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Microbial Transformation of Steroids—IX. Purification of Progesterone Hydroxylase Cytochrome *P*-450 from *Phycomyces blakesleeanus*

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Progesterone hydroxylase cytochrome *P*-450 was purified to homogeneity from *Phycomyces blakesleeanus* microsomes by a four step procedure. An M_r value of 60,000 was determined for this protein by SDS–PAGE. The DEAE–cellulose and Blue-1 MIMETIC affinity fractions gave major peaks at 452 nm in a dithionite-reduced, carbon monoxide, difference spectrum. $NaIO_4$-dependent progesterone hydroxylation was obtained by the pure enzyme without NADPH and NADPH-cytochrome *P*-450 reductase. NADPH-dependent hydroxylation required the addition of other *Phycomyces* microsomal proteins present in the Blue-1 fraction.

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INTRODUCTION

Microbiological hydroxylation can introduce chirality into molecules and activate unreactive centres of compounds where no corresponding organic synthetic reaction is available. The classic example of the replacement of multi-step chemical syntheses by "single-pot" microbiological transformation is the 11-hydroxylation of progesterone by *Rhizopus arrhizus* and *R. nigricans*, discovered by Peterson and Murray in 1952 [1]. A dozen separate reactions were replaced by this transformation, which is still used today in the industrial synthesis of corticosteroids.

Cytochrome *P*-450 is the enzyme responsible for fungal xenobiotic-steroid hydroxylation [2–5]. This enzyme is the terminal member of a membrane-bound, microsomal, electron transfer chain that donates electrons to molecular O_2, reductively cleaving the oxygen–oxygen double bond to form H_2O and an —OH group that is covalently attached to enzyme-bound substrate [6]. The eukaryotic microbial steroid hydroxylase cytochromes *P*-450 are rather unstable and consequently are extremely difficult to purify. They are readily converted to the inactive *P*-420 form on solubilization from the microsomal membrane. Hudnik-Plevnik's group and that of Janig have described, respectively, the purification of steroid 11α-hydroxylation from *Rhizopus nigricans* [5] and steroid 11β-hydroxylase from *Cochliobolus lunatus* [7]. Hydroxylations by the final enzyme preparations were not reported by either group. In late 1993 the purification of active 11-deoxycortisol-induced steroid 11β-hydroxylase cytochrome *P*-450 was reported [8].

Mycelia of the filamentous fungus *Phycomyces blakesleeanus* efficiently hydroxylate progesterone, predominantly at sites 7α and 15β [9]. Cytochrome *P*-450 was thought to be the hydroxylase because in crude extracts azole fungicides, which are known to be inhibitors of cytochrome *P*-450, inhibited progesterone hydroxylation and a characteristic dithionite-reduced, CO, difference spectrum with a maximum peak at *ca* 450 nm was produced in that fraction [10]. In this paper we describe a procedure for purifying to homogeneity this cytochrome *P*-450.

EXPERIMENTAL

Materials

Culture media were from Oxoid, Unipath Ltd, Basingstoke, Hants, U.K. All chemicals used were of analytical grade and were obtained from Sigma Chemical Co., Poole, Dorset, U.K. and BDH Ltd, Poole,

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Abbreviations: CO, carbon monoxide; HP, hydroxyprogesterone; PAGE, polyacrylamide gel.
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Dorset, U.K. Protein Assay Kit was obtained from Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts., U.K. DE-52 DEAE–cellulose was purchased from Whatman International Ltd, Maidstone, Kent, U.K.; MIMETIC Blue-1 affinity resin from Affinity Chromatography Ltd, Freeport, Ballasala, Isle of Man, U.K.; and Macrosep centrifuge concentrators from Flowgen Instruments Ltd, Sittingbourne, Kent, U.K. Kieselgel $60F_{254}$ fluorescent high-performance TLC plates were obtained from BDH Ltd., Poole, Dorset, U.K.

