous substrates and thereby offers unique opportunities for probing melanin structure at the molecular level.⁽³⁾ Various catecholamines such as L-dopa have been reported to be obligatory exogenous precursors for pigments that are then known as L-dopa melanins. The resulting melanin

products are deposited in the cell walls of *C. neoformans* and can be isolated by stepwise treatment with fungal cell wall-digesting enzymes, denaturants, proteinases, chloroform, and boiling hydrochloric acid.^(3, 11, 27) The recovered materials (known as melanin “ghosts” because they exist in structures that retain the shape of the cell) are then suitable for biophysical characterization.^(5, 11) Solid-state ¹³C NMR spectroscopic studies have highlighted the striking compositional differences between synthetic L-dopa melanins (prepared enzymatically or chemically from L-dopa) and naturally biosynthesized melanins.^(3, 5, 6) These observations have also prompted intensive investigation of the molecular structure of the natural pigments. The requirement of exogenous substrates for *C. neoformans* melanization provides a versatile platform to produce well-defined natural pigments that are ideal for solid-state NMR determinations of the molecular architecture and underlying melanogenesis pathways.

Our previous NMR studies have shown that, aside from the aromatic moieties, other major compositional contributions to the *C. neoformans* L-dopa melanin ghosts come from aliphatic constituents, including long-chain acylated methylene groups.^(5, 6) Structural studies of this melanin swelled in solvents revealed the presence of functional groups including alkanes, alkenes, alcohols, ketones and carboxylic acid esters.⁽⁶⁾ Moreover, glucose present in the cell growth medium was tracked biosynthetically to both polysaccharides and aliphatic chains in the fungal melanin ghosts.⁽⁶⁾ Although a preliminary picture of the L-dopa melanin molecular architecture has emerged from these studies, a comprehensive understanding of the pigment's molecular framework and synthesis pathways has remained unavailable.

Taking advantage of highly selective catecholamine-derived *C. neoformans* melanogenesis, melanins produced with a series of catecholamine substrates including L-dopa, methyl-L-dopa, (-)-epinephrine, and (-)-norepinephrine manifested considerable differences in terms of the yield, color of soluble and insoluble products, surface charge, stable free radical content, and other physicochemical characteristics of these acid-resistant particles.⁽³⁾ In the current study, solid-state NMR was used to rationalize these contrasting properties based on the molecular moieties present in the intact *C. neoformans* melanin pigments, with materials produced from the L-dopa precursor serving as a standard. By using this panel of systematically varying obligatory catecholamine precursors to produce a well-defined set of distinctive melanized fungal materials, the present investigation builds a molecular foundation for our understanding of natural melanogenesis pathways that include both the formation of indole-based biopolymers and their assembly with polysaccharide cell wall and/or membrane constituents within eumelanin particles.

Materials and Methods

Biosynthesis of Fungal Melanins

To compare the structural frameworks of various fungal melanins, the pigments were biosynthesized in the 24067 strain of *Cryptococcus neoformans* from four catecholamine precursors, namely L-dopa, methyl-L-dopa, (-)-epinephrine, and (-)-norepinephrine. The isolation of CN melanins for biophysical study was carried out following a method described previously,^(3,26) using a systematic set of chemical treatments that solubilizes cellular components other than melanin pigments to produce melanin cell ghosts.^(3,11,26) In the current study, the reproducibility of the protocol was tested by repeating the extraction process with two different batches of CN pigments produced with each of the above four catecholamine precursors. The protein content in the melanin ghosts was determined by standard methods.⁽²⁸⁾

