J. Steroid Biochem. Molec. Biol. Vol. 38, No. 2, pp. 249–256, 1991
Printed in Great Britain.

0960-0760/91 $3.00 + 0.00

MICROBIAL TRANSFORMATION OF STEROIDS—VII. HYDROXYLATION OF PROGESTERONE BY EXTRACTS OF *PHYCOMYCES BLAKESLEEANUS*

KELVIN E. SMITH,[1]* SHAHID A. LATIF[1]† and DAVID N. KIRK[2]
[1]School of Biological Sciences and [2]Department of Chemistry, Queen Mary and Westfield College, Mile End Road, London E1 4NS, England

(Received 6 July 1990)

Summary—Post mitochrondrial supernatants (S-12 extracts) were prepared from *Phycomyces blakesleeanus* by grinding washed and frozen mycelial cakes in fine sand and extracting the paste produced with buffer containing Tris-HCl pH 7.8 (0.1 M), EDTA (0.01 M), dithiothreitol (5 mM) and glycerol (10% v/v). The S-12 extracts, obtained in this way, reproducibly hydroxylated progesterone, producing 7α- and 15β-hydroxyprogesterone the major products of whole-cell transformation.

Cell-free progesterone hydroxylation was found to be approximately linearly dependent on extract concentration, to require reduced NADP (partly replaceable by NADH), and to be dependent on progesterone (apparent K_m calculated to be 4 mM). K^+ and Mg^{2+} were found not to be required. Maximum progesterone hydroxylation occurred after 2 h at pH 7.8 and at 24°C. Using optimum conditions S-12 extracts were capable of hydroxylating between 5 and 15% of added progesterone (0.2 mM).

Hydroxylation was found to be partially inhibited by carbon monoxide (*ca* 40%) and almost completely inhibited by azoles, ketoconazole and diconazole. The NADPH and molecular oxygen requirements were replaceable by $NaIO_4$. These findings strongly suggest that hydroxylation was being catalyzed by cytochrome *P*-450. This was confirmed by preparing progesterone-hydroxylating microsomes and Triton N-101-solubilized microsome extracts, and by obtaining a dithionite–reduced carbon monoxide-difference absorption spectrum peak at 455 nm in the solubilized microsome extracts.

INTRODUCTION

Hydroxylation of steroid hormones at the 7α skeletal site is chemically demanding and requires time-consuming, multi-step synthetic pathways which are expensive in materials and manpower. However, 7α-hydroxylated derivatives of progesterone, testosterone and androstenedione are required to stock the Steroid Reference Collection at QMW.

The literature contains a number of reports on steroid 7α-hydroxylation by microorganisms, mainly fungi [1–4], and such syntheses seemed a worthwhile alternative to chemical synthesis.

Phycomyces blakesleeanus transformed progesterone and C-19 androstane-based steroids moderately, to a complex mixture of products [5]. Only monohydroxylated derivatives were obtained from progesterone incubations, other structural modifications not being detected. The metabolites produced were 7α-hydroxyprogesterone (major metabolite) and 6β-, 14α-, 15α- and 15β-hydroxyprogesterone (minor metabolites), but yields were disappointing. On average, from 10 mg of progesterone in 100 ml of water in shake-cultures, which was the optimum substrate charge tolerated, about 2–3 mg of 7α-hydroxyprogesterone and 1.5 mg of total minor metabolites were isolated. We therefore considered the practicality of making this compound in cell-free incubations, the rationale being that concentrated preparations of steroid hydroxylase enzymes might be more efficient hydroxylators than whole cells. However, reports on microbial cell-free steroid hydroxylation are few [6–15], being confined almost entirely to those on 11α-hydroxylation by extracts of *Aspergillus* and *Rhizopus* species.

We report for the first time in this paper on progesterone 7α-hydroxylation by cell-free extracts of *P. blakesleeanus*. We have optimized conditions for hydroxylation and have found

*To whom correspondence should be addressed.
†Present address: PCSIR Laboratories, Feroze-pur Road, Lahore, Pakistan.

the steroid hydroxylase enzyme(s) to be cytochrome(s) *P*-450.

EXPERIMENTAL

Materials

Freeze-dried cultures of *P. blakesleeanus* (stain 118496) were purchased and maintained as previously described [5].

All chemicals used in this work were of analytical grade and were mostly obtained from Sigma Chemical Company, Poole, Dorset, England or BDH Ltd, Poole, Dorset, England. [4-^{14}C]progesterone (56 mCi/mmol) was purchased from Amersham International, Amersham, Bucks, England.

