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Cytochrome *P*-450-dependent 14α-demethylation of lanosterol in *Candida albicans*

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A novel assay for cytochrome *P*-450-dependent 14α-sterol demethylase of the important opportunistic fungal pathogen, *Candida albicans*, is described. The enzyme was assayed in microsomal preparations (microsomes) by measuring the incorporation of [^{14}C]lanosterol into (4,14)-desmethylated sterols. The efficacy of different cell-breakage methods was compared; desmethylated-sterol biosynthesis was maximal when cells were broken with a Braun disintegrator. The solubilization of [^{14}C]lanosterol with detergent in the assay system was essential for enzyme activity, which was enhanced considerably when microsomes were gassed with O_2. Under these conditions, there was a reciprocal relationship between the amount of radioactivity incorporated into desmethylated sterols and that lost from lanosterol. The major radiolabelled desmethylated sterol was ergosterol. The enzyme had an apparent K_m of 52.73 ± 2.80 μM and an apparent $V_{max.}$ of 0.84 ± 0.14 nmol/min per mg of protein ($n = 3$). Enzyme activity was decreased greatly when microsomes were treated with CO or the triazole antifungal ICI 153066.

INTRODUCTION

Candida albicans is a widespread opportunistic fungal pathogen responsible for superficial and systemic candidoses (Odds, 1988). Members of the recently introduced azole (*N*-substituted imidazole or triazole) class of antifungal antibiotics are used commonly in the treatment of mycoses, including candidoses (Fromtling, 1988). The mode of action of azoles is complex: they inhibit a number of membrane-bound enzymes (Uno *et al.*, 1982; Portillo & Gancedo, 1984; Mason *et al.*, 1985), and some of them affect membranes by binding to lipids (Cope, 1980; Brasseur *et al.*, 1983). However, the primary mode of action is probably the inhibition of 14α-sterol demethylase (Vanden Bossche *et al.*, 1986), an important enzyme in ergosterol (ergosta-5,7,22-trien-3β-ol) biosynthesis in fungi and in cholesterol (cholest-5-en-3β-ol) biosynthesis in mammals. Azole-treated cells accumulate 14α-methylated sterols, which disrupt membrane structure and function (Yeagle *et al.*, 1977).

The potency of azoles may be evaluated by measuring the incorporation of [^{14}C]acetate or [^{14}C]mevalonate into the 14α-methylated sterols of azole-treated cells (Van den Bossche *et al.*, 1978; Marriott, 1980; Hitchcock *et al.*, 1987). Comparison of these data with those obtained from studies on cholesterol biosynthesis has demonstrated that the mammalian 14α-sterol demethylase is much less sensitive to azole antifungals (e.g. see Marriott *et al.*, 1986; Vanden Bossche *et al.*, 1986).

Removal of the 14α-methyl group (C-32) from lanosterol (4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol) involves cytochrome *P*-450, and this process has been studied extensively by using 14α-sterol demethylase purified from rat liver (Trzaskos *et al.*, 1986) and from the yeast *Saccharomyces cerevisiae* (Aoyama *et al.*, 1987). Both enzymes catalyse the complete oxidative demethylation of 24,25-dihydrolanosterol to 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol and formic acid. The reaction requires NADPH and molecular O_2, and is inhibited by CO. Furthermore, the yeast 14α-sterol demethylase has been used to study the interaction of azole antifungals with the active site of the enzyme (Yoshida & Aoyama, 1986, 1987). The imidazole or triazole moiety binds to the sixth co-ordination position of the haem, while the substituent groups of the antifungal interact with the apoprotein of the enzyme.

In contrast, there is a paucity of information on 14α-sterol demethylase in *C. albicans*: the enzyme has never been purified or characterized in microsomal preparations. In order to investigate the 14α-demethylation process in *C. albicans*, we have developed a sensitive and simple assay for 14α-sterol demethylase with [^{14}C]lanosterol as substrate. We report now some of the properties of microsomal 14α-sterol demethylase, a key enzyme in ergosterol biosynthesis and target site for the azole antifungal antibiotics.

METHODS

Materials

Biochemicals were purchased from Sigma Chemical Co. Solvents and other chemicals were of analytical grade. DL-[2-^{14}C]Mevalonic acid (dibenzyldiethyldiamine salt; sp. radioactivity 1.89 GBq/mmol) was purchased from Amersham International, Amersham, Bucks., U.K. The triazole antifungal ICI 153066 [1-(2,4-dichlorophenyl)-1-(4-fluorophenyl)-2-(1,2,4-triazoyl)ethanol] was synthesized at ICI Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire, U.K.

Fungal strain and cultural conditions

C. albicans [Serotype A; NCPF (National Collection of Pathogenic Fungi) 3153] was maintained on Yeast Morphology agar (Oxoid) and grown in Sabouraud's glucose broth (Oxoid) essentially as described by Hitchcock *et al.* (1986).

Abbreviation used: NSF, non-saponifiable lipid fraction.

