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Purification and properties of cytochrome *P*-450-dependent 14α-sterol demethylase from *Candida albicans*

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The purification of cytochrome *P*-450-dependent 14α-sterol demethylase (*P*-450$_{DM}$) from the important opportunistic fungal pathogen, *Candida albicans*, is described. Optimal purification (875-fold) was achieved by extracting the cytochrome from microsomes with sodium cholate followed by hydroxyapatite, octyl-Sepharose and CM-Sepharose chromatographies, giving a cytochrome preparation of 17.5 nmol/mg of protein. By the use of SDS/polyacrylamide-gel electrophoresis the cytochrome was judged to be highly purified on the basis of Coomassie Brilliant Blue staining of protein. The M_r of *P*-450$_{DM}$ was estimated to be 51000. The absorption spectrum of oxidized *P*-450$_{DM}$ was characteristic of a low-spin cytochrome, and its reduced CO complex had a Soret absorption peak at 447 nm. When reconstituted in a model membrane system of dilauroylphosphatidylcholine with NADPH and O_2, *P*-450$_{DM}$ catalysed the complete 14α-demethylation of lanosterol, which was inhibited by CO. The cytochrome appeared to have a high degree of substrate specificity; it was unable to oxidize a number of xenobiotic compounds in the reconstituted assay.

INTRODUCTION

The azole (*N*-substituted imidazole or triazole) antifungal antibiotics have been used with considerable success in the treatment of superficial mycoses (Fromtling, 1988). However, the toxicity of these compounds has hampered their use in the treatment of deep-seated mycoses and life-threatening systemic infections (Hay, 1985; Fromtling, 1988). The primary mode of action of the azoles is probably the inhibition of cytochrome *P*-450-dependent 14α-sterol demethylase (*P*-450$_{DM}$; Vanden Bossche, 1985), a key enzyme in ergosterol (ergosta-5,7,22-trien-β-ol) biosynthesis in fungi and cholesterol (cholest-5-en-3β-ol) biosynthesis in mammals. The accumulation of 14α-methylated sterols in azole-treated cells is thought to disrupt membrane structure and function, leading to an inhibition of growth (Vanden Bossche, 1985). At therapeutic concentrations the efficacy of azoles is thought to reside in their greater affinity for fungal, rather than mammalian, *P*-450$_{DM}$ (e.g. see Marriott *et al.*, 1986; Vanden Bossche *et al.*, 1986).

To date, comparisons of the interaction of azoles with fungal and mammalian *P*-450$_{DM}$ have been confined to microsomes incubated with radiolabelled sterol precursors, such as acetic and mevalonic acids (Marriott *et al.*, 1986; Vanden Bossche *et al.*, 1986). In order to investigate further the structural features of azoles which govern their binding to *P*-450$_{DM}$, it will be necessary to study the detailed interaction of these compounds with enzymes purified from fungal and mammalian cells. Such an approach may help in the development of new azoles with enhanced selective activity against the fungal, rather than mammalian, enzyme. Cytochrome *P*-450$_{DM}$ has been purified to homogeneity from rat liver (Trzaskos *et al.*, 1986) and from the yeast *Saccharomyces cerevisiae* (Yoshida & Aoyama, 1984). Furthermore, the yeast enzyme has been used to study the interaction of azoles with the active site of the enzyme (Yoshida & Aoyama, 1986, 1987). In contrast, *P*-450$_{DM}$ has not been purified from pathogenic fungi, and there is a dearth of information on cytochromes *P*-450 in these organisms.

Candida albicans is an important opportunistic fungal pathogen responsible for superficial infections of the mouth, skin and vagina and is assuming greater importance as the cause of serious systemic infections, particularly in immunocompromised patients (Odds, 1988). In the present study we have purified and characterized *P*-450$_{DM}$ from *C. albicans*. The results should contribute to future studies designed to investigate the specificity of interaction of azoles with fungal *P*-450$_{DM}$.

MATERIALS AND METHODS

Materials

Biochemicals were purchased from Sigma Chemical Co.; solvents and other chemicals were of analytical grade and were purchased from BDH Ltd. [1-^{14}C]Lauric acid (sodium salt; specific radioactivity 2.15 GBq/mmol) was purchased from Amersham International, and Emulgen 913 was kindly donated by the Kao Corporation, Tokyo, Japan.

Abbreviations used: *P*-450$_{DM}$, cytochrome *P*-450-dependent 14α-sterol demethylase; SDS/PAGE, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The following sterols are referred to by their trivial names: lanosterol, 4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol; ergosterol, ergosta-5,7,22-trien-3β-ol; cholesterol, cholest-5-en-3β-ol.