Cultivation and induction of progesterone hydroxylase

P. blakesleeanus was grown for 3–4 days at 24°C in 100 ml batches of sterile medium as previously described [5].

When good mycelial growth had been obtained progesterone was added to the cultures (10 mg in 0.5 ml ethanol per flask) which were then reincubated for 12 and 16 h to amplify intracellular concentrations of progesterone hydroxylase enzyme(s). After this time, mycelia were filtered and washed thoroughly to remove adhering steroids with 1 l of sterile NaCl (0.5% w/v) followed by 1 l of sterilised distilled H_2O. In early experiments the washed cells were collected on filter paper, press-dried and then macerated. This method was later modified by including a step where washed mycelia were soaked in buffer (see next section for composition) for 30 min before re-filtering (see Results). Then the resulting mycelial cake was pressed between layers of filter paper to remove excess liquid, weighed and stored at −20°C for about 2 h or until the cells had just frozen.

Preparation of crude post-mitochondrial extracts

The initial method we used was based on the one described by Madyastha *et al.* [11]. Frozen mycelia (15 g) were mixed with pre-cooled acid-washed glass powder (15 g) and ground to a thick paste in a pre-cooled mortar and pestle, immersed in an ice-bath. The resulting paste was mixed with buffer (50 ml) [glucose (0.25 M), Tris-HCl pH 7.8 (0.1 M), EDTA (10 mM), dithiothreitol (DTT) (0.5 mM) and 10% (v/v) glycerol], and the slurry obtained was centrifuged at 4°C (1100 *g* for 20 min in an 8 × 50 ml angle head in a Sorvall RC-5B Super Speed Centrifuge). The aqueous layer was carefully decanted, re-centrifuged (12,000 *g* for 20 min), and if necessary diluted with buffer to a concentration of 20 mg/ml protein. The supernatant (S-12) obtained was used for cell-free hydroxylation assays. Aliquots were streaked onto PYG agar plates and incubated at 25°C to check for the presence of unbroken mycelia and for bacterial contamination. Hydroxylation results from contaminated extracts were ignored.

Cell-free steroid hydroxylase assays

At the start we used the method of Madyastha *et al.* [11], modified to include [^{14}C]progesterone. Cell-free incubations were performed in MF11 quick-fit glass tubes as described [11]. Incubation mix (1 ml final vol containing Tris-HCl buffer pH 7.8 (0.1 M), EDTA (10 mM), KCl (1 mM), $MgCl_2$ (1 mM), DTT (0.5 mM), 0.5 or 0.25 μCi of [4-^{14}C]progesterone (56 mCi/mM), unlabeled progesterone (0.33 μmol in 15 μl 20% Tween-80 in acetone), glucose-6-phosphate (0.68 mM), $NADP^+$ (0.22 mM) and 0.2 units of glucose-6-phosphate dehydrogenase was added to fungal extract (0.5 ml and 20 mg protein/ml). The reactions were incubated with gentle shaking for 1 h at 24°C and then terminated by adding $CHCl_3$ (1.5 ml). In later work the NADPH generating system was first replaced by reduced NADP and then by $NaIO_4$ (3 mM) (see Results for other changes). Steroids were extracted from the aqueous incubations by three successive $CHCl_3$ extractions (1.5 ml) each lasting for 5 min. The fractions were combined and evaporated to dryness.

The solid residues were dissolved in methanol (10 μl) and aliquots were dispensed onto Merck plastic-backed silica GF_{254} TLC plates. Steroids were separated by running the plates in an ether:toluene:ethyl acetate solvent (4:3:3 v/v) and they were detected by viewing the plates under u.v. light and then by autoradiography on Fuji XR-100 X-ray film. The X-ray film was placed carefully on top of the TLC plates which were left at −70°C for between 1 and 2 wk. The film was developed with Kodak LX24 developer and fixed with Kodak FX-40 fixer.

Quantitative analysis of cell-free steroid hydroxylation

To quantify hydroxylation, the TLC plates used to separate the steroid mixtures were cut into two equal parts, the bottom half containing all the transformed metabolites, the top half the

untransformed progesterone. The strips were placed into scintillation fluid [PPO (5 g) and POPOP (0.25 g)/l of toluene] and counted in a Packard Tricarb 460 liquid scintillation counter. Quenching was determined by applying known amounts of radioactivity to clean TLC strips which had been cut to sizes identical to the test strips.

