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Microbial Transformations of Steroids—VIII. Transformation of Progesterone by Whole Cells and Microsomes of *Aspergillus fumigatus*

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The filamentous fungus, *Aspergillus fumigatus*, efficiently hydroxylated exogenous progesterone producing, after 3 h of incubation, 11α- and 15β-hydroxyprogesterone as major products, 7β-hydroxyprogesterone as a minor product and trace amounts of 7β,15β- and 11α,15β-dihydroxyprogesterone. After 72 h the dihydroxyprogesterones were the sole metabolites in the culture medium. Microsomes, prepared by Ca^{2+} precipitation, catalysed only monohydroxylation of progesterone at the same sites as whole cells. Hydroxylation was dependent on NADPH (but not NADH) which was replaceable by $NaIO_4$. Hydroxylation was inhibited by carbon monoxide and by the azole fungicide, ketoconazole. Microsomes gave a dithionite-reduced, carbon monoxide difference absorbance spectrum with a peak at 448 nm and a Type-I progesterone-binding spectrum typical of cytochrome *P*450 interaction with substrate. Ketoconazole inhibition studies suggest the presence of two non-inducible cytochrome *P*450 progesterone hydroxylases, one possessing 7β site-selectivity, the other 11α/15β site-selectivity.

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INTRODUCTION

The fungi resemble higher eukaryotes in being multigenic for cytochrome *P*450 [1] and isoforms of the enzyme not only participate in endogenous biosynthetic reactions, (e.g. lanosterol 14α-demethylase [2] which catalyses the oxidative demethylation of lanosterol to 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol, a poly unsaturated intermediate in the biosynthesis of the fungal membrane sterol, ergosterol), but they also have activity in the detoxification of exogenous xenobiotics [3]. A common example of the latter reaction is the ability of filamentous fungi to perform regio- and stereo-selective hydroxylation of mammalian steroid hormones [4, 5]. Exogenous steroid transformation by these organisms usually produces mixtures of products in which either a limited number of hydroxy isomers, or their derivatives, predominates e.g. [6–9]. Significantly, the steroid metabolite pattern produced is a metabolic "fingerprint" of that organism.

The xenobiotic steroid hydroxylases of filamentous fungi are site-selective cytochromes *P*450 [10–15] that are distinct from their endogenous steroid-biosynthesis counterparts. Although the literature contains reports on steroid transformation by several *Aspergillus* species [4, 5], there are none on progesterone bioconversion by *A. fumigatus*.

Biochemical characterization of the purified isoforms of cytochrome *P*450 in *A. fumigatus* is of considerable importance because of their potential application in chemical asymmetric synthesis and in remote-site functionalization of poorly reactive, hydrocarbon-rich, compounds. In this communication we report that mycelia of *A. fumigatus* efficiently hydroxylate progesterone predominantly at sites 11α and 15β; the 7β site is also hydroxylated but much less efficiently. Microsomal fractions faithfully reproduced the hydroxylation stereochemistry of whole cells.

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EXPERIMENTAL

Materials

Culture media were purchased from Oxoid, Unipath Ltd. (Basingstoke, Hants, England).

All chemicals used in this work were of analytical grade and were obtained from Sigma Chemical Co. (Poole, Dorset, England) and BDH Ltd. (Poole, Dorset, England).

Media and cultivation of A. fumigatus

A. fumigatus was maintained on potato-dextrose agar plates and slopes [potato extract (4 g), dextrose (20 g) and agar No. 1 (15 g)/l of distilled water, pH 5.6] at 4°C until mycelia were required for transformation. The organism was sub-cultured every three months.

Steroid transformations were performed in liquid PYG medium [bactopeptone (10 g), yeast extract (5 g) and glucose (40 g)/l of distilled water].

Screening of A. fumigatus for steroid transformation

Steroid transformation was performed in a similar manner to that described previously [6]. Mycelia were grown in liquid medium until sufficient biomass had accumulated for progesterone transformation, which was performed by incubating an ethanolic solution of steroid at a final concentration of 0.2 mg ml^{-1} (and 1% ethanol). After removal of mycelia, metabolites and untransformed substrate were extracted from the aqueous medium by two vigorous shakings for at least 10 min with an equal volume of CH_3Cl. The combined extracts were evaporated to dryness and steroids were purified by reverse-phase HPLC [6].

Structural analysis of purified metabolites was performed by ^1H NMR spectroscopy on a Bruker WH400 MHz NMR spectrometer as described previously [6]. Where necessary assignments were confirmed by 2-D ^1H homonuclear correlation spectroscopy (COSY) [6].