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Organism and cultural conditions

C. albicans (serotype A; NCPF 3153) was maintained on Yeast Morphology Agar (Oxoid) essentially as described by Hitchcock *et al.* (1986).

Preparation of microsomes

Microsomes were prepared from cells broken by enzymic digestion or mechanical disruption with a Braun disintegrator, as described by Hitchcock *et al.* (1989).

Purification of cytochrome *P*-450$_{DM}$

Unless stated otherwise, all experimental work was done at 4 °C. Microsomes (1.5–2.5 g of protein) were suspended in 200 ml of 100 mM-potassium phosphate (pH 7.2) containing 1 mM-EDTA, 1 mM-dithiothreitol, 0.25 mM-phenylmethanesulphonyl fluoride, 20% (v/v) glycerol and 2% (w/v) sodium cholate (buffer A). After gentle stirring for 1 h, the solution was centrifuged at 145000 *g* ($r_{av.}$ 8.1 cm) for 1 h to pellet solid material. The supernatant was loaded directly onto a column (1.5 cm × 5 cm) of hydroxyapatite (Bio-Gel HTP; Bio-Rad) equilibrated with buffer A. The column was washed with 100 ml of buffer A before the bound haemoproteins were eluted with a step gradient of 20 ml each of 100 mM-, 150 mM- and 200 mM-potassium phosphate (pH 7.0) containing 30 mM-nicotinamide, 50 mM-*N*-acetylcysteine, 20% (v/v) glycerol and 0.2% (v/v) Emulgen 913. The elution of haemoproteins was monitored spectrophotometrically at 416 nm. The fractions eluted with 100 mM-potassium phosphate buffer (which contained *P*-450$_{DM}$) were pooled, diluted 20-fold with 20% (v/v) glycerol containing 1 mM-dithiothreitol (buffer B), and loaded onto a column (1.4 cm × 5 cm) of octyl-Sepharose CL-4B (Pharmacia) equilibrated with buffer B. After washing the column with 200 ml of buffer B containing 0.4% (w/v) sodium cholate and 200 ml of buffer B, *P*-450$_{DM}$ was eluted with buffer B containing 0.2% (v/v) Emulgen 913. The eluted cytochrome was chromatographed on columns (1.2 cm × 5 cm) of CM-Sepharose CL-6B (Pharmacia) and hydroxyapatite using exactly the same procedure as that described for the octyl-Sepharose column. The purified *P*-450$_{DM}$-containing fractions eluted from the hydroxyapatite column were concentrated using an Amicon Centricon 10 microconcentrator and centrifuged at 5000 *g* ($r_{av.}$ 6.98 cm) for 2 h. In order to remove Emulgen 913 from *P*-450$_{DM}$, the concentrate was diluted 10-fold with 100 mM-potassium phosphate (pH 7.4) containing 30 mM-nicotinamide, 5 mM-*N*-acetylcysteine and 20% (v/v) glycerol (buffer C) and concentrated as described above. This procedure was repeated twice and the concentrated *P*-450$_{DM}$ (1–2 nmol/ml) stored at −70 °C.

Analytical methods

The purity and apparent monomeric molecular mass of *P*-450$_{DM}$ were estimated by SDS/PAGE [9% (w/v) acrylamide slab gels, 2 mm thick] by using the method of Laemmli (1970). Routinely, protein was fixed in the gel with methanol/water/acetic acid (5:5:1, by vol.) and stained with this solution containing 0.5% (w/v) Coomassie Brilliant Blue R-250. The destaining solution was methanol/water/acetic acid (4:10:1, by vol.). In some experiments protein was stained with silver. In this method protein-fixed gels were washed with the following solutions made up with deionized glass-distilled water with the incubation time of each wash given in parentheses: 40% (v/v) methanol/12% (w/v) trichloroacetic acid (30 min); 10% (v/v) ethanol/5% (v/v) acetic acid (3 × 30 min); 0.06% potassium permanganate/0.02% copper(II) sulphate (30 min); 10% (v/v) ethanol (2 × 30 min); 0.1% silver nitrate (60 min); water (1 min). The protein was detected with 3% (w/v) sodium carbonate containing 0.05% (v/v) formaldehyde and the staining was stopped with a concentrated solution of citric acid added dropwise.

Cytochrome *P*-450$_{DM}$ was measured spectrophotometrically from its sodium dithionite-reduced CO difference spectrum using a Shimadzu MPS-2000 recording spectrophotometer, essentially as described by Omura & Sato (1964).