Standards of 6β-, 7α-, 14α-, 15α- and 15β-hydroxyprogesterone used to identify the *in vitro* progesterone metabolites were from a collection of hydroxylated progesterones in our laboratory. These compounds were originally synthesized by microbial progesterone transformation as described by Smith *et al.* [9, 11, 12] and their structures were assigned by 1- and 2-D ^{1}H NMR analysis as described in those papers.

Media and cultivation of P. blakesleeanus

P. blakesleeanus (strain 118496) was maintained at 4°C on potato–dextrose agar plates and slopes [potato extract (4 g), dextrose (20 g) and agar No. 1 (15 g)/l of distilled water and pH 5.6]. The organism was sub-cultured quarterly.

Preparation of P. blakesleeanus microsomes and microsomal progesterone transformation

Mycelia were grown at 22°C for 3–4 days in liquid PYG medium [bactopeptone (10 g), yeast extract (5 g) and glucose (40 g)/l of distilled water] and with gentle shaking (110 rpm) in an orbital shaker.

Homogenates were prepared from those mycelia by the technique of Ballard *et al.* [13], and microsomes were precipitated from the homogenates by the method of Kamath and Rubin [14], except that Mg^{2+} replaced Ca^{2+} as precipitant. The precipitates were collected by centrifugation and re-suspended to a protein concentration of 10 mg/ml in Buffer A (potassium phosphate buffer, pH 7.8 [0.1 M], EDTA [10 mM], DTT [0.5 mM] and glycerol [10% by vol]).

Progesterone transformation was performed essentially as previously described [10, 11]. Briefly, a suspension of microsomes (0.5 ml) was mixed with an equal volume of fresh Buffer A containing progesterone (4 mM and 0.5 μCi [4-^{14}C]progesterone) and $NaIO_4$ (6 mM). Mixtures were incubated with gentle shaking (30 rpm) in an orbital shaker for 1 h at 22°C and terminated by the addition of $CHCl_3$ (3 ml). 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. Solids were re-dissolved in methanol (50 μl) and 2 μl aliquots were spotted onto Kieselgel $60F_{254}$ fluorescent high-performance TLC plates. Samples of authentic monohydroxylated progesterones were also applied. TLCs were developed in an ethyl acetate, toluene, ether solvent mixture (4:3:3, by vol), dried in air and viewed under UV to locate the hydroxyprogesterones. These spots were then scraped from the plates mixed together and eluted in HPLC-grade methanol (1 ml). After removing silica gel, the UV absorbance of the combined hydroxylated metabolites was measured at 240 nm. Progesterone spots were similarly processed. The amounts of metabolites produced were calculated from the ratio of hydroxyprogesterones to total steroids. This method is sufficiently sensitive to permit determination of 1–2 nmol of eluted steroid in 0.5 ml of methanol measured in a 1 cm path-length cuvette. The values calculated by the UV method were confirmed by radioactive analyses.

Identification of microsomal progesterone transformation metabolites

Using HPLC and ^{1}H NMR analysis we previously showed that *P. blakesleeanus* produces 7α- and 15β-hydroxyprogesterone as major products of progesterone transformation and 6β-, 14α- and 15α-hydroxyprogesterone as more minor products [9]. Cell-free extracts were also shown to produce the same metabolites [9]. The progesterone hydroxylation products described in this communication, which were produced by microsomes and all active purified solubilized microsomal fractions, are identical to those described in our 1989 paper and were confirmed by TLC.

Purification of P. blakesleeanus progesterone hydroxylase cytochrome P-450

All purification procedures were performed at 4°C. Quantities and volumes of reagents are for purification from 100 g of mycelia.

Triton N-101 detergent (10%, by vol) was added dropwise to gently stirred suspensions of microsomes to give a final concentration of 1% (by vol). The mixture was incubated on ice for 16 h. Solubilized proteins were separated from particulate matter by centrifugation at 105,000 ***g*** for 1 h. This soluble fraction contained *ca* 85% of the total microsomal progesterone hydroxylase activity.

