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PROGESTERONE SIDE-CHAIN CLEAVAGE BY *PAECILOMYCES LILACINUS*

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Abstract—The filamentous fungus *Paecilomyces lilacinus* converts progesterone to more polar compounds by a side-chain cleavage reaction. We have investigated the role of cytochrome P-450 in this mono-oxygenase reaction and confirmed the involvement of other cytosolic enzymes. The activity was found to be NADPH-dependent and inducible by pre-exposure of mycelium to progesterone.

INTRODUCTION

Fungal biotransformations of steroids are among the earliest examples of biocatalysis to achieve stereo- and regio-specific conversion [1]. *Paecilomyces lilacinus* (Thom) Samson was previously reported to undertake the biotransformation of progesterone to more polar compounds such as 4-androstene-3,17-dione and testosterone and subsequently to D-homo-17α-oxa-4-androsten-3,17-dione [2]. The reaction of progesterone side-chain cleavage is catalysed by a cytochrome P-450 mono-oxygenase in higher eukaryotes [3] but *Cylindrocarpon radicicola* has been observed to utilize a cytosolic non-P-450 mono-oxygenase [4] for this biotransformation. The reaction proceeds from progesterone to 4-androsten-17β-ol-3-one-17-acetate where an esterase can yield testosterone [5]. Recent reports indicate that C-17 side-chain cleavage of progesterone with P-450-dependent C_{17-20}-lyase from higher eukaryotes may involve a Baeyer–Villiger rearrangement [6] in the reaction mechanism, very similar to the analogous reaction in filamentous fungi [4].

We present here studies on the microsomal and cytosolic fractions of *P. lilacinus* which were examined for activity in progesterone metabolism and for the presence of cytochrome P-450. Other fungal cytochrome P-450s are known to be involved in progesterone hydroxylation [7, 8] as well as in sterol 14α-demethylation, a step in ergosterol biosynthesis [9], and are located in the microsomal fraction.

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RESULTS AND DISCUSSION

Cell disruption studies indicated the presence of a low level of cytochrome P-450 in the microsomal fraction of *P. lilacinus*, but not in the cytosolic or mitochondrial fractions. Treatment of the mycelia with 0.1 mg ml^{-1} progesterone did not induce high levels of cytochrome P-450 as reported for other fungi [8] and a specific content of 3.5 ± 0.6 pmol mg^{-1} (microsomal protein) was observed. However, this was higher than in microsomal fractions obtained from mycelia with no prior exposure to progesterone where no cytochrome P-450 was detected. No substrate binding of progesterone to cytochrome P-450 was observed as indicated by spectral changes using microsomal fractions of other fungi with a maximum at 387 nm and a minimum at approximately 415 nm [10]. These studies suggested that cytochrome P-450 was not involved in steroid biotransformation in this fungus, which was confirmed by the activity studies. The detection of cytochrome P-450 under prior exposure to progesterone seems to reflect altered regulation of expression or turnover of cytochrome P-450 not involved in progesterone metabolism, e.g. possibly sterol 14α-demethylase. Further studies on this cytochrome P-450 may be valuable as sterol 14α-demethylase is the target of azole antifungals [11] and *P. lilacinus* is an opportunistic pathogen in immunocompromized patients [12, 13].