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Mechanical disruption of cells

Yeast cell suspensions (2×10^9 cells/ml) were prepared from late-exponential-phase cultures in the following disruption buffer: 100 mM-potassium phosphate (pH 7.4) containing 30 mM-nicotinamide, 5 mM-*N*-acetylcysteine, 5 mM-$MgCl_2$, 0.25 mM-phenylmethanesulphonyl fluoride and 20% (v/v) glycerol. Cell lysates were prepared by breaking cells at 4 °C in a Braun disintegrator (Braun G.m.b.H., Melsungen, W. Germany) operated under the conditions described by Barrett-Bee *et al.* (1986).

In some experiments cells were broken by three passages through a French pressure cell (American Instrument Co., Silver Springs, MD, U.S.A.) operated at a pressure of approx. 10^2 MPa.

Enzymic disruption of cells

Yeast cell suspensions (2×10^9 cells/ml) in 100 mM-potassium phosphate buffer (pH 7.2) containing 100 mM-EDTA and 100 mM-2-mercaptoethanol were digested with 100 units [1 unit will give ΔA_{800} of 0.001 unit/min at pH 7.5 and 25 °C, with a suspension of *S. cerevisiae* (3 mg dry wt.) as substrate in a 3 ml reaction mixture (1 cm light path)] of zymolyase 60000 (Kirin Brewery, Tokyo, Japan) by the method of Jenkins *et al.* (1983). Sphaeroplasts were harvested by centrifugation at 600 *g* ($r_{av.}$ 10.7 cm) for 5 min and resuspended in disruption buffer to a concentration of 2×10^9/ml. Breakage of sphaeroplasts was effected by using 15 strokes of a Potter–Elvehjem motor-driven homogenizer operated at approx. 200 rev/min.

Centrifugation of the cell lysates

The cell lysates from the three breakage procedures were centrifuged at 5000 *g* ($r_{av.}$ 6.98 cm) for 20 min at 4 °C to remove unbroken cells and cellular debris. Routinely, the supernatant of Braun-disintegrator lysates was centrifuged at 145000 *g* ($r_{av.}$ 8.1 cm) for 1 h at 4 °C to pellet microsomes (microsomal fractions). The pellets were resuspended in disruption buffer to give a protein concentration in the range 15–20 mg/ml and stored at −70 °C.

The protein content of cell lysates and microsomes was estimated by the method of Lowry *et al.* (1951), with a bovine serum albumin standard.

Measurement of sterol biosynthesis with [2-^{14}C]mevalonic acid

Sterol biosynthesis was assayed by the incorporation of DL-[2-^{14}C] mevalonic acid into the non-saponifiable lipid fraction (NSF) of cell lysates as described by Barrett-Bee *et al.* (1986). The NSF lipids were chromatographed on analytical t.l.c. plates of silica gel H (Merck) with toluene/diethyl ether (9:1, v/v) as the developing solvent (solvent system 1). The 4,4-dimethylated and desmethylated sterol fractions were located by autoradiography (2 h exposure) and eluted from the silica gel with diethyl ether. A portion of each fraction was taken for measurement of radioactivity by liquid-scintillation counting, and the remainder was acetylated (together with 1 mg of ergosterol as carrier) with pyridine/acetic anhydride (1:2, v/v) under N_2 at room temperature. The acetylated sterols were chromatographed on analytical t.l.c. plates of silica gel H impregnated with $AgNO_3$ (10%, w/w), with toluene/hexane (7:3, v/v) as the developing solvent. Radioactive zones were located by autoradiography, and the areas corresponding to the zones of blackening were scraped from the plate and their radioactive contents measured by liquid-scintillation counting. Steryl acetates were identified by comparison of their R_F values with those of authentic standards.

Preparation of [^{14}C]lanosterol from [2-^{14}C]mevalonic acid

[^{14}C]Lanosterol was biosynthesized from [2-^{14}C]-mevalonic acid in Braun-disintegrator lysates (at 37 °C for 3 h) containing the triazole antifungal ICI 153066 (0.1 mM) to inhibit 14α-sterol demethylase. The reaction was terminated by addition of 3 M-KOH in 90% (v/v) ethanol, and lipids were saponified by heating at 80 °C for 1 h. After cooling, the NSF lipids were extracted with light petroleum (b.p. 60–80 °C) and the solvent was removed by rotary evaporation. The residue was dissolved in a small volume of chloroform/methanol (2:1, v/v) and loaded on silica-gel H preparative t.l.c. plates. The NSF lipids were separated at 4 °C with solvent system 1. Lipids were detected under u.v. light at 350 nm, and the 4,4-dimethylated sterol fraction was identified by comparison of its R_F value with that of an authentic lanosterol standard. The 4,4-dimethylated sterol fraction was eluted from the silica gel with chloroform/methanol (2:1, v/v), reduced to dryness under a stream of N_2 and acetylated as described above. After drying under N_2, the residue was dissolved in a small volume of chloroform/methanol (2:1, v/v) and loaded on silica-gel H analytical t.l.c. plates impregnated with 10% $AgNO_3$. The chromatogram was developed at 4 °C with toluene/hexane (1:1, v/v), and the band corresponding to lanosteryl acetate ($R_F = 0.22$) was identified under u.v. light at 350 nm, by reference to an authentic standard. Lanosteryl acetate was eluted from the silica gel with chloroform/methanol (2:1, v/v) and reduced to dryness under N_2 before being saponified with 3 M-KOH in 90% (v/v) ethanol. The non-saponified sterol was extracted with light petroleum (b.p. 60–80 °C) and, after rotary evaporation, the residue was dissolved in water-saturated-chloroform/methanol (19:1, v/v) for the removal of non-lipid contaminants (Wells & Dittmer, 1963).