The solubilized proteins were precipitated by $(NH_4)_2SO_4$ (70% saturation at 0°C), harvested, re-dissolved in a minimum volume of Buffer A (*ca* 1 ml), dialysed for 16 h against that buffer and then loaded onto a DE-52 DEAE–cellulose column (10 ml bed volume) equilibrated in Buffer A. The column was washed with 10 ml Buffer A+KCl (0.25 M) and eluted in 2 ml aliquots with Buffer A+KCl (0.5 M).

The eluted DE-52 fractions containing progesterone hydroxylase activity were combined, dialysed against Buffer A, concentrated to *ca* 1 ml in a 15 ml Macrosep centrifugal concentrator and applied to a column of MIMETIC Blue-1 affinity resin (10 ml bed volume). Unbound protein was washed from the column with Buffer A and the bound protein was eluted with 25 ml Buffer A+KCl (0.5 M).

The Blue-1 fraction containing progesterone hydroxylase was dialysed, concentrated as described above and 25 μl of this concentrate was loaded per well of 15% (by wt) non-denaturing polyacrylamide gels (6×8×0.15 cm) containing Tris–HCl (0.025 M), glycine buffer, pH 7.8 (0.192 M). These gels were run at 150 V for 12 h. One lane was cut from each gel and silver stained [15]. These strips were then re-joined to their parent unstained gels to locate protein bands. Sections were cut from the unstained gels and individual proteins were electroeluted into 125 μl ammonium acetate (7.5 M) per well in a Model UEA Unidirectional Electroelutor (International Biotechnologies, New Haven Connecticut, U.S.A.). The electroeluted fractions were combined and dialysed in Buffer A.

Spectral analyses of P. blakesleeanus progesterone hydroxylase cytochrome P-450

Dithionite-reduced, CO, difference spectra were obtained as previously described [12].

SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–polyacrylamide (15% by wt) gel electrophoresis was performed according to the method of Laemmli [16] except that the running buffer contained glycerol (10%, by vol). Gels were run at 125 V for 3 h, stained with Coomassie Brilliant Blue and if necessary over-stained with silver [15].

Protein determinations

Protein concentrations were determined by a modified Bradford dye-binding procedure [17] using a Bio-Rad Protein Assay Kit.

RESULTS

Purification of progesterone hydroxylase cytochrome P-450

We have previously shown that the rate of progesterone hydroxylation catalysed by *P. blakesleeanus* microsomal cytochrome *P*-450 is linear for at least 1.5 h, and directly proportional to protein concentration. It requires either NADPH and O_2 (natural *P*-450 cycle) or a peroxy compound such as $NaIO_4$ ("peroxide shunt"-driven *P*-450 cycle) for activity. Hydroxylation is blocked by cytochrome *P*-450 inhibitors such as azole fungicides (ketoconazole) and carbon monoxide [10]. We used these properties to assay the progesterone hydroxylase activity during protein purification.

SDS–PAGE analysis of the four stages of purification of the progesterone hydroxylase cytochrome *P*-450 shows that at least 14 major proteins were solubilized by Triton N-101 treatment (Fig. 1, lane 3). Half of these were removed by DEAE–cellulose anion exchange