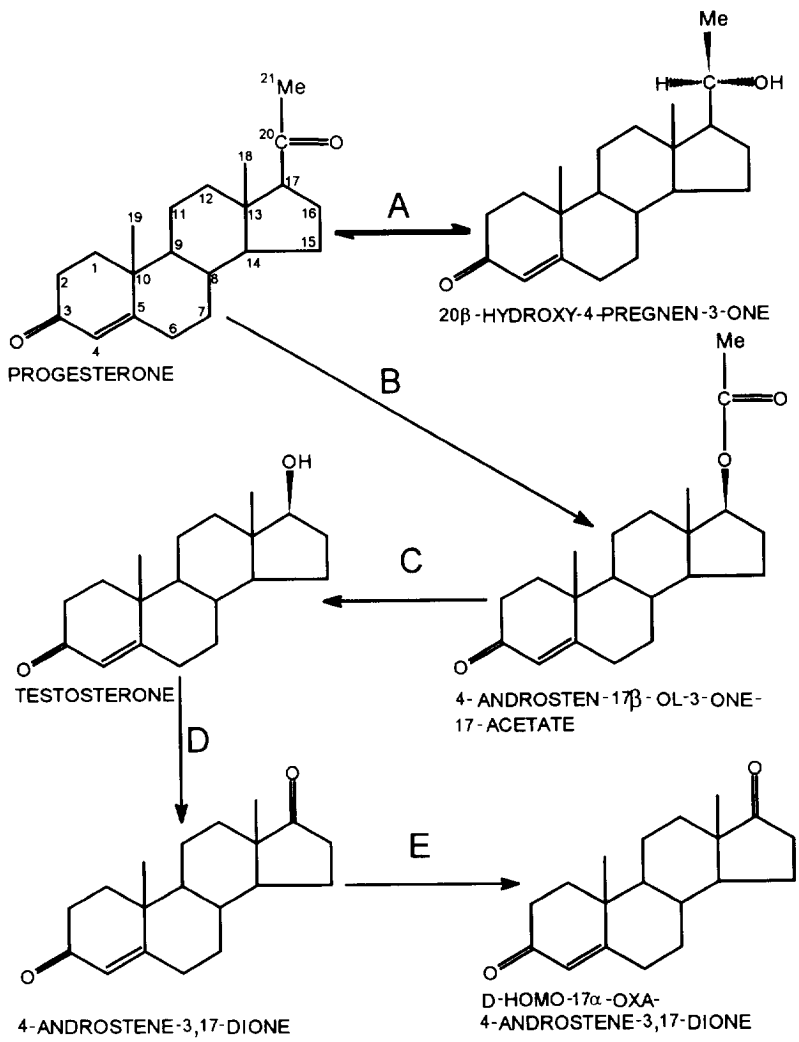
The investigation of *in vitro* progesterone side-chain cleavage activity confirmed the involvement of enzyme(s) other than cytochrome P-450. Progesterone biotransformation was detected in the cytosol and was found to be inducible by pre-exposure of mycelium to progesterone. No constitutive activity for the biotransformation

was observed and cycloheximide treatment, prior to mycelial exposure to progesterone, reduced activity by more than 90%, suggesting the activity required *de novo* protein synthesis (TLC data available on request). Table 1 shows that the activity was sensitive to heat, but not carbon monoxide, indicating the protein responsible was not a cytochrome P-450. The activity was not absolutely dependent on NADPH, and appeared to resemble the NADPH-dependent steroid mono-oxygenase characterized in *C. radicicola* by Itagaki [4]. 20β-Hydroxy-4-pregnen-3-one and 17α-hydroxy-4-pregnen-3,20-dione were also observed to be substrates for the enzyme.

Table 1. Activities of cytosolic progesterone side-chain cleavage reaction

SAMPLE	Activity (nmol min^{-1} mg^{-1})
Cytosol + NADPH	0.16 ± 0.04
Cytosol − NADPH	0.04 ± 0.006
Cytosol + NADPH with CO bubbling	0.20 ± 0.01
Heat-inhibited cytosol + NADPH	0.007 ± 0.002

The role of steroid-hydroxylating cytochrome P-450s in fungi is of interest with regard to their possible function in foreign compound metabolism. Progesterone side-chain cleavage activity has been observed in a variety of fungi and our study and that of Itagaki [4] show the biotransformation is not cytochrome P-450 mediated. This may generally be the case where fungal side-chain cleavage activity is observed. The absence of any hydroxylation of steroid by *P. lilacinus* indicates that no other cytochrome P-450s were induced to metabolize progesterone. Whether this fungus has lost the genes encoding such enzymes remains to be determined, but the evolution and regulation of the cytosolic protein observed in *P. lilacinus* will be of interest for comparison to the inducible steroid cytochrome P-450 hydroxylases.



Scheme 1. Metabolic pathway of side-chain cleavage of progesterone in filamentous fungus *Paecilomyces lilacinus*. A, 20β-dehydrogenase; B and E, monooxygenase; C, esterase; and D, 17β-dehydrogenase.