Yields of metabolites were calculated by TLC. Steroids extracted from 100 ml transformations containing exactly 10 mg of progesterone were dissolved in an accurately measured minimum volume of HPLC-grade methanol (usually 0.1–0.2 ml). An exact volume (usually 5 μl) of this solution was spotted onto Kieselgel 60 F_{254} fluorescent high-performance TLC plates which were run in 65:35, v/v ethyl acetate–petroleum ether (60–80 boiling fraction). Individual spots were located under UV, scraped from the TLC plate and eluted in HPLC-grade methanol. After removing the silica gel, the methanol was evaporated and the steroids were redissolved in 1 ml of methanol. The UV absorbance at 240 nm of the individual steroid solutions was measured and the absolute amount calculated of steroid in the methanol extract and thence in each transformation medium.

Preparation of A. fumigatus microsomes and microsomal progesterone transformation

The abrasive grinding method, using acid-washed fine sand, described by Smith *et al.* [7, 9] and the homogenization technique of Ballard *et al.* [16] for disrupting mycelia both produced active progesterone-hydroxylating microsomes. However, microsomes prepared by homogenization were virtually devoid of contaminating mitochondrial cytochrome oxidase. Microsomes were precipitated by 8 mM Ca^{2+} [17], and then resuspended in buffer A [0.1 M Tris–HCl buffer pH 7.8 10 mM EDTA, 0.5 mM dithiothreitol (DTT) and 10% (v/v) glycerol].

Progesterone transformation was performed as described by Smith *et al.* [7, 9]. Briefly, 0.5 ml of microsome suspension was mixed with an equal volume of freshly made Buffer A containing 4 mM progesterone, 0.5 μCi [4-^{14}C]progesterone and 6 mM $NaIO_4$. Mixtures were incubated, with gentle shaking (30 rpm in an orbital shaker), for 3 h at 22°C and terminated by the addition of 3 ml $CHCl_3$. Steroids were extracted by vigorous shaking for 1 min. After standing to separate the two phases, the chloroform layer was removed and evaporated to dryness. Relative yields of metabolites were calculated by TLC [18]. Equal A_{240}-absorbing units of extracted steroids in methanol were spotted onto Kieselgel 60 F_{254} fluorescent high-performance TLC plates which were processed essentially as described above. Dried steroids eluted from TLC plates were redissolved in 50 μl of methanol and radioactivity determined by scintillation counting. The values for the metabolites and substrate progesterone were summed and divided into the value for individual metabolites to give the ratio and thence percentage of metabolite produced.

Spectral studies on A. fumigatus microsomes

Reduced-carbon monoxide (CO) cytochrome *P*450 difference spectra were obtained using the method of Omura and Sato [19], and substrate binding studies used the split-cell technique as described by Kelly *et al.* [20].

RESULTS

Identification of progesterone transformation products

Progesterone was progressively transformed into polar metabolites by 3- to 4-day-old mycelia over 72 h at 26°C in PYG medium. Five identical 100 ml cultures were used for each of three separate time-point incubations. In 3 h incubations two major spots and several minor ones are visible in the monohydroxyprogesterone region of the TLC [Fig. 1(a)]. Several minor spots, each more polar than monohydroxyprogesterones, are also visible, which suggests dihydroxylation occurs even in very brief incubations. After 24 h, two of the monohydroxy metabolites had been totally converted to dihydroxyprogesterones which are