In order to check the purity of the [^{14}C]lanosterol, a sample of lanosteryl acetate was chromatographed at 4 °C on alumina impregnated with 10% $AgNO_3$ with toluene/hexane (1:3, v/v) as the developing solvent. Autoradiography of the t.l.c. plate revealed one band with an R_F value of 0.38, corresponding to that of the authentic standard. When subjected to g.l.c. as described by Hitchcock *et al.* (1986), the purified [^{14}C]lanosterol gave one peak with a retention time corresponding to that of the authentic standard. The purified [^{14}C]lanosterol was judged to be approx. 98% pure when analysed by mass spectrometry with a Kratos MS25 mass spectrometer equipped with an electron-impact source (Kratos, Manchester, U.K.). The radioactive content of the purified [^{14}C]lanosterol was measured by liquid-scintillation counting; the external-standards-ratio method was used for the determination of counting efficiency, which was 91 ± 4%. The specific radioactivity was 1.33×10^3 d.p.m./nmol.

Sterol demethylase assay with [^{14}C]lanosterol

Sterol demethylase was assayed by measuring the

incorporation of [^{14}C]lanosterol into the NSF of microsomes prepared by the Braun-disintegrator method. Routinely, the microsomes were diluted to the required protein concentration (usually 2–4 mg/ml) with disruption buffer and gassed gently for 1 min with O_2/CO_2 (19:1) before use. The assay system was similar to that used in experiments with [2-^{14}C]mevalonic acid. Briefly, [^{14}C]lanosterol (10–100 nmol: 1.3×10^4–1.3×10^5 d.p.m.) in 50μl of chloroform/methanol (2:1, v/v) containing 1 μl of Tween 80 was dispensed into test tubes, and the solvent was removed under a stream of N_2. Each tube received 50 μl of disruption buffer, containing: 1 μmol of $NADP^+$, 1 μmol of NAD^+, 3 μmol of glucose 6-phosphate, 5 μmol of ATP, 3 μmol of GSH, 0.7 unit of glucose-6-phosphate dehydrogenase, 2 μmol of $MnCl_2$, 3 μmol of $MgCl_2$ and 1 μl of dimethyl sulphoxide. The reaction was started by the addition of 940 μl of microsomes. The reaction mixture was vortex-mixed and incubated at 37 °C with shaking. The reaction was terminated by saponification, and the NSF lipids were extracted with light petroleum; the efficiency of extraction of [^{14}C]lanosterol was judged to be 85±6% by comparison with control experiments containing boiled microsomes to inhibit 14α-sterol demethylase. The NSF lipids were separated by t.l.c. and their radioactive contents measured by liquid-scintillation counting as described above.

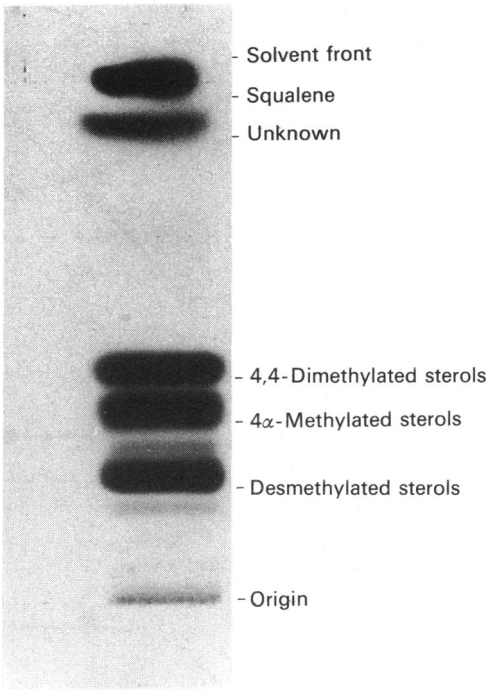

Fig. 1. Autoradiograph (16 h exposure) showing the distribution of radioactivity in the NSF lipids of a Braun-disintegrator lysate incubated with [2-^{14}C]mevalonic acid for 50 min

The NSF lipid (1.9×10^4 d.p.m.) was chromatographed on t.l.c. plates of silica gel H with toluene/diethyl ether (9:1, v/v) as the developing solvent.