The proportion of hydroxylated progesterone metabolites formed was calculated by adding together the unquenched cpm values for the total metabolites and for the untransformed progesterone and dividing them into the values for the hydroxylated products. The fractional figures obtained were then converted into the amounts of metabolites formed by multiplying them by the amount of progesterone substrate added at zero time to the incubations.

Preparation of P. blakesleeanus microsomes

Post-mitochondrial supernatants (S-12 extracts) were further centrifuged at 105,000 *g* and at 4°C for 1 h in an M.S.E. "Prep-Spin 75" ultracentrifuge to sediment microsomes. The firmly packed pellets, designated P-100 fraction (microsomal fraction), were washed and resuspended to a concentration of approx. 10 mg protein/ml in buffer solution which comprised Tris-HCl buffer pH 7.4 (0.1 M), EDTA (10 mM) DTT (0.5 mM) and glycerol (10% v/v). The resuspended pellets were tested for steroid hydroxylation using the improved hydroxylation assay system described in the Results section.

RESULTS

Disruption of fungal cells

Extracts from cells disrupted by several methods in common laboratory use were examined for their progesterone hydroxylation characteristics. The breakage methods used included ultrasonic disintegration, French-pressing, breakage by agitation with glass beads, grinding with abrasives and enzymic digestion of cell walls, either alone or in conjunction with physical breakage methods. Several mixtures of hydrolytic enzymes were used. A single batch of cells was used for these experiments to eliminate organism variability. Only the *abrasive grinding* method repeatedly produced highly-active extracts.

The composition of cell soaking and grinding buffer was found to affect S-12 progesterone hydroxylation efficiency. Table 1 summarizes the data obtained, line 1 being the activity produced by extracts of cells soaked, macerated and extracted in control buffer whose composition is given in the Experimental section. Homogenates made in Tris-HCl buffer alone were found either to be inactive or at best weakly active (line 2). Omission of DTT from control buffer (line 3) had limited effect producing extracts with 79% of the activity of those prepared with DTT. Omission of EDTA or glycerol had substantial effects reducing activity to 59% (line 4) and 58% (line 5), respectively, of control activity. Changing the buffer pH reduced extract activity. Roughly 50–60% of control (pH 7.8) extract activity was lost by preparing extracts at pH 7 and pH 8.3 (lines 7 and 8). However, omission of glucose had no effect (line 6). In summary, we found the best conditions for preparing extracts from thoroughly washed mycelia to be pre-soaking mycelia in buffer containing Tris-HCl, pH 7.8 (0.1 M), EDTA (10 mM), DTT (0.5 mM) and 10% (v/v) glycerol (buffer A) then press-drying them into cakes and *lightly* freezing them at −20°C until they had become brittle, then macerating them by vigorous grinding with an equal weight of ice-cold acid-washed abrasive for 5 min in a cold mortar and pestle immersed in an ice-bath, and extraction of the thick paste obtained with 3 volumes (based on the weight of fungal cake) of ice-cold buffer A plus 10% glycerol.

Figure 1 shows an autoradiograph of a TLC separation of $CHCl_3$-extracted material from eight S-12 extracts prepared by this method and incubated with [^{14}C]progesterone. Strong bands of approximately equal intensity of monohydroxylated progesterone, which correspond in

Table 1. Effect of composition of cell soaking and grinding buffer on progesterone hydroxylation by *P. blakesleeanus* S-12 extracts

Grinding buffer composition	Hydroxylated products produced (nmol)
0.1 M Tris-HCl pH 7.8, 0.25 M glucose, 10 mM EDTA 0.5 mM DTT and 10% glycerol (control buffer)	21.5
0.1 M Tris-HCl pH 7.8	<2.0
0.1 M Tris-HCl pH 7.8, 0.25 M glucose, 10 mM EDTA and 10% glycerol (no DTT)	17.0
0.1 M Tris-HCl pH 7.8, 0.25 M glucose, 5 mM DTT and 10% glycerol (no EDTA)	12.8
0.1 M Tris-HCl pH 7.8, 0.25 M glucose, 10 mM EDTA and 0.5 mM DTT (no glycerol)	12.5
0.1 M Tris-HCl pH 7.8, 10 mM EDTA, 0.5 mM DTT and 10% glycerol (no glucose)	20.5
Control buffer pH 7	9.3
Control buffer pH 8.3	11.1