Protein was estimated by the method of Lowry *et al.* (1951), using a bovine serum albumin standard.

Assay of reconstituted *P*-450$_{DM}$ activity with lanosterol

Lanosterol (4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol: 23 nmol) and dilauroylphosphatidylcholine (240 nmol) in 100 μl of chloroform/methanol (2:1, v/v) were dispensed into test tubes and the solvent was removed under a stream of N_2. In some experiments lipids extracted from microsomes (150 μg) by the method of Bligh & Dyer (1959) were used instead of dilauroylphosphatidylcholine. The residue was diluted with 100 μl of 0.15 M-KCl and vesicles were prepared with a sonication probe operated at low power with an amplitude setting of 1 for 5 s (150 W ultrasonic disintegrator; M.S.E., Crawley, West Sussex, U.K.). Each tube received *P*-450$_{DM}$ (0.05–0.1 nmol) and 1 unit of cytochrome *P*-450 reductase and the mixture was incubated at 30 °C for 10 min. The reaction was started with buffer C containing 1 μmol of NADPH, 20 μmol of glucose 6-phosphate and 1 unit of glucose-6-phosphate dehydrogenase (final volume 1 ml) and incubated at 30 °C with shaking. The reaction was stopped with 2 ml of 3 M-KOH in 90% (v/v) ethanol and the tubes were heated at 80 °C for 1 h. After cooling, each tube received 10 nmol of 5α-cholestane internal standard before sterols were extracted with light petroleum (b.p. 60–80 °C) and the solvent evaporated *in vacuo*. Sterols were converted to trimethylsilyl derivatives with bis(trimethylsilyl)trifluoroacetamide (Christie, 1982) and analysed by g.l.c. using a fused-silica capillary column coated with OV-17 (0.3 mm × 50 m, 0.25 μm film thickness; Phase Separations, Queensferry, Clwyd, U.K.) operated isothermally at 260 °C in a Sigma 3B gas chromatograph. The injector and detector temperatures were 300 °C and the carrier gas was N_2 with a flow rate of 2 ml/min. This method is essentially the same as that used by Aoyama *et al.* (1984) and readily separates 5α-cholestane (6.2 min; 1.0), lanosterol (26.0 min; 4.19) and the C-14 demethylation product, 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol (28.3 min; 4.56); the retention times and retention times relative to 5α-cholestane are given in parentheses. Sterols were identified by comparison of their retention times with those of authentic standards and published values (Aoyama *et al.*, 1984). Lanosterol and 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol were quantified using 5α-cholestane as internal standard with peak areas calculated by electronic integration.

In each experiment the amount of *P*-450$_{DM}$ was adjusted so as to ensure that the rate of reaction was

proportional to the amount of enzyme and was not limited by substrate concentration. Enzyme activity was calculated from the relative amount of lanosterol and 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol extracted from the reconstitution assay compared with non-enzyme controls.

Assay of reconstituted P-450$_{DM}$ activity with xenobiotic substrates

In these experiments lanosterol was replaced with xenobiotic substrates in the standard reconstitution assay. The following enzyme activities were assayed using published methods: 7-ethoxyresorufin *O*-de-ethylase (Grant *et al.*, 1988), 7-ethoxycoumarin *O*-de-ethylase (Dawson *et al.*, 1985), benzo[*a*]pyrene hydroxylase (Nebert & Gelboin, 1968), and [1-^{14}C]lauric acid hydroxylase (Parker & Orton, 1980). The final assay concentration of these substrates was 10 μM except for [1-^{14}C]lauric acid which was 100 μM. The efficacy of each assay was checked by demonstrating the appropriate enzyme activity in rat liver microsomes.

Purification of NADPH: cytochrome *c* (cytochrome *P*-450) reductase

Cytochrome *P*-450 reductase was purified from rat liver using the affinity chromatography method described by Ardies *et al.* (1987).

