Is mRNA sequestration involved in the regulation of progesterone 14α-hydroxylase cytochrome P-450 expression in 
*Mucor hiemalis*?

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**INTRODUCTION**

A common feature of filamentous fungi is their ability to biotransform steroids, often by regio- and stereoselective hydroxylation. The site of hydroxylation and the efficiency of the process are often unique and characteristic features of a fungal genus or species (Charney & Herzog, 1967; Smith, 1974; Sariaslani, 1991). Some microbial steroid transformation processes, for example 11α-hydroxylation, are so efficient and cost effective that these reactions have replaced traditional chemical routes for the synthesis of valuable pharmaceuticals such as anti-inflammatory corticosteroids (Hogg, 1992).

Fungal steroid hydroxylase enzymes are site-selective cytochrome P-450 monoxygenases. They are terminal acceptor proteins in type-I NADPH-dependent microsomal electron transport chains (Nebert et al., 1991; Durst et al., 1992).

The progesterone 11α-hydroxylases of *Rhizopus nigricans* (Breskvar & Hudnik-Plevnik, 1978) and *Aspergillus ochraceus* (Jayanthi et al., 1982; Madyastha et al., 1984; Samanta & Ghosh, 1987) are reputed to be inducible by steroid substrate, but little is known about the details of the regulation mechanisms involved. *Mucor hiemalis*, which belongs to the order *Mucorales*, an order that includes *Rhizopus* species, has been reported also to 11α-hydroxylate steroids (Charney & Herzog, 1967; Smith, 1974). Here we report a strain of *Mucor hiemalis* that expresses a cytochrome P-450-dependent enzyme which predominantly 14α-hydroxylates progesterone. Secondary hydroxylation reactions convert this 14α-hydroxyprogesterone to a complex mixture of dihydroxylated progesterones. The results of experiments on the regulation of the progesterone 14α-hydroxylase cytochrome P-450 activity is consistent with the sequestration of the mRNA encoding this hydroxylase.

**METHODS**

**Strains.** *Mucor hiemalis* was kindly supplied by Dr R. L. Evans (School of Biological Sciences, Queen Mary & Westfield College, University of London).

**Media and culture maintenance.** Cultures of *M. hiemalis* were grown to near confluence on PYG-agar plates (2%, w/v, glucose, 1%, w/v, Bacto-peptone, 1%, w/v, Bacto-yeast extract supplemented with 2%, w/v, agar) at 26 °C. They were stored on PYG agar slopes in sterile 20 ml McCartney bottles at 4 °C. The organism was sub-cultured after 6 months.

**Small-scale transformation of progesterone by *M. hiemalis***
mycelia. Mycelia were grown, with gentle shaking, for 3-4 d at 26 °C in 100 ml batches of liquid PYG broth contained in 250 ml conical flasks. Mycelia were harvested by aseptic vacuum filtration and washed with 300 ml of sterile, de-ionized, water. Following harvesting and washing, the mycelial cake was divided into two equal portions and each portion was transferred into 150 ml conical flasks containing 50 ml of sterile, de-ionized, water. Progesterone (20 mg ml⁻¹ in ethanol) was added to a final concentration of 100 µg ml⁻¹. Flasks were incubated for 6, 24, 48 and 72 h, and then the mycelia were harvested by filtration under vacuum. Steroids were extracted from 10 ml samples of filtrate by vigorously shaking twice with 1.5 vols of chloroform. This phase was collected, combined and evaporated to dryness. Solids were re-dissolved in 100 µl of HPLC-grade methanol and equal absorbance units of each sample were spotted onto Merck Kieselgel 60 F₂₅₄ fluorescent high-performance TLC plates, which were developed in an ethyl acetate/petroleum ether (65:35, v/v) solvent system. Steroids were visualized by UV absorbance and the plates photographed. Progesterone hydroxylase activity was induced by pre-incubation of mycelia with either progesterone or 14α-hydroxy-progesterone (1 and 10 µg ml⁻¹) for 16 h immediately prior to the main progesterone transformation incubation.

Structural analysis of progesterone transformation products. The structures of the purified products were determined by ¹H NMR spectroscopy on a Bruker WH 400 MHz spectrometer as previously described (Smith et al., 1988; Kirk et al., 1990).

Inhibition of nucleic acid and protein synthesis. From 10 mg ml⁻¹ stock solutions (made up with sterile de-ionized water), actinomycin D or cycloheximide were added, at a final concentration of 100 µg ml⁻¹, to mature 3-4-d-old cultures of M. hiemalis and incubated overnight (16 h). At this concentration both compounds completely inhibited mycelial growth on potato-dextrose agar plates for at least 4 d at 26 °C. Mycelia were filtered, washed and transferred into 150 ml conical flasks containing 50 ml sterile de-ionized water and substrate amounts of progesterone (100 µg ml⁻¹). The flasks were incubated for 6 h at 26 °C. Where indicated, inhibitor was also added after pre-incubation at the beginning of progesterone transformations.