Chemicals were purchased from Sigma Chemical Co., St. Louis, MO, unless stated otherwise. The fungal cells were grown in the presence of a 1 mM solution of an obligatory precursor in chemically defined media (29.4 mM KH_2PO_4 , 10 mM MgSO_4 , 13 mM glycine, 15 mM D-glucose and 3 μM thiamine) for 10 days at 30 °C in a rotatory shaker at 150 rpm. The cell pellets were obtained by centrifugation at 2000 rpm. After washing with phosphate buffered saline (PBS), the isolated fungal cells were suspended in 1.0 M sorbitol / 0.1 M pH 5.5 sodium citrate solutions and incubated with 10 mg/mL lysing enzymes (from *Trichoderma harzianum*, Sigma) for 24 h at 30 °C to remove cell walls. The resulting solution was centrifuged at 2000 rpm for ten minutes and the pellet (melanized protoplasts) was washed several times with PBS until the supernatant was nearly clear. A 20 mL aliquot of 4.0 M guanidine thiocyanate was added to form a suspension that was incubated for 12 h in a rocker (Shaker 35, Labnet, Woodbridge, NJ) at room temperature to denature proteinaceous components. The cell debris was collected and washed 2-3 times with ~20 mL PBS and suspended in 5 mL proteinase K incubation buffer (10 mM Tris-HCl, pH 8.0, 5 mM CaCl_2 , 5% SDS) with 1 mg/mL proteinase K (Boehringer Mannheim, Germany) and then incubated for 4 h at 65 °C. The recovered cell debris was washed 2-3 times with ~20 mL PBS and then subjected to lipid extraction using the Folch method⁽²⁹⁾ while maintaining the proportions of chloroform, methanol and saline solution in the final mixture as 8:4:3. After repeating the delipidation procedure three times, the final product was suspended in 20 mL of 6 M HCl and boiled (at 100 °C) for one hour to hydrolyze cellular contaminants associated with melanin. The black particles of interest that survived after HCl treatment were dialyzed against distilled water. The resulting melanin particles ('ghosts') were lyophilized for further biophysical analysis. In selected experiments, [$^{13}\text{C}_6$]-glucose was used as a sugar source.

Solid-state NMR experiments

^{13}C cross-polarization magic-angle spinning (CPMAS) NMR measurements were performed at a typical 15 kHz (± 20 Hz) spinning speed and with a recycle delay of 3 s between successive acquisitions to observe resonances from the various carbon moieties. Ramped-amplitude cross-polarization,⁽³⁰⁾ in which the proton field strength was varied linearly by ~20-50%, was implemented to compensate for inhomogeneous radiofrequency fields across the sample and improve spectral observation of melanin moieties with varying molecular mobilities. Typical 1-2 ms ^1H spin-lock times were used to transfer magnetization from ^1H to ^{13}C nuclear spin baths, and high-power heteronuclear proton decoupling (90-185 kHz) was achieved using the TPPM or SPINAL composite pulse sequences.^(31, 32) High-fidelity ^{13}C direct polarization (DPMAS) experiments were conducted at typical 15 kHz spinning speeds, with 100 s delays between successive acquisitions. Typically, 24000-36000 and 1500-2000 transients were collected for CPMAS and DPMAS experiments, respectively, on natural abundance melanin samples. Solid-state ^1H MAS NMR measurements were conducted at a typical 35 kHz (± 20 Hz) spinning speed with a recycle delay of 6 s between successive acquisitions and recording of 32-128 transients.

A Varian VNMRS NMR spectrometer operating at a ^1H frequency of 600 MHz and equipped with an HCN fastMAS probe (Agilent Technologies, Santa Clara, CA) was used to carry out ^{13}C CPMAS, ^{13}C DPMAS, and ^1H MAS experiments on the pigments (1-4 mg of powdered samples). Additional CP measurements were conducted on Bruker spectrometers (Bruker BioSpin Corp., Billerica, MA) operating at ^1H frequencies of 750 MHz (Avance II) and 900 MHz (Avance III), both equipped with 4 mm HXY probes. The reproducibility of the spectroscopic measurements was assessed by (1) obtaining replicate CPMAS spectra at 600 MHz and one additional spectrometer frequency; and (2) collecting spectra at two different rotor spinning frequencies (10 and 15 kHz). All spectral datasets were processed

with 50-200 Hz of line broadening; chemical shifts were referenced externally to the methylene ($-\text{CH}_2-$) group of adamantane (Sigma) at 38.48 ppm.⁽³³⁾

Results

Shown in Figure 1 are CPMAS ^{13}C NMR spectra for *C. neoformans* melanins synthesized in the presence of L-dopa, methyl-L-dopa, (-)-norepinephrine and (-)-epinephrine, illustrating the distinctive spectral fingerprints displayed by each of the resulting pigments. To facilitate structural comparisons among the biopolymers obtained from the four catecholamine substrates, the molecular profile of fungal melanin made with the customary exogenous L-dopa precursor was taken as a standard.