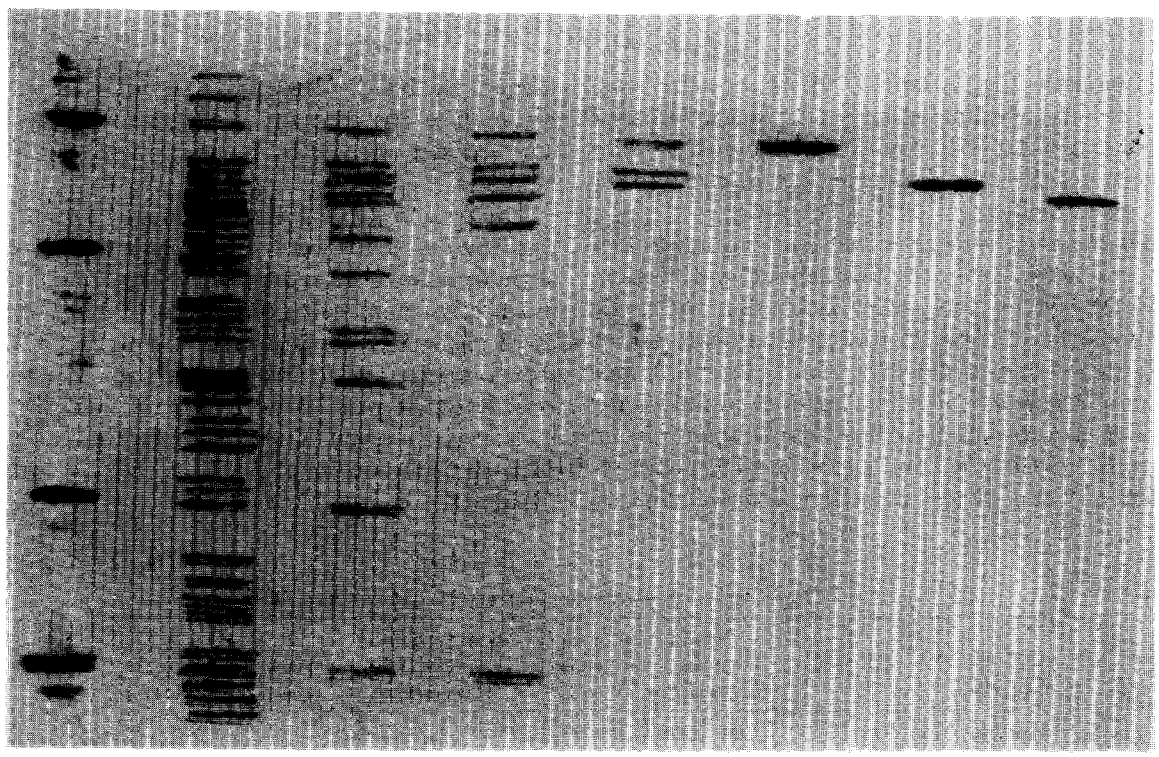

Fig. 1. SDS–PAGE of the proteins obtained from the four-stage purification of *P. blakesleeanus* microsomal progesterone hydroxylase cytochrome *P*-450. Lane 1, protein standards (M_r values $\times 10^{-3}$ of the major bands from the top of the gel: 66, 45, 24 and 15); lane 2, microsomal fraction (10 μg); lane 3, Triton N-101 solubilized fraction (1 μg); lane 4, DE-52 fraction (1 μg); lane 5, Blue-1 MIMETIC affinity column fraction (1 μg), lanes 6–8, Blue-1 protein bands 1–3, respectively (0.5 μg of each).