Heat-stress of M. hiemalis mycelia. M. hiemalis was cultured as described above and then heat-stressed for 1 h at 37 °C before transformation of progesterone at 26 °C. Optimum time and temperature for heat-stress were determined by TLC analysis of 6 h post-heat-stress progesterone transformation mixtures.

RESULTS
Progesterone transformation products of M. hiemalis

The structures of major metabolites in a possible transformation pathway of progesterone, as determined by ¹H NMR spectroscopy, are shown in Fig. 1. Readers are referred to a series of articles by Smith et al. (1988,
Fig. 2. (a) TLC of small-scale progesterone transformations by \textit{M. hiemalis} incubated for 6 (lane 1), 24 (lane 2), 48 (lane 3) and 72 h (lane 4). Spots below progesterone are transformation products. Lane 5, progesterone marker; lane 6, 14a-HP marker; lane 7, 11\alpha-HP marker. (b) TLC of large-scale 6 (lane 1) and 72 h (lane 2) progesterone transformation by \textit{M. hiemalis}. Purified metabolites are: 6\alpha, 14a-DHP (lane 3), 6\beta, 14a-DHP (lane 4), 9\alpha, 14a-DHP (lane 5), 7\alpha, 14a-DHP (lane 6), 7\alpha-HP (lane 7), 9\alpha-HP (lane 8), 15\beta-HP (lane 9), 14\alpha-HP (lane 10), 6\beta-HP (lane 11), 9\alpha-OH-14-dehydroprogesterone (lane 12), 8(9), 14-didehydroprogesterone (lane 13) and progesterone (lane 14). Spots 1–12 are identified metabolites.

Fig. 3. The effect of pre-incubation of \textit{M. hiemalis} with low concentrations of steroid prior to transformation of progesterone. (a) TLC showing transformation patterns of small-scale incubations with progesterone as inducer. Lanes 1–3 are 6 h transformations and lanes 6–8 are 72 h transformations. Lanes: 1 and 6, control pre-incubation without progesterone; 2 and 7, mycelia pre-incubated with 1.0 \mu g progesterone ml\(^{-1}\) for 16 h; 3 and 8, mycelia pre-incubated with 10 \mu g progesterone ml\(^{-1}\) for 16 h. (b) As for (a) except mycelia were pre-incubated with 14\alpha-HP. Lane 4, progesterone marker; lane 5, 14\alpha-HP marker.

1989a, b, 1991) and Kirk \textit{et al.} (1990) for the NMR details of the individual common metabolites described here. In small-scale incubations, the transformation of progesterone for 6 h produced mainly 14\alpha-hydroxyprogesterone (HP) plus traces of 7\alpha, 14\alpha-, 9\alpha, 14\alpha- and 6\beta, 14\alpha-dihydroxyprogesterone (DHP) (Fig. 2a, lane 1). After 24 h (Fig. 2a, lane 2) only 14\alpha-DHPs were visible on TLC; metabolite patterns changed little thereafter (Fig. 2a, lanes 3 and 4). In large-scale incubations several minor metabolites, mainly mono-HPs, were isolated. They were 6\beta-, 7\alpha-, 9\alpha- and 15\beta-HP. Traces of 6\alpha, 14\alpha-DHP, 9\alpha-hydroxy-14(15)-didehydroprogesterone and 8(9), 14(15)-didehydroprogesterone (see below) were also isolated (Fig. 2b). Although several other minor progesterone metabolites were produced they were isolated in insufficient chemical purity and concentration to permit structural determination. 11\alpha-HP was not detected in any incubation. A novel microbial progesterone metabolite, 8(9), 14(15)-didehydroprogesterone (structure shown in Fig. 1), i.e. pregna-4, 8\[9], 14\[15]-triene-3, 20-dione, \Delta^{8,11}-progesterone (Fig. 2b, spot 11) was produced during longer transformation times (72 h). The NMR details which led to structural assignment of compound 11 are given below.