RESULTS

Efficacy of cell breakage and sterol biosynthesis

Cellular lysates of *C. albicans* A yeast cultures were prepared either by mechanically disrupting cells with a Braun disintegrator or French pressure cell, or by enzymic digestion of the cell wall. In each case 93±5% of the cells were disrupted, as judged by haemocytometer counts, giving an S_5-cell-lysate (i.e. the supernatant obtained after centrifugation at 5000 *g* for 20 min) protein concentration in the range 10–12 mg/ml.

In cell-lysate incubations with [2-^{14}C]mevalonic acid, approx. 40% of the total radioactivity was recovered in the NSF, irrespective of the method of cell disruption. The NSF was separated by t.l.c. (Fig. 1) into desmethylated sterols (0.22), 4 α-methylated sterols (0.35), 4,4-dimethylated sterols (0.40), an unidentified lipid (0.87) and squalene (0.96), with R_F values indicated. Fig. 2 shows the results typical of an experiment comparing the incorporation of radioactivity into the NSF of lysates prepared from the same batch of cells but by using different cell-disruption procedures. In Braun-disintegrator lysates radioactivity was incorporated rapidly into squalene up to an incubation period of 20 min, after which the amount of radioactivity decreased steadily. In contrast, the radiolabel was incorporated into desmethylated and 4,4-dimethylated sterols linearly with respect to incubation time, and after an incubation period of 50 min desmethylated sterols had incorporated approx. 1.2-fold and 7-fold more radioactivity than had squalene and 4,4-dimethylated sterols respectively. Fig. 2 shows clearly that the pattern of incorporation of [2-^{14}C]mevalonic acid into the NSF of sphaeroplast and French-pressure-cell lysates was quite different from that of Braun-disintegrator lysates. For example, in the first two lysates the biosynthesis of squalene reached a plateau after an incubation period of 30 min. However, the most notable observation was that both lysates biosynthesized much less desmethylated sterols and more 4,4-dimethylated sterols compared with Braun-disintegrator lysates. In fact, after a 50 min period of incubation sphaeroplast lysates had incorporated approx. 8000 dpm of radioactivity into desmethylated sterols, whereas French-pressure-cell lysates incorporated only a trace amount (< 1000 d.p.m.) of the radiolabel. The accumulation of 4,4-dimethylated sterol precursors at the expense of desmethylated sterols in lysates indicates that the sterol-biosynthetic pathway was affected by the sphaeroplast and French-pressure-cell breakage methods. In contrast, Braun-disintegrator lysates biosynthesized readily desmethylated sterols from 4,4-dimethylated sterols, and, on the basis of this observation, the Braun-disintegrator method was chosen for further experiments with 14α-sterol demethylase in *C. albicans* preparations.

In order to identify the components of the desmethylated and 4,4 dimethylated sterol fractions, sterols were acetylated and chromatographed on $AgNO_3$-impregnated silica-gel H t.l.c. plates. Table 1 shows the probable chromatographic identities and radioactive contents of the desmethylated and 4,4-dimethylated sterol components obtained from a Braun-disintegrator lysate. Ergosteryl acetate was the major sterol of the desmethylated sterol fraction, accounting for approximately half of the total amount of radioactivity loaded on the t.l.c. plate. Besides ergosteryl acetate, there were smaller amounts of two unidentified components. A relatively large proportion of the radioactivity was recovered at the origin of the t.l.c. plate and is probably the breakdown product(s) of a labile desmethylated sterol. In contrast, only a small amount of the radio-