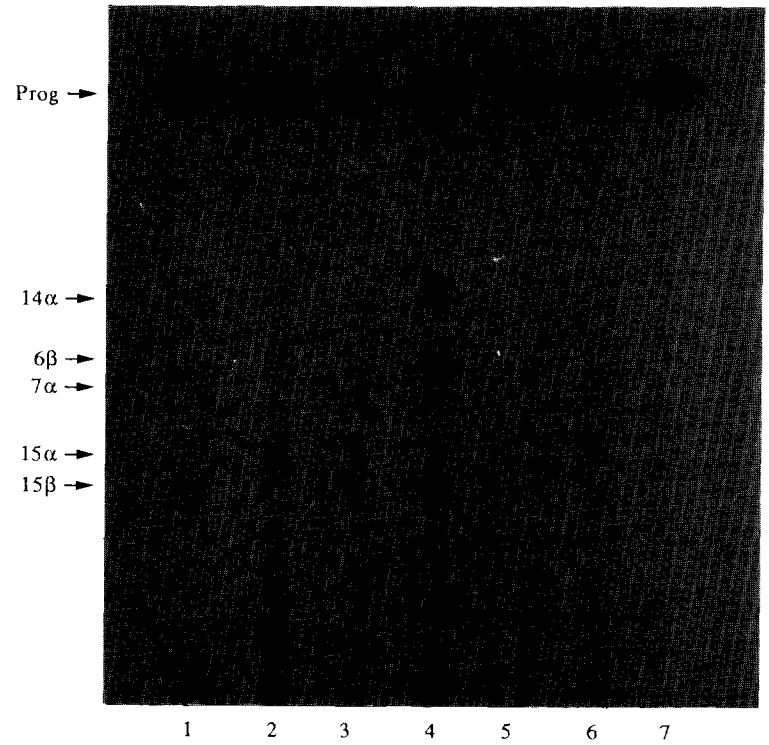

Fig. 2. TLC showing progesterone hydroxylase activity during purification of *P. blakesleeanus* microsomal progesterone hydroxylase cytochrome *P*-450. Lane 1, intact microsomes (1 mg protein); lane 2, Triton N-101-solubilized microsomal proteins (1 mg protein); lane 3, DE-52 fraction (0.25 mg protein), lane 4, Blue-1 fraction (0.4 mg protein); lane 5, PAGE Band-1 protein (0.02 mg protein); lane 6, PAGE Band-2 protein (0.02 mg protein) and lane 7, PAGE Band-3 protein (0.02 mg protein). The identity of the individual metabolite spots is shown on the left of the TLC.

chromatography (Fig. 1, lane 4). After the third step, Blue-1 affinity chromatography, 3 major proteins remained (Fig. 1, lane 5). Finally, the individual proteins were separated by non-denaturing gel electrophoresis (Fig. 1, lanes 6–8).

P. blakesleeanus progesterone hydroxylation site selectivities were retained throughout purification, which is shown by comparing incubations of crude microsomes (Fig. 2, lane 1) and purified progesterone hydroxylase gel band 2 (Band 2, Fig. 1) in Fig. 2, lane 6. It is noteworthy that all active fractions produced the same five hydroxyprogesterone metabolites as in whole-cells (i.e. 6β-, 7α-, 14α-, 15α- and 15β-hydroxyprogesterone) and in approximately equal quantities, judged by TLC (Fig. 2). The ratio of these five metabolites was constant in all these fractions (Fig. 2) and was not affected by hydroxylation with $NaIO_4$ or NADPH.

The data in Table 1 are an average of three separate purifications. They were reproducible and showed only slight variation from experiment to experiment. A typical purification from 100 g of mycelia (Table 1) produced *ca* 25 mg of microsomal protein containing 1330 units of total progesterone hydroxylase activity. The final yield was 90 μg of pure Band-2 protein and 57 units of hydroxylase activity. These data represent an apparent 11.6-fold purification and a 4.3% yield of hydroxylase (Table 1).

Table 1. *Purification of progesterone hydroxylase cytochrome P-450*

Procedure	Total activity (μmol HP/h)	Total protein (mg)	Specific activity (μmol HP/h/ mg protein)	Yield (%)	Purification
Mycelia		10^5			
Microsomes	1330	24.8	54	100	1
Triton N-101 supernatant	*	12.4			
DE-52 column	83	1.2	69	6.2	1.3
Blue-1 column	31	0.25	125	2.3	2.3
PAGE Gel Band 2	57	0.09	628	4.3	11.6

*Trace amounts of Triton N-101, which always remain in this fraction after $(NH_4)_2SO_4$ precipitation of detergent solubilized proteins, prevent accurate measurement of progesterone hydroxylase activity.