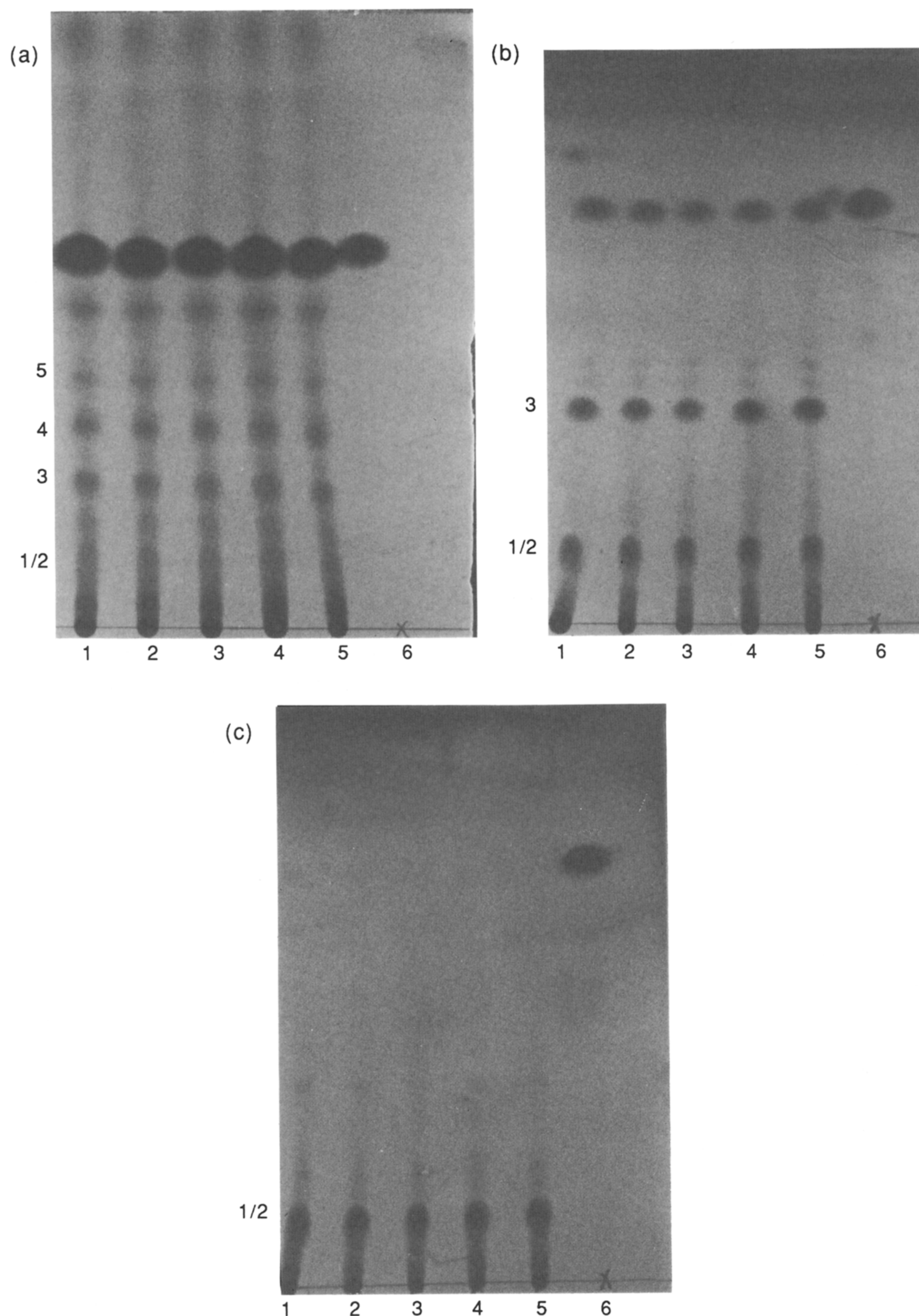

Fig. 1. Progesterone transformation by five replicate cultures of *A. fumigatus*. Mycelia were grown in liquid PYG medium for 4 days before the addition of progesterone to a final concentration of 0.1 mg.ml^{-1}. Incubations were for (a) 3 h; (b) 24 h; and (c) 72 h. Lanes 1–5, *A. fumigatus* progesterone transformation mixtures; lane 6, a marker of authentic progesterone. The metabolites are spot 1, 11α,15β-dihydroxyprogesterone; spot 2, 7β,15β-dihydroxyprogesterone; spot 3, 11α-hydroxyprogesterone; spot 4, 15β-hydroxyprogesterone; and spot 5, 7β-hydroxyprogesterone.

Table 1. ^{1}H-NMR data for progesterone and some monohydroxyprogesterones, and A. fumigatus progesterone transformation products