RESULTS

Microsomal cytochrome *P*-450

We have shown that sterol biosynthesis in *C. albicans* microsomes is influenced by the method of cell disruption (Hitchcock *et al.*, 1989); the incorporation of [^{14}C]lanosterol into ergosterol in microsomes was optimal as long as cells were broken in a liquid CO_2-cooled Braun disintegrator. In view of this situation, the Braun disintegrator method was chosen for the routine preparation of microsomes for P-450$_{DM}$ purification. However, a drawback with this procedure is that microsomes are contaminated with cytochrome oxidase in fragments of broken mitochondria. These interfere with the measurement of cytochrome *P*-450 in the reduced CO difference spectrum (Fig. 1). The cytochrome oxidase spectrum has a trough in the range 441–445 nm, thus precluding the measurement of cytochrome *P*-450, a phenomenon reported for other fungi (Kappeli, 1986). In order to eliminate cytochrome oxidase from microsomal preparations, cells were digested enzymically and intact mitochondria removed by differential centrifugation. The resulting microsomes were used to quantify cytochrome *P*-450 in the reduced CO difference spectrum, which had a Soret peak at 447 nm and was free from cytochrome oxidase (Fig. 1*b*). The specific content of total cytochrome *P*-450 in microsomes was 0.02±0.001 nmol/mg of protein (mean±S.E.M. of experiments on 10 separate batches of exponential-phase cultures).

Purification of P-450$_{DM}$ from microsomes

An important aspect of the purification of P-450$_{DM}$ from *S. cerevisiae* (Yoshida & Aoyama, 1984) and rat liver (Trzaskos *et al.*, 1986) was its enrichment from solubilized microsomal proteins by $(NH_4)_2SO_4$ and poly(ethylene glycol) fractionation. In contrast, we found that these procedures resulted in the complete denaturation of *C. albicans* cytochrome *P*-450 to cytochrome *P*-420. Instead, solubilized microsomal proteins were loaded directly onto an hydroxyapatite column, and three peaks of haemoproteins were eluted with increasing concentrations of potassium phosphate containing 0.2% (v/v) Emulgen 913 (Fig. 2). Pooled samples of each peak had a Soret absorption maximum of 447 nm in the

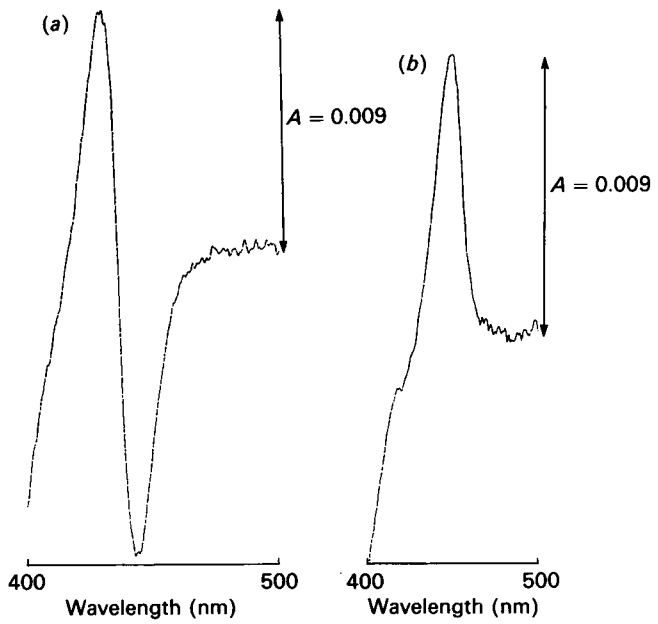

Fig. 1. Effect of different breakage procedures on the reduced CO difference spectrum of microsomes

Spectra were recorded using microsomes derived from cells broken (*a*) by Braun disintegration, or (*b*) by enzymic digestion. The protein contents of (*a*) and (*b*) were 2 mg/ml and 5.5 mg/ml, respectively. The *P*-450 concentration of (*b*) was 0.1 nmol/ml.

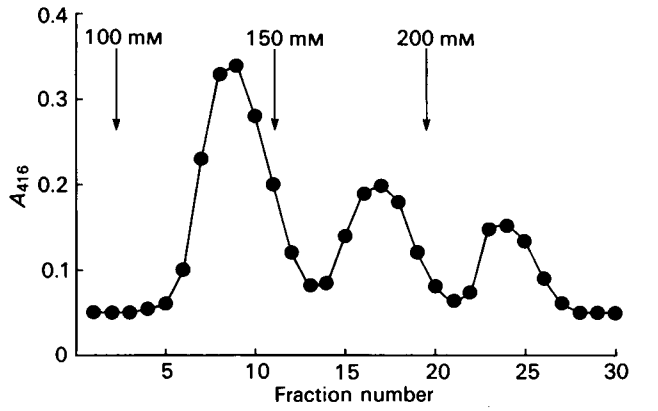

Fig. 2. Elution profile of cytochromes *P*-450 from hydroxyapatite

Microsomal proteins extracted with sodium cholate were applied to a column of hydroxyapatite, equilibrated with buffer A. The column was washed with 100 ml of equilibration buffer before haemoproteins were eluted with a step gradient of 100 mM-, 150 mM- and 200 mM-potassium phosphate (pH 7.0) containing 0.2% (v/v) Emulgen 913. Fractions (2 ml) were pooled according to 416 nm absorbance and assayed for 14α-sterol demethylase activity. The column flow rate was 0.3 ml/min.