The CPMAS ^{13}C spectrum of that pigment is notably different from the L-dopa substrate in terms of peak positions and line widths, demonstrating metabolic transformation of the precursor. As noted in our earlier report,⁽⁵⁾ the ^{13}C spectrum of *C. neoformans* L-dopa melanin shows not only the expected aromatic and/or olefinic carbon resonances (110-160 ppm), but also signals attributable to long-chain aliphatic methylene groups ($(\text{CH}_2)_n$, 20-40 ppm), oxygenated aliphatic carbons (CH_nO , 60-80 ppm), and carboxyls (COO , 170-173 ppm). The appearance of 30 ppm, 24 ppm, and 15 ppm resonances constitutes a pattern typical of $(\text{CH}_2)_n\text{CH}_2\text{CH}_3$ alkyl chains.

To optimize spectral resolution and sensitivity, the CPMAS ^{13}C NMR data were collected at various magnetic field strengths, rotor spinning frequencies, and temperatures. Modestly better spectral resolution was obtained at higher magnetic fields; both aliphatic and carboxyl resonances were somewhat more prominent in the ^{13}C NMR spectra at higher spinning speeds; the overall signal-to-noise ratio improved at lower temperatures.

In the L-dopa melanin spectrum, the broad appearance of the aromatic/olefinic region could arise from proximity of particular aryl groups to unpaired electrons, structural disorder typical of an amorphous material, and/or overlap of signals from slightly dissimilar polymeric building blocks. It is possible to rule out this degree of broadening for the individual resonances, because numerous resolved aromatic signals are present in the 110-150 ppm region of the ^{13}C NMR spectra obtained at higher magnetic field strengths (Supplemental Figure S1) and for previously produced melanins from $[2,3-^{13}\text{C}_2]$ -L-dopa and $[\text{ring-}^{13}\text{C}_6]$ -L-dopa substrates.^(5, 6) Whereas NMR results reported previously for the fungal melanin produced with $[\text{U-}^{13}\text{C}_6]$ -L-dopa support preservation of the intact phenyl ring, analysis of the pigment derived from $[2,3-^{13}\text{C}_2]$ -L-dopa indicates cyclization of most sidechain carbons into the an indole-type aromatic structure. Although a single indole-like moiety itself would display sharp resonances, the broad aromatic spectral features of the fungal melanin support conversion of the L-dopa precursor to an indole-based polymer with a variety of aromatic inter-unit covalent connection patterns.^(6, 8) Moreover, the observation of new melanin ghost signals at 25-85 ppm implicates aliphatic structural elements that were not present in the corresponding L-dopa precursor.

Analogous comparisons of aromatic/olefinic regions of the ^{13}C spectra of methyl-L-dopa, norepinephrine, and epinephrine with their respective pigments demonstrate metabolic transformation of the various precursors, respectively, into structurally different polymeric forms (Figure 1). For methyl-L-dopa, the broad aromatic spectral envelope centered at 128 ppm is attributed to conversion of the precursor ring and sidechain to heterogeneous indole-based aromatic structures, much as observed for L-dopa. Conversely, the absence of prominent spectral features between 110 and 160 ppm for the biopolymers produced with epinephrine and norepinephrine demonstrates a significant divergence in biosynthetic pathways with respect to the L-dopa melanin standard. This absence of prominent

unsaturated carbon moieties could arise from cleavage of the precursor aromatic ring structures, 'bleaching' (broadening) by free radicals present in norepinephrine melanin samples, or formation of soluble materials that are removed during isolation of the melanin ghosts (presented below).