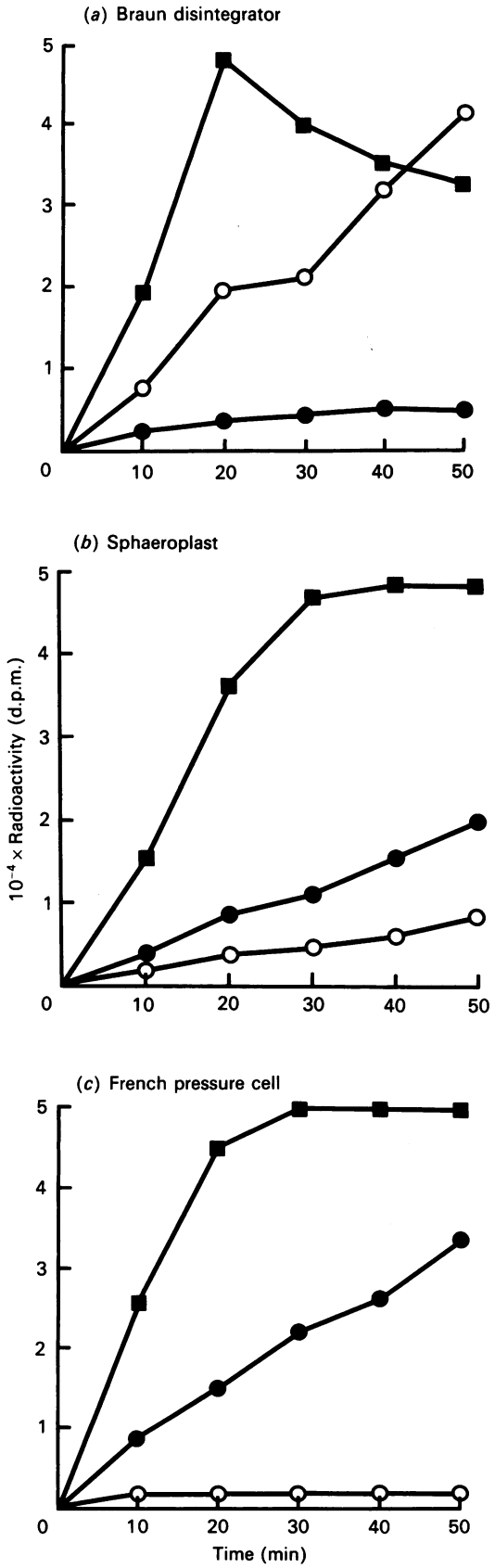

Fig. 2. Effect of different breakage procedures on incorporation of [2-^{14}C]mevalonic acid into the NSF of cell lysates

Values are means of triplicate determinations, which varied by < 5%: ■, squalene; ○, desmethylated sterols; ●, 4,4-dimethylated sterols.

Table 1. Probable chromatographic identities of the acetates of 4,4-dimethylated and desmethylated sterols obtained by incubation of microsomes with [2-^{14}C]mevalonic acid

The desmethylated and 4,4-dimethylated sterol fractions from t.l.c. on silica gel H were acetylated and chromatographed on silica-gel H t.l.c. plates impregnated with $AgNO_3$, with toluene/hexane (7:3, v/v) as the developing solvent. Each experiment was done in duplicate, and the results given are means of two values which varied by < 10%. The amount of radioactivity associated with each fraction is expressed as a percentage of the total radioactivity.

Sterols	Probable identity	R_F	Radioactivity (d.p.m.)	Radioactivity (%)
4,4-Dimethylated	4,4-Dimethyl-24,25-dihydrosteryl acetates	0.30	1761	36.8
	4,4-Dimethyl-Δ^{24}-steryl acetates	0.23	2592	54.2
	Origin	0.00	432	9.0
Desmethylated	Unknown	0.12	2976	15.5
	Ergosteryl acetate	0.10	9331	48.7
	Unknown	0.07	1075	5.6
	Origin	0.00	5760	30.1

Table 2. Effect of different treatments on microsomal 14α-sterol demethylase

Values are the means of triplicate determinations, which varied by < 10%. Tweens were used at 0.1% (v/v), and O_2/CO_2 at 19:1.

Treatment	14α-Sterol demethylase activity (nmol/min)
Untreated	0.00
Tween 40	0.02
Tween 80	0.10
Tween 80, O_2/CO_2	0.45
Tween 80, O_2/CO_2, CO	0.05
Tween 80, O_2/CO_2, ICI 153066 (10 μM)	0.00

activity from the 4,4-dimethylated sterol fraction remained at the origin. The major radioactive components of this fraction had the chromatographic properties of 4,4-dimethyl-24,25-dihydro- and 4,4-dimethyl-Δ^{24}-steryl acetates.

Measurement of 14α-sterol demethylase activity with [^{14}C]lanosterol

The activity of 14α-sterol demethylase in microsomes was assayed by measuring the incorporation of [^{14}C]-lanosterol into desmethylated sterols, and a number of factors that influence this activity are shown in Table 2. Enzyme activity could be detected only when the detergents Tween 40 or Tween 80 were added to the

cofactor solution to give a final assay concentration of 0.1% (v/v). Tween 80 increased 14α-sterol demethylase activity approx. 5-fold compared with Tween 40, but enzyme activity could be enhanced even further in microsomes that had been gassed with O_2/CO_2 (19:1) before their incubation with cofactors and radiolabelled substrate. This procedure was considered adequate for measuring 14α-sterol demethylase activity in *C. albicans*, and was used for the remainder of the experiments reported in this paper.

In some experiments microsomes were treated with CO to inhibit cytochrome *P*-450. This resulted in a marked decrease in the rate of metabolism of lanosterol as measured by the amount of unmetabolized [^{14}C]-lanosterol recovered from the 4,4-dimethylated sterol fraction on t.l.c. plates. Furthermore, 14α-sterol demethylase activity was abolished completely when the triazole antifungal ICI 153066 (at a concentration of 10 μM) was added to the assay system.