Table 1. Summary of the purification of P-450$_{DM}$ from microsomes

Procedure	Protein (mg)	P-450 (nmol)	Specific content (nmol/mg of protein)	Recovery (%)
Microsomes	970	19.4	0.02	100
Hydroxyapatite	16	10.9	0.68	56.2
Octyl-Sepharose	2.8	8.3	2.96	42.8
CM-Sepharose	0.14	2.4	17.14	12.3
Hydroxyapatite	0.12	2.1	17.50	10.8

reduced CO difference spectrum. However, it is important to note that the major peak, which was eluted with 100 mM-potassium phosphate, was the only P-450 to contain 14α-sterol demethylase activity in the reconstituted assay. On the basis of this observation P-450$_{DM}$ was purified further using octyl-Sepharose, CM-Sepharose and hydroxyapatite chromatographies.

The results of a typical purification are shown in Table 1. Cytochrome oxidase contamination of the microsomes precluded the measurement of cytochrome P-450. Therefore, the specific content value of 0.02 nmol/mg of protein shown in Table 1 refers to the cytochrome oxidase-free microsomes derived from enzymically-digested cells, described above. Cytochrome oxidase also hampered the routine measurement of cytochrome P-450 in the solubilized protein fraction. However, parallel experiments with cytochrome oxidase-free microsomes demonstrated that approx. 60% of the total amount of cytochrome P-450 was solubilized with sodium cholate. In contrast to cytochrome P-450, cytochrome oxidase did not adhere to the first hydroxyapatite column, and consequently, P-450$_{DM}$ could be quantified accurately during its purification.

Besides the incomplete solubilization of microsomal cytochrome P-450, the rather low recovery of P-450$_{DM}$ (10%) reflects the fact that the cytochrome was weakly bound to the CM-Sepharose cation exchange resin, under the conditions employed; unbound cytochrome washed from the resin could be recovered on either octyl-Sepharose or hydroxyapatite columns. The binding of P-450$_{DM}$ to CM-Sepharose increased slightly when the pH of the running buffer was lowered from 7 to 6. However, this resulted in denaturation of the protein to cytochrome P-420. Therefore, a running buffer at pH 7 was used for the routine purification of P-450$_{DM}$ with CM-Sepharose. Despite the rather low recovery, P-450$_{DM}$ was purified 875-fold with respect to microsomes giving a specific content of 17.5 nmol/mg of protein. The enrichment of P-450$_{DM}$ with each procedure in the purification was demonstrated by analysis of the proteins by SDS/PAGE followed by staining with Coomassie Brilliant Blue or silver (Figs. 3a and 3b). Cytochrome P-450$_{DM}$ was purified to homogeneity as judged by Coomassie Brilliant Blue staining, which is the criterion for electrophoretic purity of other cytochromes P-450$_{DM}$ (Yoshida & Aoyama, 1984; Trzaskos *et al.*, 1986). Minor protein contaminants of purified P-450$_{DM}$ preparations were detected only when polyacrylamide gels were stained with silver, which is 100-fold more sensitive to protein than is Coomassie Brilliant Blue (Ragan, 1986). The M_r of P-450$_{DM}$ was estimated to be 51000 by comparison of its mobility with those of standard proteins. On the basis of this M_r value the cytochrome was judged to be 89% pure.

Spectral properties of purified P-450$_{DM}$

The absolute absorption spectra of P-450$_{DM}$ are shown in Fig. 4. The oxidized cytochrome absorbed at 552 and 528 nm with a Soret maximum at 417 nm indicating that it was in the low-spin state. The Soret peak shifted approx. 8 nm toward the blue end of the spectrum when the cytochrome was reduced with sodium dithionite. When the cytochrome was gassed with CO, the reduced CO difference spectrum had a Soret peak at 447 nm in common with microsomal cytochrome P-450.