In the aliphatic spectral region, the methyl-L-dopa substrate again displays similar transformations to L-dopa: the sharp aliphatic signals (20-65 ppm) are replaced in the biosynthesized pigment by relatively broad resonances from oxygenated and long-chain aliphatic carbons (COO, CH_nO, (CH₂)_n). The strong pigment signals at 172 ppm could originate from cellular components other than the catecholamines, particularly for epinephrine and norepinephrine precursors that lack carboxyl groups. To explore this possibility, the metabolic fate of glucose in the growth medium was tracked independently via the ¹³C NMR spectrum of L-dopa melanin produced with [U-¹³C₆]-glucose, which was dominated by COO (carboxyl), C=C (unsaturated carbons), CH_nO, and (CH₂)_n (bulk methylene) resonances derived from the sugar (Figure 2, top). Those resonances were then identified in the spectra of L-dopa melanin produced with unlabeled glucose (Figure 2, middle) and of both norepinephrine and epinephrine melanins produced with [U-¹³C₆]-glucose (Supplemental Figure S2). Moreover, the aliphatic resonances in materials derived from all four precursors display similar behavior when the CPMAS ¹³C NMR spectra are acquired with delayed proton decoupling:⁽³⁴⁾ resonances from rigid protonated carbons in the oxygenated aliphatic region (50-90 ppm) are attenuated, and both nonprotonated carboxyl carbons (172 ppm) and highly mobile long chain aliphatic groups (20-40 ppm) are retained (Supplemental Figure S3).

The ¹³C NMR spectra also made it possible to compare the content of major functional groups in each of the four melanin ghosts. DPMAS ¹³C NMR data (Supplemental Figure S4) confirmed the provisional observations from CPMAS spectra and provided quantitative estimates of the aliphatic-to-aromatic ratios in L-dopa melanin and methyl-L-dopa melanin ghosts from integrated signal intensities in their 0-50 and 90-160 ppm regions, respectively. As compared with the ratio for the L-dopa standard (0.60±0.02), the methyl-L-dopa melanin ghosts have fewer aliphatics (0.29±0.02). Moreover, the predominance of pigment constituents in the melanin ghosts recovered from CN cells was established, in analogy with prior reports for hair melanins⁽³⁵⁾, through aromatic-to-carbonyl ratios derived from peaks centered at 129 and 170 ppm, respectively. The ratios were estimated as 7.2 and 5.4, respectively, for the L-dopa and methyl-L-dopa CN melanins produced in the 24067 strain and 6.4 for L-dopa melanin from the H99 cell line, ruling out significant spectral contributions from associated proteins.

Finally, the contrast between insoluble melanins produced with L-dopa and epinephrine was sharpened by examination of their ¹H MAS NMR spectra (Figures 3 and S5). Whereas the L-dopa and methyl-L-dopa ghosts exhibited a broad aromatic envelope (4-8 ppm) and sharp chain methylene resonances (~ 2 ppm) analogous to the ¹³C CPMAS spectra, epinephrine and norepinephrine produced insoluble products with ¹H spectra dominated by sharp aliphatic resonances. These variations in the proportion of aromatic moieties are likely to depend on the polymerization pathway, efficacy of pigment bonding to cell-wall constituents, and surface charge and paramagnetic properties of the particles.