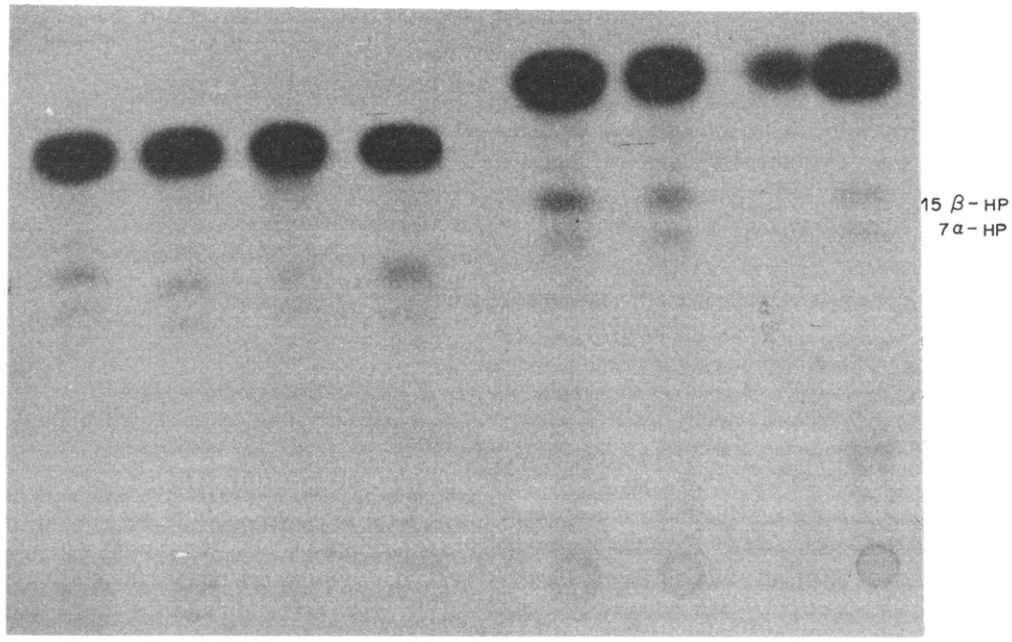

Fig. 1. Autoradiograph of the progesterone transformation products produced by eight cell-free extracts of *P. blakesleeanus* prepared by the method summarized in the Results section— "Disruption of fungal cells".

position to authentic standards of 7α- and 15β-hydroxyprogesterone, are clearly visible on the autoradiograph below the [^{14}C]progesterone spots. Little, if any, 6β, and 15α-hydroxyprogesterone are visible.

Effects of preincubating P. blakesleeanus with progesterone on steroid hydroxylase activity of S-12 extracts

Steroid 11α-hydroxylase in *Aspergillus* and *Rhizopus* has been shown to be an inducible enzyme whose intracellular concentration substantially increases upon preincubation [7, 12]. We found *P. blakesleeanus* S-12 extracts to have equal activity whether prepared from progesterone preincubated cells or non-preincubated cells. In this organism steroid 7α-hydroxylase must be a constitutive enzyme and not inducible or amplifiable.

Optimizing cell-free steroid hydroxylation assays

Using a previous method [11], repeated attempts at hydroxylation with freshly prepared S-12 extracts from newly-grown batches of mycelia, showed little significant hydroxylation activity even in 8 h incubations. Since the NADPH generating system might not have been providing sufficient reducing equivalent for the hydroxylases and that the progesterone solution (in 20% Tween-80 detergent in acetone) might be inhibitory, we decided to add progesterone in ethanol and to replace the NADPH generating system with excess (over progesterone) of NADPH. NADPH concentration was found to be strongly stimulatory up to a concentration of 1.26 mM, the highest tested (Fig. 2A, solid circles). NADH was less effective (Fig. 2A, solid squares). Addition of equal amounts of NADPH and NADH (0.42 mM each) had no synergistic effect (Fig. 2, legend). In fact 0.42 mM NADPH alone was more stimulatory than the mixture.

Both molecular oxygen and NADPH requirements in fungal steroid hydroxylation are replaceable by peroxy compounds such as $NaIO_4$ [9, 12] which are thought to function by oxidizing steroid-bound high-spin ferric cytochrome *P*-450 directly to a hydroperoxo ferric haem intermediate [16]. $NaIO_4$ strongly supported hydroxylation, but at higher concentrations than NADPH (Fig. 2B). These $NaIO_4$ results provide supportive evidence for cytochrome *P*-450 being the hydroxylase enzyme (see below).