Compound	4-H	17α-H	18-H	19-H	21-H	CHOH
(a) NMR reference data for progesterone and some authentic derivatives used for the identification of progesterone metabolites produced by the A. fumigatus						
Progesterone	5.73	2.54	0.67	1.20	2.13	—
7β-Hydroxy-	5.78	2.49	0.71	1.23	2.14	3.47 (7α-H)
progesterone	(0.05)	(−0.05)	(0.04)	(0.03)	(0.01)	(2.41)
11α-Hydroxy-	5.74	2.57	0.71	1.33	2.14	4.04 (11β-H)
progesterone	(0.01)	(0.03)	(0.04)	(0.13)	(0.01)	(2.58)
15β-Hydroxy-	5.74	2.49	0.94	1.22	2.15	4.33 (15α-H)
progesterone	(0.01)	(−0.05)	(0.27)	(0.02)	(0.02)	(2.61)
7β,15β-Dihydroxy-	5.79	2.45	0.97	1.26	2.16	3.65 (7α-H)
progesterone						4.48 (15α-H)
	(0.06)	(−0.09)	(0.30)	(0.06)	(0.03)	(2.59, 7α-H)
						(2.76, 15α-H)
11α,15β-Dihydroxy-	5.75	2.52	0.98	1.35	2.16	4.07 (11β-H)
progesterone[a]						4.32 (15α-H)
	(0.02)	(−0.02)	(0.31)	(0.15)	(0.03)	(2.61, 11β-H)
						(2.60, 15α-H)
(b) NMR data for the A. fumigatus progesterone transformation metabolites 1–5						
Compound 1	5.77	2.51	0.98	1.35	2.15	4.09 (11β-H)
(11α, 15β-DHP)[b]						4.31 (15α-H)
	(0.02)	(−0.01)	(0.00)	(0.00)	(−0.01)	(0.02, 11β-H)
						(−0.01, 15α-H)
Compound 2	5.79	ND	0.98	1.26	2.17	3.64 (7α-H)
(7β,15β-DHP)						4.48 (15α-H)
	(0.00)		(0.01)	(0.00)	(0.01)	(−0.01, 7α-H)
						(0.00, 15α-H)
Compound 3	5.76	2.57	0.72	1.34	2.15	4.06 (11β-H)
(11α-HP)	(0.02)	(0.00)	(0.01)	(0.01)	(0.01)	(0.02)
Compound 4	5.76	2.50	0.96	1.23	2.16	4.34 (15α-H)
(15β-HP)	(0.02)	(0.01)	(0.02)	(0.01)	(0.01)	(0.01)
Compound 5	5.77	ND	0.72	1.22	2.16	3.47 (7α-H)
(7β-HP)	(−0.01)		(0.01)	(−0.01)	(0.02)	(0.00)

In (a) δ relative to Me_4Si. Increments relative to progesterone in parentheses; in (b) chemical shifts relative to authentic hydroxyprogesterones in parentheses [*cf* data in (a)].

[a] δ calculated from summed individual group increments, relative to progesterone, of 7β-, 11α- and 15β-monohydroxyprogesterone.

[b] HP, hydroxyprogesterone; DHP, dihydroxyprogesterone. ND, not detectable.

strongly UV-absorbing spots near the origin of the TLC [Fig. 1(b)]. By 72 h, progesterone transformation was complete [Fig. 1(c)].

^{1}H-NMR spectra of compounds purified from those three incubations confirmed all the metabolites to be hydroxylated progesterones. Androstene-based compounds were not detected showing C 17–C 20 lyase activity to be absent in this organism.

Three monohydroxylated progesterones; 7β- (compound 5), 11α- (compound 3), and 15β-hydroxyprogesterone (compound 4); and two dihydroxylated progesterones; 7β,15β- (compound 2), and 11α,15β-dihydroxyprogesterone (compound 1) were readily identified from their NMR spectra (Table 1). All retained the characteristic 4-H (s* or narrow d, δ *ca* 5.76–5.79) and 21-H_3 (s, δ *ca* 2.15–2.17) signals confirming retention of the pregnane side-chain during incubation.

*s, singlet; d, doublet; t, triplet; m, multiplet etc.

The positions of hydroxylations, and other substitutions, were determined from the distinctive features of the individual spectra, which are briefly summarized in the paragraphs below and in Table 1(b).

Compound 1. 11α,15β-Dihydroxyprogesterone. This compound has spectral similarities to both 11α- and 15β-monohydroxyprogesterone (compounds 3 and 4, respectively) which are described below. An authentic standard of this dihydroxy compound is not available. Thus, values for the chemical shifts of individual "fingerprint" signals for compound 1 [Table 1(b)] were calculated on the basis of additivity of the respective counterpart substituents of authentic 11α- and 15β-monohydroxyprogesterone [Table 1(a)], and are in line with expectation. COSY analysis confirmed the structure assignment.

Compound 2. 7β,15β-Dihydroxyprogesterone. The features of both 7β and of 15β hydroxylation (see below) were visible in compound 2 [21], but due to the

mutual deshielding effects of the two hydroxyls, which results from their close spatial proximity, both mid-field CHOH methine proton signals were shifted slightly to low-field than in the respective monohydroxyprogesterones. Again, COSY analysis confirmed the structure assignment.

Compound 3. 11α-Hydroxyprogesterone. A broad multiplet centred at δ 4.06 for the 11β proton; an exceptionally low-field 1β-H signal at δ 2.68, shifted down-field compared to progesterone by the close proximity of 11α-OH; a large shift to low-field of the 19-H_3 signal (δ 1.34) by *ca* 0.14 ppm; and a small but significant shift to low-field of *ca* 0.05 ppm of the 18-H_3 (δ 0.72) are the distinguishing features of compound 3, which are shared by authentic 11α-hydroxyprogesterone [21]. Compound 3 and authentic 11α-hydroxyprogesterone co-migrated on TLC and were co-retained on HPLC.