The results of a typical experiment with 50 nmol of [^{14}C]lanosterol and 3.9 mg of microsomal protein in the standard assay are shown in Fig. 3. When measured by either disappearance of 4,4-dimethylated sterol or production of desmethylated sterol, the rate of metabolism was constant at approx. 0.5 nmol/min for incubation periods up to 50 min, after which the metabolic rate slowed gradually. About 80% of the lanosterol added to the standard assay was converted into desmethylated sterols after an incubation period of 100 min. Radioactivity was detected only in the bands corresponding to 4,4-dimethylated sterols and desmethylated sterols, and at the origin (approx. 1% of the total amount of radioactivity) on t.l.c. plates from incubations up to 60 min (Fig. 4). However, when the incubation was extended to 100 min, radioactivity was also recovered from the area separating 4,4-dimethylated sterols from

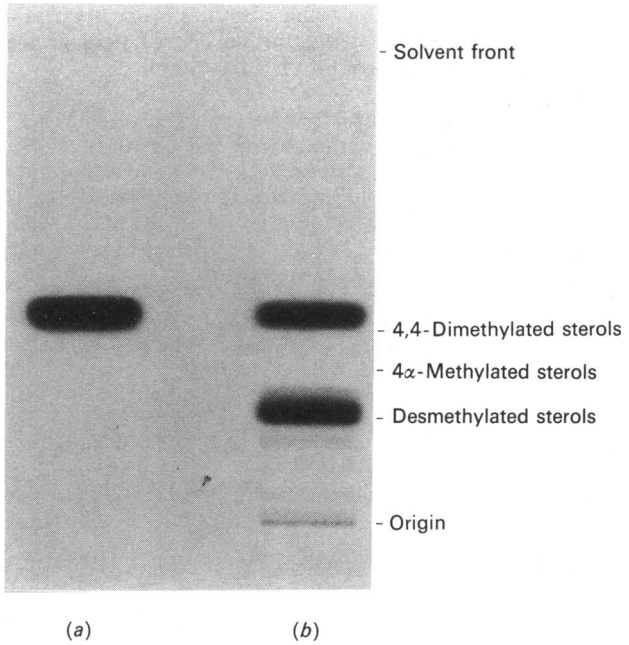

Fig. 4. Autoradiograph (8 h exposure) showing the distribution of radioactivity in the NSF lipids after incubation of microsomes with [^{14}C]lanosterol

Microsomes were incubated with cofactor solution and 50 nmol of [^{14}C]lanosterol for 50 min. After saponification with 3 M-KOH, the NSF lipids were extracted with light petroleum and chromatographed on t.l.c. plates of silica gel H, with toluene/diethyl ether (9:1, v/v) as the developing solvent. Track (*a*), NSF lipid (63921 d.p.m.) extracted from an incubation with boiled microsomes to inhibit enzyme activity; track (*b*), NSF lipid (64250 d.p.m.) extracted from an incubation with untreated microsomes.

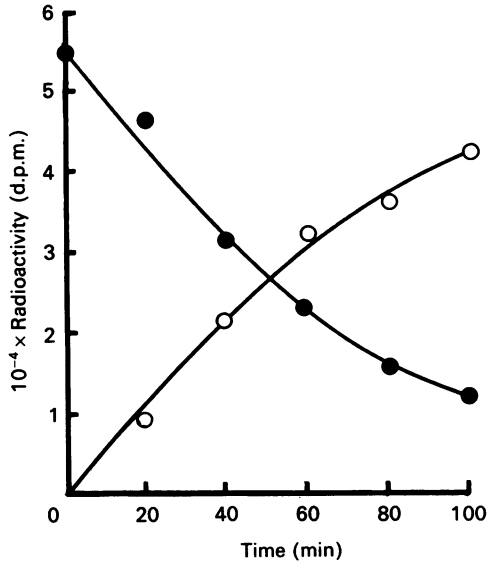

Fig. 3. Time course of [^{14}C]lanosterol conversion into [^{14}C]desmethylated sterols by microsomes

Values are means of triplicate determinations, which varied by < 10%: ●, 4,4-dimethylated sterols; ○, desmethylated sterols.

desmethylated sterols on t.l.c. plates (4α-methylated sterol fraction), which accounted for about 5% of the total amount of radioactivity added to the assay system. Analysis of the acetates of the desmethylated sterol fraction by t.l.c. on $AgNO_3$-impregnated silica gel gave essentially the same results as those obtained when sterol biosynthesis was measured with [2-^{14}C]mevalonic acid: the most abundant desmethylated sterol product had the chromatographic properties of ergosterol acetate, with smaller amounts of the two unidentified components.