The time course of $NaIO_4$-driven progesterone hydroxylation by S-12 extracts incubated at 24°C is shown in Fig. 3A. Hydroxylation was approximately linear for 60 min when maximum transformation, about 5% of added substrate, was obtained. The amount of hydroxy products produced remained almost constant up to 8 h,

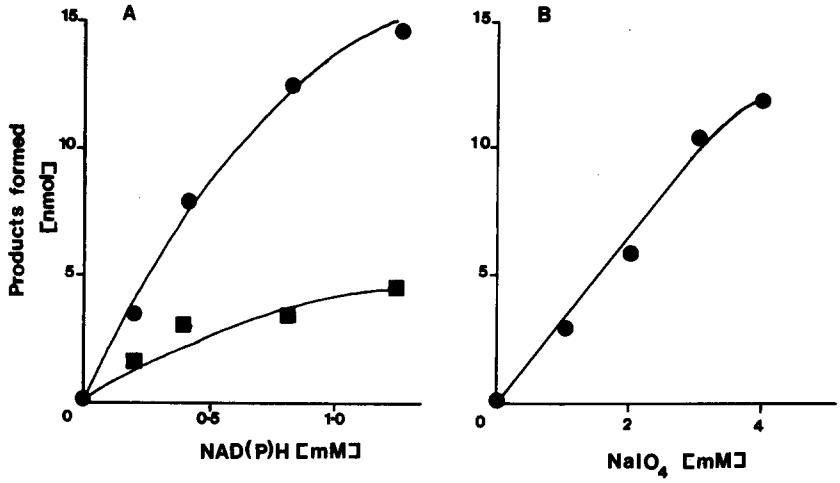

Fig. 2. Effect of NADPH, NADH and $NaIO_4$ concentration on progesterone hydroxylation by S-12 extracts of *P. blakesleeanus*: (A) the effect of NADPH and NADH concentration, circles being NADPH and squares NADH. Incubations containing equal amounts of NADPH and NADH (0.42 mM) yielded 6 nmol of hydroxylated progesterone products; and (B) the effect of $NaIO_4$ concentration.

suggesting that extracts lose activity fairly quickly.

Temperature markedly affected hydroxylation (Fig. 3B). The optimum was found to be 24°C. Considerable loss of activity was obtained when incubations were at 37°C (58% decline) and 18°C (44% decline). The pH of the incubation mixture also noticeably affected product formation, optimum hydroxylase activity was obtained between pH 7.4 and 7.8 (Fig. 3C). Above pH 7.8, activity was sharply reduced, being at pH 8.6 only 22% of the optimum. It was also substantially reduced at pH values below 7, being 45% of the optimum activity at pH 6.6.

The effects of varying substrate concentrations are shown in Fig. 4. All progesterone concentrations tested stimulated hydroxy metabolite formation. Hydroxylation was linear up to 0.2 mM progesterone, about the highest concentration usable in the incubation mixtures (Fig. 4A). Calculations showed that in our incubations the hydroxylase had an apparent K_m for progesterone of 4 mM.

Hydroxyprogesterone production was found to be dependent on S-12 extract concentration added to the incubations over a wide range from 0.3 to 2.7 mg of protein per incubation (Fig. 4B). On the other hand K^+ and Mg^{2+} had no effect on the reaction at sub-physiological concentrations (up to 2 mM Mg^{2+} and 10 mM K^+), but were slightly inhibitory at higher concentrations (results not shown).

The supposed inhibitory effect of detergent on hydroxylation noted earlier was confirmed by the findings that 1.2% (w/v) final concentrations of Tween or CHAPS severely inhibited hydroxylation, product formation declining 60 and