Compound 4. 15β-Hydroxyprogesterone. This compound is distinguished by the exceptional shift to low-field of the 18-H_3, (δ 0.96) compared to progesterone (δ 0.67), the lowest position occupied by this signal in a monohydroxylated progesterone. A moderately resolved multiplet at δ 4.34; singlets for 21-H_3 at δ 2.16 and for 19-H_3 at δ 1.23 coincided with counterpart signals of authentic 15β-hydroxyprogesterone [21] [Table 1(a)]. Compound 4 and 15β-hydroxyprogesterone have identical HPLC retention times and TLC R_f values.

Compound 5. 7β-Hydroxyprogesterone. This fraction was not chemically pure and was isolated in sub-milligram amounts. A broad multiplet for 7α-H at δ 3.47, the only signal of a methine proton of a CHOH system, apart from the 12α-H of 12β-hydroxyprogesterone (δ *ca* 3.45 [21]) to occupy this mid-field region of the spectrum; small but significant shifts to low-field of the 4-H (δ 5.77), 18-H_3 (δ 0.72) and 19-H_3 (δ 1.22) compared to progesterone, and contrasting the much larger shift to low-field of the 18-H_3 in 12β-hydroxyprogesterone, characterize compound 5 as 7β-hydroxyprogesterone [21]. HPLC and TLC properties of compound 5 match those of authentic 7β-hydroxyprogesterone [21].

Yields of progesterone metabolites

A. fumigatus hydroxylated exogenous progesterone extremely efficiently. After 3 h of incubation 11α- and 15β-hydroxyprogesterone were the major metabolites produced which accounted for approx. 39% of added substrate. Trace amounts, $<5\%$ each, of 7β-hydroxyprogesterone and of the two dihydroxylated progesterones were also isolated from this incubation (Table 2, column 1). After 24 h the amount of 11α-hydroxyprogesterone produced had further increased and accounted for *ca* 33% of added progesterone, whereas the concentration of 15β-hydroxyprogesterone had already fallen to below accurately measureable levels due to it being converted, by rehydroxylation, into 7β,15β- and 11α,15β-dihydroxyprogesterone. In this incubation the dihydroxyprogesterones together accounted for about 30% of added progesterone (Table 2, column 2). 7β-Hydroxyprogesterone could barely be detected then (Table 2, column 2). By 72 h monohydroxy metabolites had been replaced by their dihydroxylated counterparts. Approximately 74% of substrate progesterone had been converted into these two products (*ca* 48% 11α,15β- and 26% 7β,15β-dihydroxyprogesterone) (Table 2, column 3).

Table 2. Yields of hydroxyprogesterone metabolites isolated from A. fumigatus progesterone transformations

Compound (numbering shown in Fig. 1)	Yield of metabolite (mg per 10 mg progesterone) 3 h	24 h	72 h
11α,15β-Dihydroxyprogesterone (compound 1)	0.39	1.69	4.81
7β,15β-Dihydroxyprogesterone (compound 2)	0.36	1.37	2.54
11α-Hydroxyprogesterone (compound 3)	2.22	3.26	Trace
15β-Hydroxyprogesterone (compound 4)	1.74	Trace	ND
7β-Hydroxyprogesterone (compound 5)	0.37	Trace	ND

ND, not detectable.

Spectral studies on A. fumigatus microsomes

The reduced-CO difference absorbance spectrum of *A. fumigatus* microsomes, prepared from cultures 18 h after inoculation of spores, is shown in Fig. 2. The level of cytochrome *P*450 has been typically observed at 19 pmol mg^{-1} protein [16], but with the introduction of a double centrifugation step to pellet mitochondria and three washes of the microsomal pellet, a spectral maximum at 448 nm can be observed instead of the 451 nm peak reported previously. This shift is the likely consequence of the removal of cytochrome oxidase contamination, a common problem in fungal cytochrome *P*450 preparation [22] and was also observed following preparation of *A. fumigatus* microsomal cytochrome *P*450 by grinding mycelia. Substrate-binding