The acetates of the 4,4-dimethylated sterol fraction were separated by t.l.c. on $AgNO_3$-impregnated silica gel into 4,4-dimethyl-Δ^{24}- and 4,4-dimethyl-24,25-dihydrosteryl acetates (Table 3). The most predominant 4,4-dimethylated sterols were of the Δ^{24}-type, accounting for approx. 77% of the total amount of radioactivity loaded on t.l.c. plates. In some experiments the 4,4-dimethyl-Δ^{24}-steryl acetates were further purified by t.l.c. on $AgNO_3$-impregnated alumina, and the results are included in Table 3. Lanosteryl acetate was the major radioactive component, and a much smaller amount of an unidentified component was also detected. A consistent observation was that t.l.c. on alumina resulted in the degradation of a relatively large amount of the steryl acetates, as judged by the accumulation of breakdown product(s) at the origin on t.l.c. plates. This phenomenon precluded a meaningful analysis of the 4,4-dimethyl-

Table 3. Probable chromatographic identities of the acetates of 4,4-dimethylated sterol fraction derived from incubations of microsomes with [^{14}C]lanosterol

The 4,4-dimethylated sterol fraction from t.l.c. on silica gel H was acetylated and chromatographed on silica-gel H t.l.c. plates impregnated with $AgNO_3$, with toluene/hexane (7:3, v/v) as the developing solvent. The 4,4-dimethyl-Δ^{24}-steryl acetates were re-chromatographed on alumina plates impregnated with $AgNO_3$, with toluene/hexane (1:3, v/v) as the developing solvent. Each experiment was done in duplicate, and the values given are the means of two values which varied by < 15%. The amount of radioactivity associated with each fraction is expressed as a percentage of the total radioactivity.

Sterols	Probable identity	R_F	Radioactivity (d.p.m.)	(%)
4,4-Dimethylated	4,4-Dimethyl-24,25-dihydrosteryl acetates	0.30	1237	10.7
	4,4-Dimethyl-Δ^{24}-steryl acetates	0.23	8863	76.6
	Origin	0.00	1469	12.7
4,4-Dimethylated-Δ^{24}	Lanosteryl acetate	0.38	2090	62.3
	Unknown	0.13	174	5.2
	Origin	0.00	1080	32.2

24,25-dihydrosteryl acetates, which comprised a small proportion of the 4,4-dimethylated sterol fraction. However, despite this situation, the results given in Table 3 indicate clearly that the predominant component of the 4,4-dimethylated sterol fraction was unmetabolized [^{14}C]lanosterol.

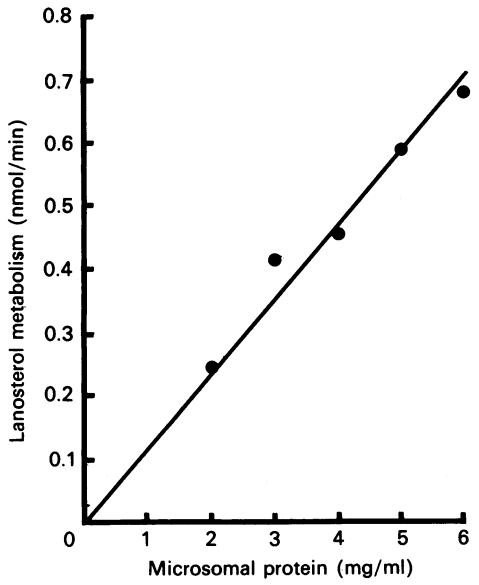

Fig. 5. Effect of microsomal protein concentration on 14α-sterol demethylase activity

The amount of microsomal protein was varied in 40 min incubations. Values are means of triplicate determinations, which varied by < 10%.

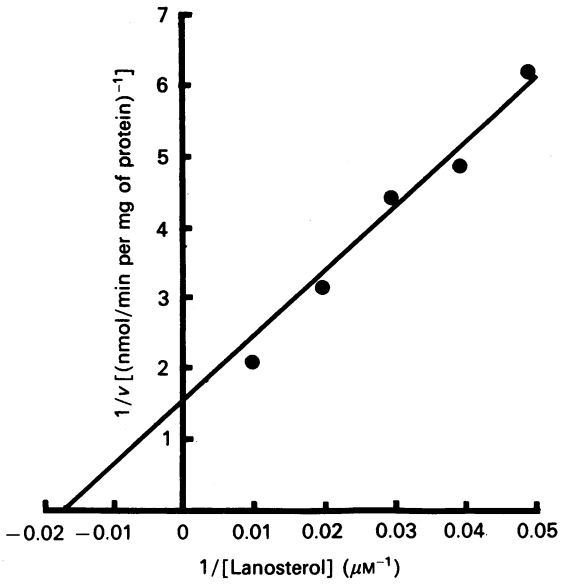

Fig. 6. Lineweaver–Burk analysis of microsomal 14α-sterol demethylase

Values are means of triplicate determinations, which varied by < 10%.

As shown in Fig. 5, the rate of lanosterol metabolism was linearly dependent on the amount of microsomal protein in an incubation also containing 50 nmol of lanosterol. In subsequent experiments with different concentrations of lanosterol, the amount of microsomal protein was adjusted so as to ensure that the incorporation of [^{14}C]lanosterol into desmethylated sterols was linear with respect to incubation time and microsomal protein. Under these conditions Lineweaver–Burk plots (Fig. 6) gave an apparent K_m of 52.73 ± 2.80 μM and an apparent $V_{max.}$ of 0.84 ± 0.14 nmol/min per mg of protein ($n = 3$ separate batches of exponential-phase cultures).

