Melanogenesis in *Cryptococcus neoformans*

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Melanogenesis in *Cryptococcus neoformans* begins with the oxidation of dihydroxyphenylalanine by the enzyme phenol oxidase. The succeeding steps are very rapid. Two intermediates, dopachrome and 5,6-dihydroxyindole, have been isolated and characterized by high performance liquid chromatography. A pathway of melanin formation in *C. neoformans* is proposed, based on the presence of these intermediates.

INTRODUCTION

Cryptococcus neoformans is a pathogenic fungus which grows preferentially in the central nervous system. It causes a disease that has a high rate of fatality among the mycoses, ranging from 19 to 37% (Butler *et al.*, 1964; Hammerman *et al.*, 1974). Recent reports have shown that cryptococcosis is frequent among patients with acquired immuno-deficiency syndrome (AIDS). In a recent survey, 8.6% of AIDS victims in Western countries were found to develop cryptococcosis (Zuger *et al.*, 1986) and in African AIDS patients, it is the most commonly observed opportunistic infection (Quinn *et al.*, 1986).

C. neoformans is the only true pathogen among 19 species described in the genus *Cryptococcus* (Rodrigues de Miranda, 1984). It is unique among members of the genus in that it grows well at 37 °C and produces a melanin like pigment when grown on diphenolic compounds (Staib, 1962). The latter property is used extensively as the basis for the identification of this yeast (Cooper & Silva-Hutner, 1985). The enzyme responsible for melanogenesis has been identified as membrane-bound phenol oxidase and has been studied in detail by Polacheck *et al.* (1982). Melanin formation was demonstrated as an essential virulence factor in *C. neoformans* (Rhodes *et al.*, 1982). A special histopathological stain showed that melanin was produced by this fungus in the brain (Kwon-Chung *et al.*, 1981; Ro *et al.*, 1987). In this paper we report on a possible pathway for melanin formation in *C. neoformans*.

Melanin biosynthesis from dihydroxyphenylalanine (DOPA) in *C. neoformans* proceeds through a series of oxidation–reduction reactions. There is strong evidence that the first step of the pathway (DOPA→dopaquinone) is catalysed by the enzyme phenol oxidase (Polacheck *et al.*, 1982). The subsequent reactions in melanogenesis according to the classical Mason–Raper scheme occur extremely rapidly (Mason, 1955). The only two intermediates that are relatively stable in this scheme are dopachrome and 5,6-dihydroxyindole. In order to examine whether these compounds are intermediates of melanogenesis in *C. neoformans*, the products of the cryptococcal phenol oxidase reaction were analysed by high performance liquid chromatography (HPLC).

Abbreviation: DOPA, dihydroxyphenylalanine.

METHODS

Synthesis of dopachrome and 5,6-dihydroxyindole. Since these compounds are unstable, and can be spontaneously auto-oxidized, they are not commercially available. Dopachrome was synthesized by the exposure of DOPA or ^{14}C -labelled DOPA to silver oxide as described by Körner & Pawelek (1980). 5,6-Dihydroxyindole was prepared from dopachrome by reduction with 2-mercaptoethanol (2-ME). Dopachrome (0.75 mg), 0.5 M-sodium phosphate buffer pH 8 (250 μl) and 20 mM-2-ME (100 μl) were incubated in a final volume of 1 ml for about 30 min at 37 °C until colourless. 5,6-Dihydroxyindole was stable on ice for at least 8 h. Both dopachrome and 5,6-dihydroxyindole were detected by HPLC on an octadecyl silane (ODS) column, as previously described (Körner & Pawelek, 1980; Pawelek *et al.*, 1980) and by their respective absorbance maxima: 305, 475 nm; 275, 298 nm.

Detection of dopachrome and 5,6-dihydroxyindole. Melanogenesis products were analysed by HPLC. Samples (50 μl) were injected into a high pressure liquid chromatograph (Waters Associated). The absorbance of the eluents was monitored at 254 and 280 nm. The separations were done with a Zorbax ODS column (6.2 mm $\times$ 25 cm, DuPont Instruments). The elution was done with a 40 min concave gradient no. 3 from sodium phosphate buffer (50 mM, pH 6.5) to 100% (v/v) methanol. The flow rate was 0.8 ml min $^{-1}$. The eluent was collected in 1 ml fractions with an automatic fraction collector. The retention times of authentic samples of dopachrome and 5,6-dihydroxyindole were also determined. The radioactivity of the fractions was measured in a Beckman scintillation counter, and their absorbance maxima were determined by scanning spectroscopy from 150 to 500 nm. The use of a concave gradient instead of the linear gradient used by others (Körner & Pawelek, 1980; Pawelek *et al.*, 1980) allowed improved separation between DOPA, dopachrome and 5,6-dihydroxyindole.

Phenol oxidase preparation and assay conditions. These were as described previously (Polacheck *et al.*, 1982). The reaction mixture contained 1 mM-L-DOPA (Sigma) or 50 μCi L-[3- ^{14}C]DOPA (specific activity 58 mCi mmol $^{-1}$,

Fig. 1. HPLC analysis of the products of the phenol oxidase reaction after various incubation periods. DOPA (1 mM) was incubated with cryptococcal phenol oxidase; at 0 (a), 15 (b), 30 (c) and 60 (d) min, samples were taken for analysis by HPLC. The 15, 19 and 29 min peaks represent DOPA, dopachrome and 5,6 dihydroxyindole, respectively, as determined by comparison with authentic samples.