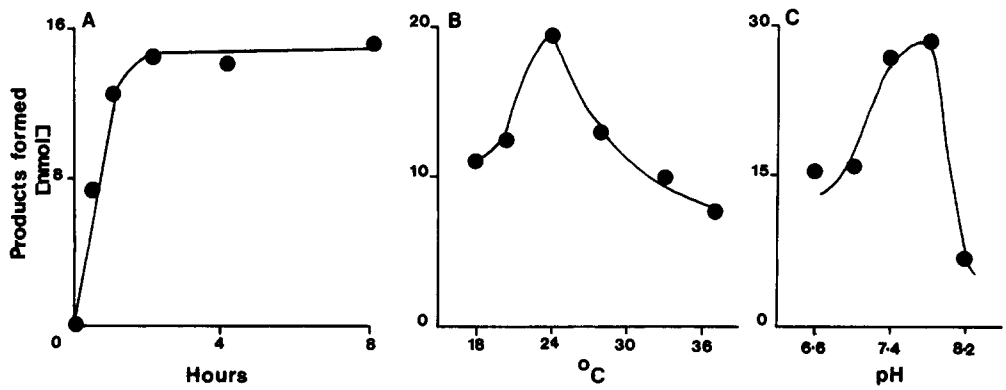

Fig. 3. Time course and effect of temperature and pH on progesterone hydroxylation by S-12 extracts of *P. blakesleeanus*: (A) the progress curve; (B) the temperature curve; and (C) the pH curve.

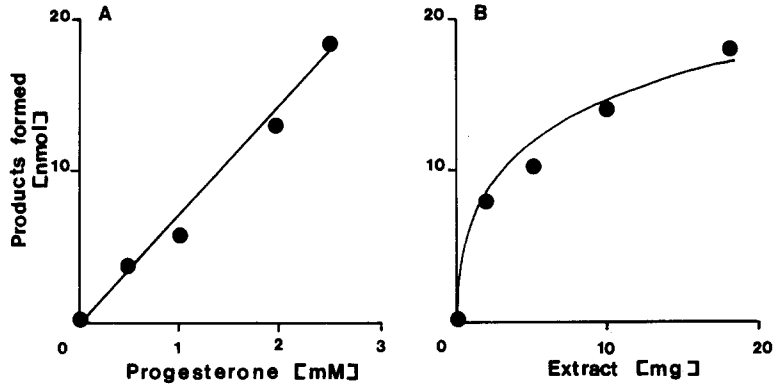

Fig. 4. Progesterone and extract concentration response curves on progesterone hydroxylation by S-12 extracts of *P. blakesleeanus*: (A) the effect of progesterone concentration; and (B) the effect of extract concentration.

50%, respectively, in their presence (Table 2). These detergent concentrations might be sufficiently high to partly solubilize the membrane to which the cytochrome *P*-450 steroid hydroxylase enzyme(s) is thought to be bound. Alternatively the secondary and tertiary structure of the enzyme might be disrupted.

The nature of steroid hydroxylase enzymes in P. blakesleeanus

Although site-selective hydroxylation of steroids by filamentous fungi has been known for many years, the enzymology of the process has not been well characterized. Recent work with steroid 11α-hydroxylating fungi [6, 7, 9–12] suggests that the reaction is accomplished by haemoproteins analogous to mammalian cytochromes *P*-450. To ascertain if these enzymes are the hydroxylases in *P. blakesleeanus* we tested our S-12 extracts with proven cytochrome *P*-450 inhibitors, carbon monoxide and azole fungicides [11].

Cell-free progesterone hydroxylation was found to be inhibited by up to *ca* 40% by bubbling carbon monoxide through ice-cold S-12 extracts for 5 min (7.8 nmol hydroxyprogesterones being produced compared with 12.9 nmol hydroxyprogesterones in untreated controls) showing this particular extract to contain a mixture of ferrous and ferric cytochrome *P*-450. Ketoconazole and diniconazole substantially inhibited progesterone hydroxylation over a ten-fold concentration range of 0.94 to 9.4 mM (Fig. 5).

Table 2. Effects of CHAPS or Tween-80 detergents on progesterone hydroxylation by S-12 extracts of *P. blakesleeanus*

Detergent concentration (% w/v)	Hydroxylated products produced (nmol)
Control, no detergent added	12.5
CHAPS	
0.1	8.2
0.5	5.8
1.0	5.8
1.2	5.0
Tween-80	
0.5	7.0
0.7	8.0
1.0	6.5
1.2	6.0

Sub-cellular localization of P. blakesleeanus steroid hydroxylases

Steroid hydroxylases have been shown to be membrane-bound cytochrome *P*-450 enzymes in all types of organism investigated to date [17] and to be pelletable by high-speed centrifugation. The results of progesterone transformation experiments performed with different subcellular fractions are presented in Table 3. The data unequivocally show the hydroxylase activity to be in the P-100 fraction, i.e. the membrane-containing microsome sub-cellular fraction. Neither the mitochondrial fraction nor the post-microsomal supernatant had detectable