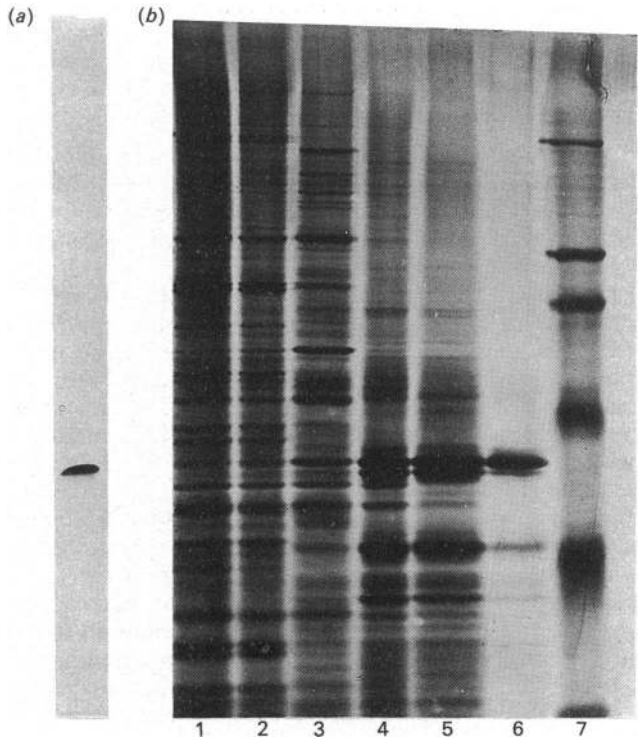

Fig. 3. Analysis by SDS/PAGE of proteins from each procedure in the purification of P-450$_{DM}$

SDS/PAGE was done as described by Laemmli (1970) using 9% (w/v) acrylamide slab gels and 1.0 μg of protein. (*a*) Purified P-450$_{DM}$ stained with Coomassie Brilliant Blue. (*b*) Proteins stained with silver: lane 1, microsomal proteins; lane 2, solubilized microsomal proteins; lane 3, first hydroxyapatite eluate; lane 4, octyl-Sepharose eluate; lane 5, CM-Sepharose eluate; lane 6, second hydroxyapatite eluate containing purified P-450$_{DM}$; lane 7, M_r standard proteins: carbonic anhydrase, 29000; egg albumin, 45000; bovine albumin, 66000; phosphorylase b, 97400; β-galactosidase, 116000; myosin, 205000.

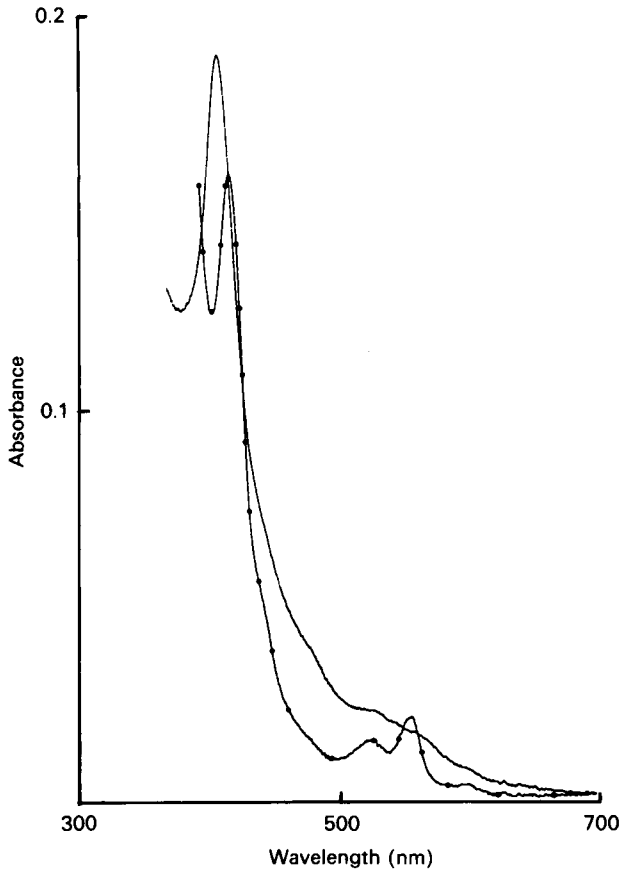

Fig. 4. Absolute absorption spectra of P-450$_{DM}$

Cytochrome P-450$_{DM}$ was diluted in buffer C to a concentration of 0.1 nmol/ml and the absolute absorption spectra recorded; ●——●, oxidized form; ——, sodium dithionite-reduced form.