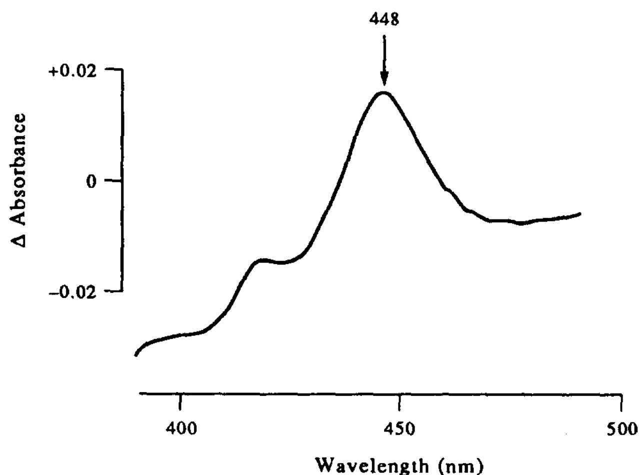

Fig. 2. Typical reduced-CO difference absorbance spectrum of *A. fumigatus* microsomes isolated from 18 h germinated spores. The cuvette contained 1.25 mg microsomal protein.

studies provided clear evidence of progesterone as a substrate of *A. fumigatus* cytochrome *P*450. Addition of progesterone resulted in a typical Type-I binding spectrum (Fig. 3) indicative of binding of progesterone to a cytochrome P450 active site and similar to spectra obtained between yeast cytochrome *P*450 and benzo[*a*]pyrene [20]. A maximum absorbance was observed at 387 nm, although a distinct minimum at approx. 420 nm was not clear. The additional absorbance maximum at 367 nm is also characteristic of benzo[*a*]pyrene substrate binding.

Progesterone hydroxylation by A. fumigatus microsomes

When microsomes, prepared by abrasive grinding, were incubated for 3 h at room temperature (22°C) with progesterone and $NaIO_4$, the substrate was converted into metabolites that were identified by TLC as being 7β-, 11α- and 15β-hydroxyprogesterone, i.e. the same three monohydroxy metabolites produced in whole-cell 3 h transformations. Comparison of relative yields of these metabolites from microsomal fractions obtained from mycelia 18 h after inoculation of spores showed variability. Moreover, unlike microsomes from mature mycelia those prepared from germinated spores failed to produce 7β-hydroxyprogesterone (results not shown) suggesting this activity to be different to that of the $11\alpha/15\beta$-hydroxylase (see below).

Boiling the microsomes totally inhibited progesterone hydroxylation. We found that the $NaIO_4$ requirement for activity was replaceable by NADPH and O_2, but not by NADH. NADPH- and $NaIO_4$-driven microsomal incubations produced identical sets of metabolites. Hydroxylation was completely inhibited by bubbling CO through microsome suspensions for 2 min prior to the addition of steroid (Table 3). These data show the progesterone hydroxylase(s) to be cytochrome(s) *P*450, particularly when considered together with the data on substrate-binding and ketoconazole inhibition.

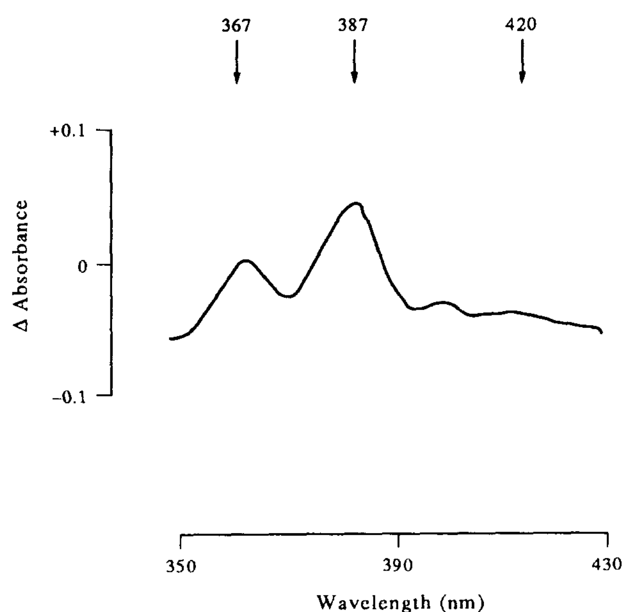

Fig. 3. Typical substrate-binding spectrum observed on addition of 68 μg ml^{-1} progesterone to a microsomal fraction containing 65 pmol cytochrome *P*450.

Table 3. The effect of NAD(P)H, $NaIO_4$, CO and ketoconazole on progesterone hydroxylation by A. fumigatus microsomes

Addition	Hydroxy metabolites produced (% of total steroid eluted from TLC)
3 mM NADPH (no $NaIO_4$)	13.3
3 mM NADH (no $NaIO_4$)	ND
3 mM $NaIO_4$ (no NAD[P]H)	10.3
No NAD(P)H or $NaIO_4$	ND
50 μM Ketoconazole + 3 mM $NaIO_4$	ND
Microsomes bubbled 2 min CO before addition of $NaIO_4$ & progesterone	ND
No progesterone + 3 mM $NaIO_4$	ND
Microsomes boiled for 1 min	ND

1.6 mg of microsomal protein was present per 1 ml incubation. ND, not detectable.