Table 2. Requirements for progesterone hydroxylation by Band-2 protein

(a) The effect of $NaIO_4$, NAD(P)H and cytochrome P-450 inhibitors on progesterone hydroxylation by Band-2 protein

Incubation	Progesterone hydroxylation (μmol HP/h/mg protein)
Control (progesterone+Band-2+$NaIO_4$)	628
Progesterone omitted	n.d.
$NaIO_4$ omitted	n.d.
3 mM NADPH replaced $NaIO_4$	n.d.
3 mM NADH replaced $NaIO_4$	n.d.
Control+35 μM ketoconazole	n.d.
Control+carbon momoxide	n.d.
Control, Band-2 boiled	n.d.

(b) The effect of Band-1 and Band-3 protein on progesterone hydroxylation catalysed by Band-2 protein with 3 mM NADPH and progesterone and without $NaIO_4$

Incubation	Progesterone hydroxylation (μmol HP/h/mg protein)
Band-1	n.d.
Band-2	n.d.
Band-3	n.d.
Bands-1+2+3	434
Bands-1+2+3: 3 mM NADH replaced NADPH	n.d.
Bands-1+2	n.d.
Bands-1+3	n.d.
Bands-2+3	n.d.

n.d., not detectable.

Identification of Band-2 as cytochrome P-450

Band-2 protein required $NaIO_4$ for progesterone hydroxylation [Table 2(a)], NAD(P)H could not be substituted. Ketoconazole (35 μM) and carbon monoxide both totally inhibited hydroxylation [Table 2(a)]. Band-1 and Band-3 proteins had no hydroxylase activity [Table 2(b)] but, significantly, when both these proteins were mixed with Band-2 protein, progesterone hydroxylation was obtained with NADPH, but not with NADH [Table 2(b)]. No other combination of these three proteins catalysed the NADPH-dependent hydroxylation.

Insufficient Band-2 protein was obtained for cytochrome *P*-450 difference spectroscopy. Instead, this analysis was performed on the DE-52 and Blue-1 fractions. We obtained the expected spectra containing a major peak of absorbance at 452 nm (Fig. 3). This form of cytochrome *P*-450 comprised over 80% of the total protein in the DE-52 fraction and *ca* 70% in the Blue-1. In both spectra a secondary peak, typical of denatured cytochrome *P*-450, is visible at 424 nm (DE-52 fraction) and 422 nm (Blue-1 fraction), which is slightly more abundant in the case of the Blue-1 fraction.

DISCUSSION

The involvement of cytochrome *P*-450 in microbial steroid hydroxylation was first demonstrated in 1977 when the inducible progesterone 11α-hydroxylase of *Rhizopus* microsomes was shown to be inhibited by carbon monoxide. These same microsomes, after dithionite reduction, gave a peak at 450 nm in a difference-spectrum [5]. Since then microsome fractions capable of *in vitro* steroid hydroxylation have been prepared from various filamentous fungi including *Aspergillus ochraceus* (steroid 11α site-selectivity) [2–4], *A. fumigatus* (11α/15β) [2], *Botryosphaeria obtusa* (7β) [11], *C. lunatus* (11β) [7], *Curvularia lunata* (11β) [8]. Crude cell-free extracts by *P. blakesleeanus* (7α/15β) [9, 10] also hydroxylate progesterone.

Procedures have been published for the purification of these cytochromes *P*-450 from *R. nigricans* [5], *C. lunatus* [7] and *C. lunata* [8] and for the partial purification of the *A. fumigatus* enzyme [18]. It is noteworthy that steroid hydroxylation by the final protein preparations was not shown or described for either the *R. nigricans* or *C. lunatus* proteins. Furthermore, reduced, CO, cytochrome *P*-450 difference spectra and inhibitor studies were not performed. For the purified *C. lunata* enzyme, hydroxylation of 11-deoxycortisol was reported, but no supporting experimental evidence was provided [8]. Thus, questions remain regarding the purity and activity of these preparations.