Discussion

Structural Basis of Catecholamine Melanin Physical Characteristics

We reported previously that *C. neoformans* melanins generated *in vitro* with a panel of four catecholamine substrates (L-dopa, methyl-L-dopa, norepinephrine, and epinephrine) had distinctive yield, color of soluble and insoluble products, surface charge, stable free radical

content, and other physical features.⁽³⁾ With solid-state ^{13}C NMR profiles including both aromatic/alkene and aliphatic components of the intact fungal pigments now in hand, it is possible to interpret this physicochemical diversity of catecholamine-derived pigments in terms of their molecular-level structural differences. For instance, L-dopa and methyl-L-dopa generated black colored cells and melanin ghosts, whereas epinephrine and norepinephrine produced smaller amounts of lightly pigmented (tan or brown) cells and ghosts.⁽³⁾ These latter types of ghost coloration, which indicate diminished multi-wavelength light absorption capability, can be correlated with the greatly diminished proportions of aromatic components evidenced in NMR spectra of the pigments derived from epinephrine and norepinephrine as compared to L-dopa and methyl-L-dopa. The lower aromatic-to-aliphatic ratios also correlate with thinner “ghost” shells observed by transmission electron microscopy for the pigments generated from norepinephrine and epinephrine, in comparison with L-dopa and methyl-L-dopa.⁽³⁾

As noted above, another distinguishing feature among fungal melanins produced with the four catecholamine substrates concerns the presence of paramagnetic centers and concomitant EPR activity.^(3, 27) The insoluble melanin ghosts from the cell-wall associated pigments made with L-dopa, methyl-L-dopa and norepinephrine exhibited EPR activity, whereas the pigment produced with epinephrine had no significant EPR signal under the same experimental conditions.³ Thus, it is possible to associate strong EPR activity with the presence of prominent aromatic resonances in solid-state ^{13}C NMR spectra, i.e., to link the presence of aromatic character in the insoluble fungal melanin ghosts to their paramagnetic properties. However, the indolequinone and semiquinone intermediates thought to be responsible for the EPR activity of the mature pigment^(2, 8, 17, 36) are not evidenced in our NMR spectra, which lack resonances at 185 ppm even for isotopically enriched L-dopa melanins.^(5, 6)

Finally, we sought a molecular rationale for the (negative) surface charge of various ghost particles, which controls their ability to form suspensions in aqueous solutions⁽³⁾ and has been attributed in part to the formation of polysaccharide capsules.⁽³⁷⁾ Zeta potential measurements of melanized cells produced with the catecholamine substrates showed significantly larger negative charges relative to non melanized controls, with substantial negative surface charge measured for all four catecholamine-derived melanin ghosts.⁽³⁾ Since both aliphatic and aromatic moieties appeared in ^{13}C NMR spectra of the various pigment assemblies, the surface charge could arise from either or both types of constituents. However, the finding of negative surface charge for all four catecholamine melanins implicates the polar oxygenated aliphatic molecular structures that are common to all of their melanin ghosts.

Origin and Development of Aliphatic Components in Melanized Fungal Cells

Although melanin biosynthesis has been portrayed as proceeding via oxidative polymerization of L-dopa, our spectroscopic analyses underscore the incomplete nature of this description in fungal cellular media. Along with biochemical studies^(3, 10, 37), scanning electron microscopy⁽³⁸⁾ revealed a close association between polysaccharide capsules and deposited melanin particles in the fungal cell walls^(38, 39). Notably, the higher aromatic-to-carbonyl NMR peak ratios and considerably lower cross-polarization efficiency for aromatics in ^{13}C CPMAS indicate the absence of any significant proteinaceous residues in the recovered L-dopa and methyl-L-dopa melanin ghosts. In addition to the expected ^{13}C NMR resonances from indole-based biopolymers, substantial aliphatic spectral contributions are evident for *C. neoformans* ghosts derived from all four catecholamine precursors (Figure 1) – a general observation that encompasses even insoluble epinephrine- and norepinephrine-based materials that lack prominent aromatic structural components.