Properties of reconstituted P-450$_{DM}$

The enzyme activity of purified P-450$_{DM}$ in the reconstituted system was assayed by measuring the conversion of lanosterol to its C-14 demethylated product, 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol. A number of factors that influenced this activity in experiments with saturating amounts of P-450 reductase are shown in Table 2. There was an absolute requirement for P-450$_{DM}$, cytochrome P450 reductase and lipid. Enzyme activity was increased 2.5-fold when microsomal lipid was replaced with dilauroylphosphatidylcholine, but this activity could be abolished completely if the cytochrome was gassed with CO. Enzyme activity was reduced markedly when Emulgen 913 was added to the reconstituted assay at a final concentration of 0.02% (v/v). In view of the fact that this detergent was used in the purification of P-450$_{DM}$, care was taken to ensure that each batch of purified P-450$_{DM}$ was washed thoroughly with detergent-free buffer before the cytochrome was used in reconstitution experiments.

On the basis of the results shown in Table 2, dilauroylphosphatidylcholine and 1 unit of cytochrome P-450 reductase were chosen for further experiments with P-450$_{DM}$ in the reconstituted assay. Under these conditions lanosterol metabolism was linear with time over 60 min. There appeared to be only one reaction

Table 2. Effect of different treatments on reconstituted P-450$_{DM}$

Lanosterol was metabolized aerobically by P-450$_{DM}$ in a reconstituted system containing P-450$_{DM}$, P-450 reductase, dilauroylphosphatidylcholine and NADPH. Lanosterol and its C-14 demethylated metabolite were analysed by g.l.c., as described in the Materials and methods section. Values are the means of triplicate determinations which varied by < 10%. Abbreviations: reductase, cytochrome P-450 reductase; DLPC, dilauroylphosphatidylcholine.

Treatment	14α-sterol demethylase activity (nmol/min per nmol of P-450$_{DM}$)
P-450$_{DM}$/reductase/microsomal lipid	0.18
P-450$_{DM}$/reductase/DLPC	0.45
P-450$_{DM}$/reductase/DLPC/CO	0
P-450$_{DM}$/reductase/DLPC/ Emulgen 913	0.05
P-450$_{DM}$/reductase	0
Reductase/DLPC	0
P-450$_{DM}$/DLPC	0

product which had the chromatographic properties of 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol. The same was true when the incubation period was extended to 5 h, when approx. 70% of the lanosterol had been metabolized by P-450$_{DM}$. Cytochrome P-450$_{DM}$ purified from four separate batches of exponential-phase cultures had a mean(±S.E.M.) enzyme activity of 0.29±0.06 nmol of lanosterol metabolized/min per nmol of P-450$_{DM}$.

The ability of P-450$_{DM}$ to oxidize other lipophilic substrates was tested by replacing lanosterol with several xenobiotic compounds in the reconstituted assay. The enzyme was unable to oxidize 7-ethoxyresorufin, 7-ethoxycoumarin, benzo[*a*]pyrene or lauric acid, even after an extended incubation period of 16 h.

DISCUSSION

Cytochrome P-450$_{DM}$ is an important enzyme in ergosterol biosynthesis and key target site for the azole antifungal antibiotics. In the present study, *C. albicans* strain 3153 was used as a source of cytochrome P-450$_{DM}$ for the following reasons: we have investigated the properties of microsomal P-450$_{DM}$ using this strain (Hitchcock *et al.*, 1989); and *C. albicans* serotype A (of which strain 3153 is a representative) is the serotype most commonly isolated from patients (Odds, 1988). The total content of cytochrome P-450 in *C. albicans* 3153 microsomes (0.02 nmol/mg of protein) is much lower than that of other *C. albicans* strains (0.074–0.1 nmol/mg of protein; Vanden Bossche *et al.*, 1987), *S. cerevisiae* (0.063 nmol/mg of protein; Yoshida & Aoyama, 1984) and rat liver (3.3 nmol/mg of protein; Trzaskos *et al.*, 1986). However, despite this situation, P-450$_{DM}$ was purified from microsomes and separated from at least two other cytochromes P-450 on the basis of enzyme activity in the reconstituted assay. In common with the present study, hydroxyapatite chromatography has been used to resolve different species of P-450 (including P-450$_{DM}$) purified from rat liver (Trzaskos *et al.*, 1986). Interestingly, both the fungal and rat liver cytochromes P-450$_{DM}$ displayed essentially the same properties during

hydroxyapatite chromatography, being eluted with 90–100 mM-potassium phosphate containing detergent.