DISCUSSION

The results of the present study demonstrate that ergosterol biosynthesis in *C. albicans* cell lysates is dependent on the method of cell disruption. The incorporation of [2-^{14}C]mevalonic acid into desmethylated sterols was optimal provided that cell lysates were prepared at 4 °C with the Braun disintegrator. In some experiments the temperature of the lysate was allowed deliberately to exceed 4 °C (8–12 °C), with the loss of enzyme activity and accumulation of 4,4-dimethylated sterol precursors. The same was true when yeasts were disrupted with a French pressure cell, which was unable to maintain the low temperature of the lysate. Although sphaeroplasts were disrupted at approx. 4 °C, the digestion of yeast cell walls to form sphaeroplasts was at room temperature, which may have been responsible for the low amount of enzyme activity. Together, these results suggest that the metabolism of 4,4-dimethylated sterols to ergosterol is sensitive to increases in temperature during cell breakage. In view of this situation care was taken to standardize the Braun-disintegrator

disruption procedure to ensure that ergosterol biosynthesis was reproducible between batches of cells; the amount of [2-^{14}C]mevalonic acid incorporated into the 4,4-dimethylated sterol fraction was always low and reproducible, and it was concluded that the method was adequate for measuring 14α-sterol demethylase in cell lysates and microsomes.

The solubilization of [^{14}C]lanosterol with the detergents Tween 40 or Tween 80 in the cofactor solution was essential for the measurement of 14α-sterol demethylase activity in experiments with microsomes. In fact, Tween 80 has been used in similar experiments with 14α-sterol demethylase in plant (Rahier & Taton, 1986) and rat liver (Gibbons *et al.*, 1979) microsomes.

Oxygen is one of the substrates in the oxidative demethylation of lanosterol catalysed by 14α-sterol demethylase (Aoyama *et al.*, 1984; Trzaskos *et al.*, 1986) and, as expected, oxygenation of the microsomes enhanced the conversion of lanosterol into desmethylated sterols. Under these conditions there was a reciprocal relationship between the amount of radioactivity incorporated into desmethylated sterols and that lost from lanosterol, the major component of the 4,4-dimethylated sterol fraction (Fig. 3). In addition, a small amount of radioactivity was incorporated into a lipid component with an R_F value on silica-gel H t.l.c. plates corresponding to that of 4α-methylated sterols, which are metabolic precursors of ergosterol (Marriott, 1980). However, we could detect this component only in experiments where the rate of 14α-sterol demethylase activity slowed with time, when approx. 80% of the [^{14}C]lanosterol had been metabolized to desmethylated sterols. A chromatographic analysis of the desmethylated sterol fraction demonstrated that the major radiolabelled sterol product was ergosterol, when either [2-^{14}C]mevalonic acid or [^{14}C]lanosterol was used as a source of radioisotope. In contrast, zymosterol is the most abundant sterol biosynthesized from [^{14}C]lanosterol in broken-cell preparations of *S. cerevisiae* (Alexander *et al.*, 1974). The identity of the large amount of radioactivity detected at the origin on $AgNO_3$-impregnated t.l.c. plates is not known, but it has been ascribed to acidic breakdown products of the metabolic precursors of ergosterol (Alexander *et al.* 1974).

C. albicans cytochrome *P*-450-dependent 14α-sterol demethylase is sensitive to CO, like the same enzyme in *S. cerevisiae* (Aoyama *et al.*, 1984), the plant *Zea mays* (Rahier & Taton, 1986) and rat liver (Gibbons *et al.*, 1979; Trzaskos *et al.*, 1984). Furthermore, the mean apparent K_m value of 52.73 μM for the *C. albicans* enzyme compares closely with a value of 50 μM for the rat liver enzyme tested with 24,25-dihydrolanosterol in microsomal preparations (Gibbons *et al.*, 1976; Trzaskos *et al.*, 1984); there are no results for other fungi for comparison.

Cytochrome *P*-450-dependent 14α-sterol demethylase purified to homogeneity from *S. cerevisiae* (Aoyama *et al.*, 1987) and rat liver (Trzaskos *et al.*, 1986) is responsible for the complete oxidative demethylation of 24,25-dihydrolanosterol to 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol and formic acid, via three mono-oxygenation steps (i.e. $CH_3 \rightarrow CH_2OH \rightarrow CHO \rightarrow HCO_2H$). The inhibition of [^{14}C]lanosterol metabolism with CO or ICI 153066 in *C. albicans* microsomes indicates that the first oxidative step of the demethylation reaction is cytochrome-*P*-450-dependent. However, the role of this cytochrome in the subsequent oxidations is not known. The purification of cytochrome *P*-450-dependent 14α-sterol demethylase from *C. albicans* should resolve the full extent of the contribution of this enzyme to 14α-demethylation.

In conclusion, the present study is an important advance in the measurement and characterization of 14α-sterol demethylase in *C. albicans*, and should prove useful in the evaluation of azole antifungal agents which work by inhibiting the enzyme.

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