0.215 GBq mmol⁻¹; Amersham) ml⁻¹, 0.05 M-phosphate buffer pH 6.5 and 50 µl of enzyme in a total volume of 1 ml. The mixture was incubated at 25 °C for up to 3 h. Samples of 100 µl were removed periodically, filtered through a 0.2 µm disposable filter to remove any melanin that had formed, and analysed for dopachrome and 5,6-dihydroxyindole content by HPLC as described above. Appropriate controls containing 10 mM-NaCN or boiled enzyme were tested simultaneously.

RESULTS

Analysis of the intermediates of melanogenesis by HPLC

The products formed during melanogenesis were analysed by HPLC after various periods of incubation of DOPA with phenol oxidase. Authentic samples of DOPA, dopachrome and 5,6-dihydroxyindole were run in parallel with each experiment; these had retention times of 15, 19 and 29 min, respectively (data not shown). The production of dopachrome and 5,6-dihydroxyindole from DOPA, assayed by continuous monitoring at 280 nm, is shown in Fig. 1. Monitoring at 254 nm gave the same results (data not shown). The large off-scale peak of DOPA (15 min) decreased while the peaks of dopachrome and 5,6-dihydroxyindole (19 and 29 min, respectively) increased during the incubation of DOPA with the phenol oxidase. All three peaks were clearly visible at 60 min (Fig. 1*d*). The absorbance maxima of each product were measured after elution from the column. The 19 min peak showed absorbance maxima at 305 and 475 nm, the same as the values obtained with the authentic dopachrome standard. The 29 min peak showed absorbance maxima at 275 and 298 nm, identical to those determined for the authentic 5,6-dihydroxyindole standard.

In another experiment radiolabelled DOPA was used as the substrate and the incubation lasted for 3 h (Fig. 2). An authentic sample of radiolabelled DOPA eluted after 16 min. The DOPA peaks disappeared with increasing incubation time, while those of dopachrome and 5,6-dihydroxyindole (19 and 29 min respectively) increased. The radioactivity peaks for the authentic radiolabelled standards used in each experiment (data not shown) corresponded exactly with the radioactivity peaks for the unknowns. When appropriate controls containing NaCN or boiled enzyme were tested, neither a decrease in the DOPA peak nor an increase in the

Fig. 2. Effect of cryptococcal phenol oxidase on DOPA. ¹⁴C-labelled DOPA (1 mM) was incubated with the cryptococcal phenol oxidase for 3 h. The products of the reaction were analysed by HPLC as described in the text. The peaks at 19 and 29 min represent dopachrome and 5,6-dihydroxyindole, respectively. (—), *A*₂₈₀; (×—×), radioactivity.

Fig. 3. Possible scheme for melanogenesis in *C. neoformans*.

peaks of dopachrome and 5,6-dihydroxyindole were observed (data not shown). This indicates that the production of these melanin precursors under the assay conditions was due to the phenol oxidase activity.

Colour changes during melanogenesis

We observed systematic colour changes during the oxidation of DOPA to melanin [red/orange $\rightarrow$ yellow (occasionally) $\rightarrow$ purple $\rightarrow$ black]. According to Mason (1955) red corresponds to dopachrome with absorbance maxima at 305 and 475 nm, yellow to indole-5,6-quinone and purple to melanochrome (maxima at 300 and 540 nm), while melanin has general absorbance and is black.

DISCUSSION

These data led us to suggest the scheme shown in Fig. 3 for melanogenesis in *C. neoformans*. This scheme is modified slightly from that suggested for melanin biosynthesis in melanoma cells (Körner & Pawelek, 1980; Pawelek *et al.*, 1980) in that it starts from DOPA instead of tyrosine, which cannot be used as a substrate for the cryptococcal phenol oxidase. Both schemes are based on the classic Mason–Raper scheme for melanogenesis (Mason, 1955). According to our proposed scheme an enzyme is needed only for the oxidation of DOPA to dopaquinone. It is generally assumed that the amount of active phenol oxidase is the major rate-limiting factor in

this process (Polacheck *et al.*, 1982). The succeeding steps can occur spontaneously through autoxidation (Körner & Pawelek, 1980; Pawelek *et al.*, 1980; Mason, 1955).

Although we have indicated the presence of the two melanin pathway intermediates (Polacheck *et al.*, 1982), this is the first detailed presentation of melanogenesis in *C. neoformans*. The present description of the melanin pathway was based on HPLC analysis, typical absorbance maxima of the intermediates and sequential colour change.

It is apparent that many of the labile intermediates have not yet been detected due to their extreme instability or their low concentration. However, we believe that the modified Mason–Raper scheme represents a preferred pathway of melanin synthesis in *C. neoformans*. This pathway is fundamentally different from the pentaketide pathway, which is known to occur in dematiaceous human pathogenic fungi that, like *C. neoformans*, are considered as neurotropic organisms (Geis *et al.*, 1984; Taylor *et al.*, 1987).

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