Using SDS/PAGE, the M_r value of $P\text{-}450_{DM}$ was estimated to be 51000, in common with the rat liver cytochrome (Trzaskos *et al.*, 1986) but smaller than that of *S. cerevisiae* which gave a value of 58000 under the same experimental conditions (Yoshida & Aoyama, 1984). Interestingly, the M_r of *C. albicans* $P\text{-}450_{DM}$ is less than that predicted from the nucleotide sequence (approx. 58000) of the $P\text{-}450_{DM}$ gene (Lai & Kirsch, 1989). This disparity might reflect post-translational modification of $P\text{-}450_{DM}$ and/or an inherent limitation of SDS/PAGE in estimating the M_r of the protein. Like the rat liver and *S. cerevisiae* cytochromes, *C. albicans* $P\text{-}450_{DM}$ appeared to be electrophoretically pure after staining with Coomassie Brilliant Blue, suggesting that only one isoenzyme of *P*-450 is responsible for the 14α-demethylation reaction. The presence of very small amounts of contaminating proteins, revealed by staining with silver, is to be expected from the purification of a membrane-bound protein, and there are no data on other purified $P\text{-}450_{DM}$ proteins for comparison. Repeated attempts to remove these contaminants from $P\text{-}450_{DM}$ by using different detergents (e.g. Emulgen 911 and Triton X-100) and potassium phosphate gradients in the column chromatography procedures were unsuccessful. The absolute absorption spectrum of oxidized $P\text{-}450_{DM}$ is characteristic of the cytochrome in the low-spin state, indicating that it was purified free from endogenous substrate (Jefcoate, 1986). Like the rat liver (Trzaskos *et al.*, 1986) and *S. cerevisiae* (Yoshida & Aoyama, 1984) cytochromes, *C. albicans* $P\text{-}450_{DM}$ is included in the *P*-448 group of cytochromes *P*-450 because its reduced CO complex has a Soret maximum of 447 nm.

The results shown in Table 2 demonstrate clearly that 14α-sterol demethylase activity is catalysed by a *P*-450-dependent mono-oxygenase: enzyme activity was dependent on phospholipid, *P*-450 reductase and NADPH and was inhibited by CO. The preference for dilauroylphosphatidylcholine over microsomal lipid shown by $P\text{-}450_{DM}$ has been described for other reconstituted *P*-450-dependent enzymes (Ingelman-Sundberg, 1986). As with the rat liver (Trzaskos *et al.*, 1986) and *S. cerevisiae* (Aoyama *et al.*, 1984, 1987) enzymes, reconstituted *C. albicans* $P\text{-}450_{DM}$ appears to catalyse the entire process of 14α-demethylation, consisting of three mono-oxygenase steps, to a metabolite with the chromatographic properties of 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol. Although this enzyme activity was reproducible between different batches of purified cytochrome, it is approximately 10-fold and 20-fold lower than that of reconstituted rat liver and *S. cerevisiae* $P\text{-}450_{DM}$, respectively. A possible explanation for this phenomenon is that rat liver *P*-450 reductase was used in the reconstituted assay. The intention was to use *C. albicans* *P*-450 reductase but we have been unable to obtain an active preparation of the enzyme. An alternative explanation is that lanosterol is not the major endogenous substrate for $P\text{-}450_{DM}$. Cultures of *C. albicans* can alkylate the C-24 position of lanosterol (Fryberg *et al.*, 1975) and it is possible that 24-methylene-24,25-dihydrolanosterol is the preferred substrate for $P\text{-}450_{DM}$ *in vivo*. On the basis of the results obtained with xenobiotics in the reconstituted assay, it would appear that $P\text{-}450_{DM}$ is specific in its oxidation of lanosterol. This observation is in agreement with results for the rat liver cytochrome (Trzaskos *et al.*, 1986) and is consistent with the high degree of substrate specificity displayed by other constitutive cytochromes *P*-450 responsible for catalysing biosynthetic reactions (Jefcoate, 1986). At present it is not clear whether the other cytochrome *P*-450 fractions separated by hydroxyapatite chromatography are breakdown products of $P\text{-}450_{DM}$ (which are unable to metabolize lanosterol), or whether they are distinct classes of the cytochrome with different substrate specificities, in common with those of mammalian cells.

In conclusion, the present study is the first demonstration of the purification of $P\text{-}450_{DM}$ from a fungal pathogen. The enzyme should provide a valuable basis for an investigation of the interaction of azole antifungals with its active site, so as to provide the necessary background against which the structure–activity relationships of these compounds can be assessed. This approach may help in the development of new azoles with improved activity against *C. albicans* $P\text{-}450_{DM}$.

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