There can be no doubt that our *P. blakesleeanus* progesterone hydroxylase is a cytochrome *P*-450 enzyme and that hydroxylation activity was retained during purification. Band-2 protein, which contains the hydroxylase activity (Fig. 1), has an M_r of 60,000, which is in the range expected for cytochrome *P*-450. In $NaIO_4$-dependent incubations hydroxylation required Band-2 protein only, but in NADPH catalysed reactions Band-1 and Band-3 proteins were also required showing the expected need for cytochrome *P*-450 reductase in natural systems (Table 2).

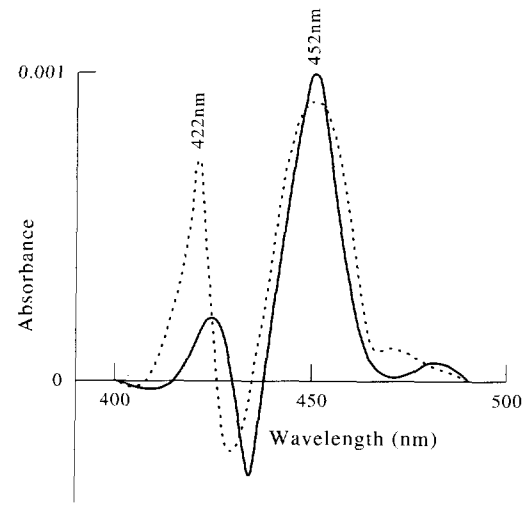

Fig. 3. Dithionite-reduced, CO, difference spectra of *P. blakesleeanus* progesterone hydroxylase cytochrome *P*-450; solid line, DE-52 fraction; dashed line, Blue-1 affinity column fraction.

The increase in total progesterone hydroxylase activity between Blue-1 fraction and Band-2 protein (Table 1, lines 5 and 6) is reproducible and suggests inhibition (natural or experimentally introduced) in cruder fractions.

Our results strongly point to a single steroid hydroxylase cytochrome *P*-450 in *P. blakesleeanus* that hydroxylates progesterone at multiple sites, rather than five individual but virtually identical isoenzymes, because we obtained only a single protein band on SDS–PAGE, on native gel electrophoresis, and on isoelectric focussing gels (pI 6.7). Secondly, the ratios of the individual hydroxyprogesterone isomers produced by the active protein fractions during purification were constant throughout. Multi-site hydroxylation by xenobiotic-metabolizing cytochromes *P*-450 has been widely reported in the literature and theoretical kinetic models to explain broad hydroxylation-site specificity have been published [19 and references therein].

We calculate, from the data in Table 1, a minimum yield of at least *ca* 1.3 nmol of cytochrome *P*-450 per 100 g of mycelia (25 mg microsomal protein) and thence a turnover of the enzyme of at least 400–500 nmol of hydroxyprogesterone metabolites produced per h per nmol cytochrome *P*-450 (*ca* 7–8/min). A turnover number of this magnitude is quite respectable for cytochrome *P*-450 and it is an order of magnitude better than the 0.6/min reported for *Bacillus megaterium* steroid 15β-hydroxylase cytochrome *P*-450 (*P*-450$_{meg}$) [20]. The turnover number of the *Phycomyces* enzyme shows that the pure protein catalyses multiple cycles of hydroxylation.

The microsomal sub-cellular location of the *Phycomyces* progesterone hydroxylase, its M_r 60,000, and its strict NADPH specificity in natural steroid hydroxylation, in preference to NADH, are the properties expected of a cytochrome *P*-450 in a eukaryotic Type-I system [6]. Type-I structural organisation was found by Suzuki *et al.* [8] for the steroid 11β-hydroxylase system of *C. lunata*, and it is in contrast to the Type-II organisation found in bacteria and mitochondria and which was claimed for the *Rhizopus* progesterone 11α-hydroxylase cytochrome *P*-450 system [5]. Absolute proof of this requires purification and characterization of the electron transfer proteins.

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