Among the metabolites of progesterone biotransformation we also found certain amounts of 20β-hydroxy-4-pregnen-3-one which indicate a 20β-hydroxy steroid dehydrogenase activity in the cytosol. This activity is presumably competing with the side-chain degrading enzymes as it has been described for the analogous reaction [14] (Scheme 1).

As side-chain cleavage of progesterone is of interest for the preparation of therapeutic steroids the purification of the cytosolic protein described here, together with its cloning and overexpression, may result in biotechnological application. The steroid metabolites, e.g. 4-androstene-3,17-dione may be useful for the production of androgens, estrogens and D-homo-17α-oxa-1,4-androstadiene-3,17-dione, which is approved for the treatment of mammary cancer [15] as well as 14α-hydroxyandrostanes which have promising anticancer properties [1].

EXPERIMENTAL

Strain cultivation. Paecilomyces lilacinus (Thom) Samson MUCL 38701 was a hygromycin-resistant strain isolated in our laboratory. It was maintained on agar slants as described previously [16]. Seven-day-old cultures were used to inoculate 100 ml of the YPG medium; 3% (w/v) glucose, 0.3% (w/v) Difco yeast extract, 2% (w/v) Bactopeptone in the 250-ml Erlenmeyer flasks. Inoculation took place by scraping the culture from the slants into sterile medium (15 ml) which was subsequently transferred to the flasks. Incubation was carried out on an orbital shaker (180 rpm) at 27° for 24 hr. Culture (60 ml) was used to inoculate 2 l of YPG medium in 5-l Erlenmeyer flasks which were incubated at 27°, 110 rpm for 20 hr. These cultures were used for biotransformation and extraction of microsomal and cytosolic cell frs.

Progesterone biotransformation and cell breakage. Approximately 8 g wet wt of mycelium was resuspended in 100 ml buffer A (0.75 mM Na_3PO_4, 0.21 mM EDTA, 0.04 mM reduced glutathione, pH 5.5) and incubated at 27°, 180 rpm with or without 0.1 mg ml^{-1} (final concn) progesterone for 3 hr. Mycelia (20 g wet wt) were harvested by filtration, washed twice with H_2O and resuspended in 20 ml ice-cold buffer B (50 mM potassium phosphate, 1 mM reduced glutathione, 1 mM EDTA, pH 7.7). Cells were homogenized and the microsomal, cytosolic frs and mitochondrial frs were prepared by subcellular fractionation [11]. Cytochrome P-450 content was assessed using reduced carbon monoxide difference spectrophotometry [17] and substrate binding by the split cuvette technique [10]. Protein content was measured using the Sigma bichinchoninic acid protein assay kit.

Progesterone metabolism. Progesterone *in vitro* metabolism was investigated using [^{3}H]progesterone (specific activity 3.40 TBq mmol^{-1}). Progesterone (0.33 μmol) containing 0.04 MBq [^{3}H]progesterone was incubated with NADPH (1.2 μmol) in buffer C (0.7 ml). This buffer contained 50 mM potassium phosphate, 1 mM reduced glutathione, 1 mM EDTA, 6 mM $MgCl_2$, 20% (v/v) glycerol, pH 7.6. Reaction was initiated by addition of 2 mg of protein with incubation at 27°, 120 rpm for 120 min and terminated by addition of 3 ml $CHCl_3$. Extraction and sepn of steroid metabolites on TLC were performed as described previously [18] with additional TLC chromatography with C_6H_6–Me_2CO (4:1). Products were cut from the plates and soaked in scintillation fluid prior to radioactivity determination by scintillation counting. In the examination of substrate specificity 20β-hydroxy-4-pregnen-3-one and 17α-hydroxy-4-pregnen-3,20-dione were used as substrates (0.1 mg ml^{-1} final concn). These compounds were added 3 hr after mycelia were exposed to progesterone in buffer A. Extraction of steroid metabolites from mycelia was performed as described previously [19]. Tests for inducibilty of proteins involved addition of cycloheximide [18]. The involvement of cytochrome P-450 in progesterone metabolism *in vitro* was determined by examining carbon monoxide inhibition and heat inactivation [20]. Biotransformation products were detected by TLC as described previously [18, 19] and radioactivity measured as described above. The specific activity was defined as the sum, in nmol, of progesterone converted into 4-androstene-3,17-dione and testosterone per minute per milligram of protein.

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