\textbf{Compound 11} (Fig. 2b, Spot 11). 8(9), 14-Didehydro-
The low polarity of compound 11, the lowest of the metabolites purified, which is shown by its close proximity on TLC to progesterone, its long retention time on reverse-phase HPLC and the absence of a mid-field 

**Effect of ketoconazole on steroid transformation in** *M. hiemalis*

In 6 h incubations of *M. hiemalis* with 47 µM ketoconazole (25 µg ml⁻¹), progesterone hydroxylation was markedly reduced and with 188 µM ketoconazole (100 µg ml⁻¹) it was completely inhibited (Fig. 4).
Progesterone 14α-hydroxylase regulation in *M. hiemalis*

Fig. 6. TLCs showing the effect of 100 μg actinomycin D ml⁻¹ on progesterone hydroxylation by *M. hiemalis* in 6 h transformation incubations with 100 μg progesterone ml⁻¹. Lanes: 1, non pre-incubated 6 h progesterone transformation incubation without actinomycin D (control); 2, as for lane 1 except mycelia were pre-incubated overnight with 1 μg progesterone ml⁻¹; 3, as for lane 2 except 14α-HP replaced progesterone in the pre-incubation; 4, as for lane 1 except both the pre-incubation and 6 h transformation incubation contained actinomycin D; 5, as for lane 4 except the pre-incubation also contained 1 μg progesterone ml⁻¹; 6, as for lane 5 except the pre-incubation contained 14α-HP; 7, as for lane 1 except mycelia were pre-incubated with actinomycin D; 8, as for lane 7 except the pre-incubation also contained 1 μg progesterone ml⁻¹; 9, as for lane 8 except 14α-HP replaced progesterone in the pre-incubation; 10, progesterone marker; 11, 14α-HP marker; 12, 9α,14α-DHP marker; 13, 6β,14α-DHP marker.

cycloheximide inhibition (cf. Fig. 5, lanes 4–6 with 1–3). When cycloheximide was present only during pre-incubation, progesterone 14α-hydroxylase activity was detected in a subsequent transformation (Fig. 5, lane 7), even in the absence of progesterone or 14α-HP induction (Fig. 5, lanes 8 and 9).

14α-HP is required as a substrate for the production of the respective 14α-dihydroxylated derivatives by progesterone 6β-, 7α- and 9α-hydroxylases. In a 6 h transformation an increased production of DHPs was seen when progesterone was replaced by 14α-HP (cf. Fig. 5, lanes 1 and 10). These data do not allow differentiation between a requirement for a threshold concentration of 14α-HP before secondary hydroxylation commences or 14α-HP-dependent induction of the 6β-, 7α- and 9α-hydroxylases.

Effects of actinomycin D on progesterone hydroxylation

Actinomycin D in both the pre-incubation and transformation incubation (Fig. 6, lanes 4–6) stimulated progesterone transformation in similar fashion to overnight pre-incubation with 1 μg 14α-HP ml⁻¹ (Fig. 6, lane 3). Production of 14α-HP and the DHPs was also obtained when actinomycin D was added only during pre-incubation (cf. Fig. 6, lanes 7–9 with 1, 2 and 4–6). The presence of steroid during pre-incubation with actinomycin D did not enhance the levels of steroid hydroxylase cytochrome P-450 activities (cf. Fig. 6, lane 4 with 5 and 6). Identical results were obtained if actinomycin D was
replaced with 100 μg ml⁻¹ of the transcription inhibitor ethidium bromide (data not presented) or by heat-stress for 1 h at 37 °C (Fig. 7), showing that our data are not artefacts of actinomycin D or toxic chemicals.

**DISCUSSION**

**Progesterone metabolite production**

A possible three-step pathway of progesterone transformation in *M. hiemalis* is given in Fig. 1, showing that progesterone is predominantly hydroxylated regio- and stereoselectively at a number of positions to give a range of mono- and dihydroxylated metabolites. Therefore, does *M. hiemalis* possess a single progesterone hydroxylase (i.e. 14α-hydroxylase), which is capable of multi-site hydroxylation, or are there multiple forms of hydroxylase enzyme each possessing its own unique hydroxylation site-selectivity? In the latter case 14α-HP might act as an inducer of the expression of these genes. Biochemical characterization of pure progesterone 14α-hydroxylase is required to distinguish these options but progesterone 9α hydroxylation is readily explained. The 9α and 14α hydrogens, meta to each other on the α face of ring C, are in close spatial proximity. Movement and/or slippage of loosely bound substrate in the hydroxylase active site would result in hydroxylation at both sites. Minor amounts of 9α-hydroxy metabolites often accompany their 14α-hydroxy counterparts in complex transformation mixtures.

Two novel unsaturated progesterone metabolites, 9α-hydroxy-14-dehydroprogesterone (data not presented) and 8(9),14(15)-didehydroprogesterone, the latter possibly produced by two consecutive dehydrations of 9α,14α-DHP, were isolated from 72 h progesterone incubations. As far as we are aware neither compound has been identified previously as a progesterone metabolite of *Mucor*.