The inducibility of progesterone hydroxylase(s) was tested by comparing metabolite synthesis by microsomes prepared from 4-day-old progesterone pre-incubated mycelia (10 μg ml^{-1} for 16 h) with that of non preincubated controls. Pre-incubation was without effect (Table 4) proving that in this organism progesterone hydroxylase(s) is(are) constitutive (i.e. non-inducible). Also, the fact that virtually identical amounts of metabolites were produced by identical quantities of microsomes (Table 4) shows the hydroxylase(s), are not even amplifiable in mature mycelia. However, the difference in 7β-hydroxyprogesterone production by microsomes of mature mycelia and 18 h germinated spores suggests that this hydroxylase is distinct from both 11α- and 15β-hydroxylase (see below) and may be subject to different gene expression control.

Cycloheximide is a potent inhibitor of protein synthesis in *A. fumigatus*. When mycelia were pre-incubated with progesterone (10 μg ml^{-1}) and the inhibitor (5 μg ml^{-1}), subsequent progesterone hydroxylation by microsomes was substantially diminished (Table 4).

Table 4. The effect of pre-incubation of A. fumigatus mycelia with progesterone and cycloheximide on microsomal progesterone hydroxylation

Condition	Hydroxy metabolites produced (% of total steroid eluted from TLC)
Control, no progesterone pre-incubation	32
Progesterone pre-incubated mycelia	30
Progesterone and cycloheximide pre-incubated mycelia	10
Cycloheximide pre-incubated mycelia	6

The concentration of progesterone in the pre-incubation was 10 μg ml^{-1} and cycloheximide, 5 μg ml^{-1}; 1.5 mg of microsomal protein was present per 1 ml incubation.

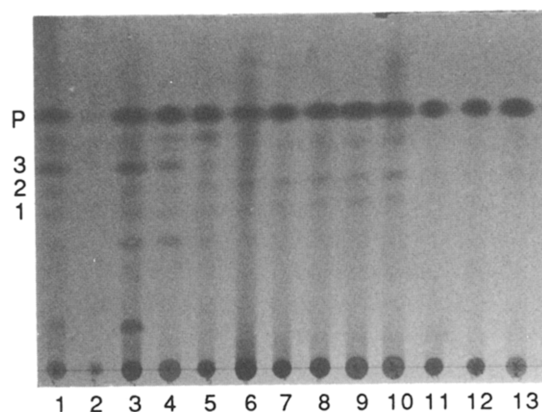

Fig. 4. The effect of ketokonazole on the inhibition of progesterone hydroxylation by non-pre-incubated microsomes (1.6 mg ml^{-1}). Lane 1, no ketoconazole; lane 2, no progesterone; lane 3, 0.01 μM ketoconazole; lane 4, 0.1 μM ketoconazole; lane 5, 0.5 μM ketoconazole; lane 6, 1 μM ketoconazole; lane 7, 2 μM ketoconazole; lane 8, 4 μM ketoconazole; lane 9, 6 μM ketoconazole; lane 10, 8 μM ketoconazole; lane 11, 10 μM ketoconazole; lane 12, 12.5 μM ketoconazole and lane 13, 15 μM ketoconazole. Spot 1, 11α-hydroxyprogesterone; spot 2, 15β-hydroxyprogesterone; spot 3, 7β-hydroxyprogesterone.

Controls, pre-incubated with cycloheximide alone, gave identical results (Table 4). We conclude from these data that progesterone hydroxylase activity must turnover fairly rapidly in the membrane.

Sterol 14α-demethylase cytochrome *P*450 is known to be inhibited by low concentrations of the azole fungicide, ketoconazole [23]. Figure 4 and Table 5 show that in *A. fumigatus*, microsomal progesterone hydroxylation was totally inhibited by ketoconazole concentrations greater than 12.5 μM (Fig. 4, lane 12; Table 5). Significantly, 7β-hydroxylation was much more sensitive to azole inhibition than either 11α- or 15β-hydroxylation, which were inhibited essentially in parallel and sharply between 8 and 12.5 μM ketoconazole (Fig. 4, lanes 10–12). On the other hand, 25% inhibition of 7β-hydroxylation was observed at ketoconazole concentrations as low as 0.1 μM and virtually 100% inhibition at 2 μM ketoconazole (Fig. 4, lanes 4 and 7). Thus, IC_{50} values of *ca* 0.5 and 10–12.5 μM respectively for ketoconazole inhibition of microsomal 7β-hydroxylation and 11α/15β-hydroxylation are one and two orders of magnitude higher than the counterpart value for inhibition of sterol 14α-demethylase cytochrome *P*450 [23]. No difference in azole sensitivity was observed for microsomes prepared from progesterone pre-incubated cells (results not shown). These data are also consistent with the progesterone hydroxylase(s) in *A. fumigatus* being cytochrome(s) *P*450, in agreement with the spectral data described above.