Likewise, aliphatic moieties were observed for hair^(35, 40, 41), sepioid^(8, 41) and neuromelanins.⁽⁴²⁾

These findings indicate tight association or covalent bonding of the pigments to other cellular materials, which are therefore retained after the enzymatic and chemical treatments used to produce melanin ghosts.⁽⁴⁻⁶⁾ Thus, it is imperative to delineate the contributions of cellular constituents other than obligatory catecholamines to the production of insoluble materials. Using [U-¹³C₆]-glucose to obtain NMR spectra of L-dopa melanin such as that illustrated in Figure 2, the polysaccharide, chain methylene and chain methine resonances (20-80 ppm; 128 ppm) are shown to arise primarily from glucose metabolites in the chemically defined media used to grow the fungal cells with the obligatory L-dopa⁽⁶⁾ precursor. The unsaturated carbons appearing at 128 ppm are contained in fatty acyl chains, as evidenced by ¹H-¹³C multiple-bond connections reported in high-resolution MAS NMR spectra of melanins swelled in DMSO.⁽⁶⁾ Possible proteinaceous materials^(35, 43) derived from glucose are ruled out spectroscopically by the absence of prominent peaks in the 155-158 ppm spectral region for either natural abundance or isotopically enriched L-dopa melanins.

This hypothesis regarding the origin of the aliphatic constituents in the catecholamine-derived CN melanins is supported and extended by two observations reported herein: (1) concordance of [U-¹³C₆]-glucose derived L-dopa melanin NMR signals with numerous aliphatic features found in all unenriched (¹²C) pigment samples; (2) similar nonprotonated and mobile protonated aliphatic carbons identified by delayed decoupling ¹³C NMR⁽³⁴⁾ for both [U-¹³C₆]-glucose derived L-dopa melanin (Supporting Figure S3) and pigments made with each of the four ¹²C precursors (not shown). Additionally, a small portion of [2,3-¹³C₂]-L-dopa has been shown to contribute to *C. neoformans* melanin aliphatic signals at 30-40 ppm.⁽⁵⁾ Identification of the molecular moieties involved in pigment interactions with the cell wall or lipid membrane materials is ongoing in our laboratories.

Our results also highlight an important dependence of pigment – cell associations on the choice of fungal growth media, as observed occasionally for different batches of *C. neoformans* melanin produced in the 24067 strain and illustrated in Figure 2 with H99 cells. Compared to L-dopa-melanin synthesized in the standard 24067 strain, this latter pigment was isolated in greater amounts but also displayed a different pattern of resonances in the oxygenated aliphatic region (CH_nO, 40-80 ppm) of the ¹³C NMR spectrum. Since the same L-dopa obligatory precursor and isolation protocols were used to generate both melanins, the final products should maintain similar structural characteristics. Additionally, the exhaustive chemical treatments used to isolate the melanin ghosts will remove any contaminations or loosely associated components, leaving only the strongly attached chemical moieties. Accordingly, the altered appearance of oxygenated aliphatic carbon spectral features indicates variations in metabolic pathways, degree of polymerization, and/or deposition of the pigment within the fungal cellular materials.

Pathways for Melanin Biosynthesis

The results obtained herein for *C. neoformans* melanogenesis using exogenous substrates of systematically varying chemical structure allowed us to develop a mechanistic rationale for molecular differences among the resulting pigments. This organism exhibits several notable biosynthetic features as compared with other melanotic pathogenic fungi such as *Wangiella dermatitidis* and *Aspergillus spp.* that synthesize melanin-like pigments from endogenous substrates.^(44, 45) Laccases, a class of copper-containing oxidase enzymes for which expression and activity are regulated by numerous genes, catalyze melanin synthesis in *C. neoformans*.^(3, 27, 46) Incubating the fungus with phenolic substrates leads to melanization via laccase reactions that can oxidize *p*- or *o*-diphenols, yielding contrasting products.⁽⁴⁷⁻⁴⁹⁾

The oxidation of *o*-diphenols (phenol rings with hydroxyl groups in the 2,3- or 3,4-positions) results in the deposition of insoluble pigment in the cell wall. Conversely, the oxidation of *p*-diphenols (phenol rings with hydroxyl groups in the 1,4- and 2,5- positions) produces soluble pigments that diffuse into the medium.⁽⁴⁷⁻⁴⁹⁾ By this reasoning, all four catecholamine (*o*-diphenol) substrates used herein should produce insoluble *C. neoformans* pigments.