Fungal sterol 14α-demethylase cytochrome P-450 (Ballard et al., 1990) and *Phycomyces blakesleeanus* progesterone 7α-hydroxylase cytochrome P-450 (Smith et al., 1991) are inhibited by azole fungicides such as ketoconazole. The inhibition of progesterone transformation in *M. hiemalis* with ketoconazole (Fig. 4) confirms that the progesterone hydroxylases are cytochromes P-450. However, azole inhibition of didehydroprogesterone production does not show whether the dehydratase activity is cytochrome P-450-mediated because production of this metabolite would automatically be blocked by azole inhibition of progesterone 9α and 14α hydroxylation, the precursor reactions to dehydration.

**Cytochrome P-450 transcriptional regulation by cycloheximide**

Low concentrations of cycloheximide increase the expression of rat cytochrome P-450 gene *CYP1A1* (Hamilton et al., 1992) and the chicken P-450 genes *CYP2H7* and *CYP2H2* (Lusska et al., 1992), a phenomenon known as super-induction. A labile, negative-regulatory protein is thought to bind to these genes preventing transcriptional initiation. An identical cycloheximide stimulatory effect was observed in the expression of the *Neurospora crassa* cytochrome P-450 gene *CYP54* (CI-1) (Attar et al., 1989; Grotewold et al., 1989). Super-induction did not occur in our system because cycloheximide prevents progesterone 14α hydroxylation when present throughout pre-incubation and transformation.

**Regulation of progesterone 14α-hydroxylase expression in *M. hiemalis***

Substrate stimulation of steroid 11α-hydroxylation cytochrome P-450 gene expression has been described in *Rhizopus nigricans* (stolonifer) (Breskvar & Hudnik-Plevnik, 1978) and *Aspergillus ochraceus* (Jayanthi et al., 1982; Madyastha et al., 1984; Samanta & Ghosh, 1987) but, in the absence of detail, the mechanism of the process remains unknown. We also find that substrate stimulates progesterone 14α-hydroxylase activity in pre-incubated *M. hiemalis* mycelia with progesterone, although it is important to note that this phenomenon is not universal in filamentous fungi (unpublished data).

If progesterone 14α-hydroxylase cytochrome P-450 gene transcription is induced by substrate, expression of this gene will be inhibited by blocking either transcription or translation even in the presence of substrate. To our surprise cycloheximide had diametrically opposite effects on progesterone hydroxylation to that of actinomycin D and heat-shock. Whilst, as expected, the former inhibited hydroxylation, the latter stimulated it. These data are consistent with the presence of latent hydroxylase mRNA whose expression is normally repressed in cells not exposed to steroid. Repression might be achieved through sequestration of the mRNA by a labile, bound protein(s). This mechanism of regulation has been described in other systems (Hentze, 1991; Lorenzi et al., 1992; Porter & Coon, 1991). When cycloheximide is present throughout pre-incubation and transformation, all protein synthesis, including that of steroid hydroxylase enzymes, is inhibited. Similarly, when actinomycin D is present throughout, transcription is totally blocked, including that of the gene encoding the sequestering protein. However, under this latter condition the stored progesterone 14α-hydroxylase mRNA is released for translation when the labile sequestering protein dissociates from the mRNA. The rapid appearance of progesterone 14α-hydroxylase activity in actinomycin-treated cells is indicative of the short half-life of the sequestering protein, hence our appellation, labile. It is noteworthy that identical effects of cycloheximide and actinomycin D have been reported on the expression of the aryl hydrocarbon hydroxylase cytochrome P-450, *CYP1A1*, in cultured mouse hepatocytes (Nemoto & Sakurai, 1991).

Heat-stress induces the expression of heat-shock genes but inhibits the expression of the vast majority of other genes. Since steroid hydroxylase cytochromes P-450 are
not heat-shock proteins, our heat-stress data lend additional support to the mRNA sequestration regulation model.

The sequestration model proposed permits progesterone induction of P-450 protein by one of two methods (Fig. 8). Either, steroid represses \( lsp \) mRNA transcription and hence \( lsp \) protein production which, in turn, results in the rapid availability of P-450 mRNA for translation, or steroid releases P-450 mRNA from sequestration by directly binding to \( lsp \) protein.

Only quantification of the individual mRNA species involved in progesterone 14\( \alpha \)-hydroxylase expression can distinguish the correct mechanism of regulation of this cytochrome P-450, a task which we are currently performing.

ACKNOWLEDGEMENTS

The NMR spectroscopy was performed by Mr Peter Haycock of the ULIRS WH-400 NMR service at Queen Mary & Westfield College. We are indebted to the late Professor David N. Kirk (Chemistry Department, QMW) for his help in confirming our assignment of the structure of pregna-4, 8(9),14(15)-triene-3,20-dione. T.H.N.A. acknowledges the award of a SERC studentship.

REFERENCES


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Received 2 December 1993; revised 14 January 1994; accepted 20 January 1994.