DISCUSSION

The genus *Aspergillus* performs efficient hydroxylation of exogenous steroids [4, 5]. Many strains transform a broad range of steroid substrates, including androstane-, oestrane- and pregnane-based substrates, and they also produce complex product mixtures in which a limited number of metabolites usually predominates. Thus, hydroxylation at sites 7β, 11α and 15β have been reported previously in *Aspergillus*, although not for *A. fumigatus*, and not with pregnane-based substrates. In this organism, 11α is the predominant site of progesterone hydroxylation (Table 2), and the amount of this isomer produced after both 3 and 24 h of incubation far exceeded that of all other metabolites produced. Likewise, 11α is the major hydroxylation site reported for many species of *Aspergillus* [4, 5]. By contrast, the 15β-hydroxy isomer appears to be a better substrate than 11α-hydroxyprogesterone for secondary hydroxylation. Yields of 15β-dihydroxy isomers predominate over their 11α-dihydroxy counterparts and 7β,11α-dihydroxyprogesterone was either not produced or was present in amounts too low to detect.

Hydroxylation data (Fig. 4 and Tables 3–5) show that *A. fumigatus* progesterone transforming activity is preserved during microsome isolation and that the monohydroxylation pattern of these preparations faithfully mirrors that of whole cells. Moreover, NADPH and molecular O_2, the physiological substrates for microsomal steroid hydroxylation, are replaceable by peroxy compounds such as $NaIO_4$, which use a shortened form of the cytochrome *P*450 hydroxylation cycle known as the "peroxide shunt" [24]. As expected, microsomes hydroxylate progesterone far less efficiently than whole cells. Therefore, it is not surprising that dihydroxy metabolites were not detected in cell-free incubations; the concentration of monohydroxyprogesterones synthesized during the first phase of incubation was probably insufficient to permit secondary hydroxylation. It is noteworthy that with microsomes from mature mycelia 7β-hydroxyprogesterone rather than the 11α-hydroxy isomer predominates (Fig. 4, Table 5).

The ketoconazole inhibition data (Fig. 4, Table 5) is evidence for at least two cytochrome *P*450 progesterone hydroxylase activities in *A. fumigatus*, although inhi-

Table 5. Inhibition of A. fumigatus microsomal progesterone hydroxylation by ketoconazole

Product	Ketoconazole concentration (μM)											
(% of steroid eluted from TLC)	None	0.01	0.1	0.5	1.0	2.0	4.0	6.0	8.0	10.0	12.5	15.0
Untransformed progesterone	60	59	69	71	77	86	82	80	80	95	100	100
7β-Hydroxyprogesterone (spot 3)	21	21	15	11	3	<1	<1	<1	0	0	0	0
11α-Hydroxyprogesterone (spot 1)	10	11	8	9	10	7	9	10	10	5	<1	0
15β-Hydroxyprogesterone (spot 2)	9	9	8	9	10	7	9	10	10	<1	<1	0

1.6 mg microsomal protein was present per 1 ml incubation.

bition data, in the absence of enzyme purification, is not absolutely definitive. Thus a totally unambiguous answer to the question of the number of progesterone hydroxylases in *A. fumigatus* must await purification of the proteins themselves, which is currently being performed in our laboratories.

Limited data are available on the regulation of gene expression of steroid hydroxylase cytochromes *P*450 in filamentous fungi. The steroid 11α-hydroxylase cytochrome *P*450 of *Rhizopus nigricans* and *A. ochraceus* are reputed to be inducible by substrate [11–14]. In *A. fumigatus* the opposite is the case, i.e. all three progesterone hydroxylase activities seem to be constitutive in mature mycelia, as appear to be the 11α- and 15β-hydroxylase activities in young spore germlings. The control of expression of cytochrome *P*450 progesterone hydroxylase genes awaits elucidation as must the true physiological role of these cytochromes *P*450 in *A. fumigatus*, their relevance in determining azide antifungal tolerance or any effects when present as a human pathogen.

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