What roles are indole-based intermediates expected to play in pigment formation? Several biosynthetic schemes have been proposed in which eumelanins are composed of indolic monomers such as 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA).⁽⁷⁾ For example, oxidative degradation analyses of *Sepia* melanin suggested that the pigment is a copolymer containing DHI (~25%) and DHICA (~75%) as primary structural units.⁽⁸⁾ In the Mason-Raper scheme for *C. neoformans* melanization (Figure 3), formation of indole-based polymers is initiated by oxidative cyclization of the L-dopa precursor,⁽⁶⁾ which leads to the formation of an indole-based dopachrome intermediate.^(7, 50) Subsequently, dopachrome undergoes either decarboxylation to form DHI^(7, 50) or enzyme-catalyzed ketoenol tautomerization to produce DHICA.⁽⁵¹⁾ Both DHI and DHICA, as indole-like intermediates, are then thought to produce melanin via oxidative polymerization.^(7, 13, 50) Experimental support for such cyclic aromatic building blocks has come from solid-state NMR spectra of *C. neoformans* melanin ghosts formed from selectively ¹³C-enriched L-dopa obligatory precursors such as [2,3-¹³C₂]-L-dopa and [ring-¹³C₆]-L-dopa.^(5, 6) Therefore, our working hypothesis is that successful pigment formation is driven by formation of a stable aromatic indole-based intermediate and subsequent polymerization.

The generality of this mechanistic framework has been tested against our melanogenesis results for four structurally related catecholamines. As compared with L-dopa melanin, the similar cellular pigmentation⁽³⁾ and molecular architecture evidenced by solid-state ¹³C NMR spectra of the insoluble melanin ghosts from methyl-L-dopa (Figure 1) suggest that the latter structural precursor is polymerized oxidatively in *C. neoformans* via an analogous indole-like cyclic intermediate such as α -methyl-L-dopachrome (Figure 4).⁽⁵²⁾ As *o*-diphenols, epinephrine and norepinephrine could undergo an oxidative cyclization process comparable to that proposed for synthetic eumelanins,^(53, 54) so these two latter catecholamine substrates would be anticipated to follow an analogous ring-closing pathway to yield indole-like intermediates.^(53, 54) However, *C. neoformans* melanization with norepinephrine and epinephrine precursors did not yield strong aromatic resonances in their solid-state ¹³C or ¹H NMR spectra (Figures 1, S1, and 3).

In the context of the proposed mechanism, the putative intermediates formed by the norepinephrine and epinephrine ring closures would lack the stability conferred by electron delocalization in the aromatic indole-based intermediates (Figure 4) or corresponding tautomeric structures^(44,45) that can lead to the formation of fused indole-based polymeric networks. Additionally, the entropically favorable decarboxylation of dopachrome (or α -methyl-L-dopachrome), which form DHI (or α -methyl-DHI) building blocks of the melanin heteropolymer,⁽⁵¹⁾ are precluded for norepinephrine and epinephrine starting materials. Finally, the formation of stable aromatic structures such as DHI from the latter two putative fused-ring intermediates (Figure 4) would require energetically expensive steps: removal of a hydroxyl group from either of the five-membered nitrogen-containing rings or cleavage of an N-C bond in the epinephrine intermediate. As an alternative pathway that competes with indole cyclization and formation of cross-linked aromatic units, the intrinsically more reactive *o*-quinone forms of the epinephrine and norepinephrine catecholamines may react with thiol or amine nucleophiles in the cellular media.⁽⁵⁵⁾ By this reasoning, a mechanistic framework based on the Mason-Raper scheme⁽⁵⁰⁾ can provide a rationale for the

development of the indole-based macromolecular networks of the *C. neoformans* melanin ghosts from these four obligatory catecholamine precursors. Beyond the previously noted specification of o-diphenol structures, the precursors of these fungal melanins may thus require a sidechain that cyclizes to form a stable indole-type intermediate which can promote subsequent polymerization and development of aromatic structural networks.

To complete our mechanistic picture of *C. neoformans* melanin formation, it is necessary to augment the Mason-Raper pathway with information about the glucose- and L-dopa-derived aliphatic constituents of the insoluble acid-resistant melanin ghosts. Stated differently, the heterogeneous structural profile of fungal pigments comprised of diverse aromatic and aliphatic constituents shows that simple extended or aggregated polymeric structural models based on the study of synthetic melanin^(7, 13, 24) are not sufficient to describe the molecular architecture of natural fungal pigments. As reported previously for L-dopa melanin^(5, 6, 8) and underscored for several catecholamine precursors herein, glucose supplied as the sole sugar source in the fungal cell medium is incorporated into the polysaccharide cell walls and long-chain acylated aliphatic structures of the resulting melanin ghosts.^{6,(56)} To resist enzymatic and acid degradation, these aliphatic materials must be tightly associated with or covalently linked to the main aromatic structure of the melanin ghosts.⁽³⁸⁾ Additionally, a minor fraction of the L-dopa precursor is converted biosynthetically to acid-resistant aliphatic structures,^(5, 6, 8) indicating an alternative metabolic pathway that could account for the small amounts of insoluble materials⁽³⁾ containing aliphatic moieties produced from norepinephrine and epinephrine substrates. Taken together, the NMR-based structural information obtained for intact catecholamine-derived pigments in the solid state provides vital insight into *in vitro* melanogenesis pathways of *C. neoformans*.

Conclusions

By monitoring the metabolic fates of a group of structurally related obligatory catecholamine precursors with solid-state ¹³C NMR of the resulting *C. neoformans* melanin ghosts, it was possible to redefine the molecular prerequisites for the formation of pigments with contrasting physical and consequent physiological characteristics. The structural resemblance between fungal melanin ghosts obtained with L-dopa and methyl-L-dopa supports common cyclization mechanisms for their aromatic (indole-based) domains and close association of the resulting polymers with aliphatic cellular constituents. Analogous metabolic transformations are precluded for norepinephrine and epinephrine substrates, for which the failure to produce insoluble indole-based pigment networks is indicated by the absence of strong aromatic resonances in their respective solid-state NMR spectra and can be rationalized within the Mason-Raper conceptual framework proposed for melanin synthesis. Nonetheless, an understanding of *in vitro C. neoformans* melanogenesis is also informed by consideration of closely associated polysaccharide and fatty acyl constituents that originate from both glucose in the cellular growth medium and from the catecholamine precursors. The present study identifies the factors controlling the development and molecular architecture of this complex pigment-cell assembly, prompting future efforts to identify the functional groups that link them and ultimately guiding drug discovery efforts related to eumelanin associated virulence and resistance to tumor therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
CPMAS	cross polarization magic-angle spinning
DPMAS	direct polarization magic-angle spinning

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Figure 1. 150 MHz CPMAS solid-state ^{13}C spectra of catecholamine precursors and the resulting *C. neoformans* melanins obtained by *in vitro* biosynthesis. Chemical shift differences among phenol carbons in the precursors (~140-150 ppm) are attributed to variations in hydrogen-bonding networks and crystal packing in the solid state.

Figure 2.

Solid-state CPMAS ¹³C spectra of L-dopa *C. neoformans* melanins produced, as noted, with different isotopically enriched glucose sources and cell lines. The ¹³C spectra were acquired at 188 MHz (top and middle) and at 225 MHz (bottom). The resonances at 128 ppm are normalized to one another.

Figure 3. Solid-state MAS ^1H spectra of *C. neoformans* (CN) melanins produced from natural abundance L-dopa, methyl-L-dopa, norepinephrine and epinephrine, acquired at a ^1H frequency of 600 MHz. A spinning rate of 35 kHz was used to acquire spectra for all materials except the norepinephrine melanin, which was examined at 10 kHz.

Figure4.
Proposed intermediates for biosynthesis of *C. neoformans* melanins from obligatory catecholamine substrates.