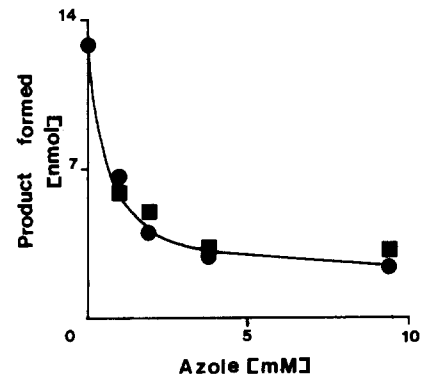

Fig. 5. Inhibition of progesterone hydroxylation in S-12 extracts of *P. blakesleeanus* by ketoconazole and diconazole. Solid circles are ketoconazole inhibition and solid squares are diconazole inhibition.

activity. Addition of cytosol to microsomes did not enhance progesterone hydroxylation (results not shown), showing all components necessary for hydroxylation to be microsome-bound.

A dithionite-reduced–CO (carbon monoxide) difference absorption spectrum of *P. blakesleeanus* microsomal membranes solubilized for 16 h in 1% Triton N-101 and then concentrated, showed a peak at 455 nm (characteristic of *P*-450) and shoulders at about 430 and 480 nm (Fig. 6). These detergent- solubilized extracts are capable of progesterone 7α- and 15β-hydroxylation (Table 3).

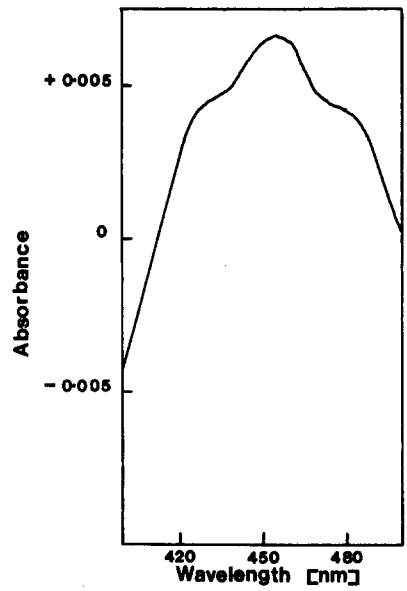

Fig. 6. Dithionite-reduced-carbon monoxide difference absorption spectrum of Triton N-101 solubilized *P. blakesleeanus* microsomal membranes. The sample was zeroed at 500 nm.

DISCUSSION

Production of rare monohydroxylated steroids, including 7α-hydroxyprogesterone, by cell-free extracts of steroid-hydroxylating fungi would have potential advantages over whole-cell transformations of reduced costs, a need for less sophisticated apparatus and reduced usage of biohazardous reagents in product purification if high product yields could be produced.

P. blakesleeanus cell-free extracts that hydroxylate progesterone mainly at skeletal sites 7α and 15β, the major sites of whole-cell hydroxylation, may be quickly and inexpensively made from under 10 g of mycelia obtainable from about 100 ml of 3-day grown cultures. Moreover replacement of expensive NADPH, and its regeneration system, by $NaIO_4$ makes cell-free steroid hydroxylation an inexpensive procedure.

However, on average a minimum of 5% and a maximum of about 15% of added progesterone substrate was found to be converted into hydroxylated products, i.e. 10–30 nmol hydroxyprogesterones (approx. 3–9 μg) per 1 ml assay containing 0.2 mM progesterone. Scaling-up incubations to 100 ml would be expected to produce 0.3–0.9 mg of products which is only 10–30% of that given by whole-cells in 100 ml transformations. Clearly, at present, there would be no advantage in making 7α-hydroxysteroids by cell-free incubations using S-12 extracts unless the efficiency of hydroxylation could be substantially increased and more concentrated preparations of hydroxylase enzymes employed. Increasing the amount of substrate in the incubation is not a practical solution due to limited solubility. It would be difficult to increase cell-free steroid hydroxylation efficiency by any means except addition of more enzyme to incubation mixtures because the optimum incubation conditions we used for progesterone 7α-hydroxylation by *P. blakesleeanus* S-12 extracts, apart from those required for NADPH regeneration, are close to those for steroid 11α-hydroxylation by *Aspergillus* cell-free extracts [9, 11, 12].

Table 3. Progesterone hydroxylation by sub-cellular fractions of *P. blakesleeanus*

Fraction	Hydroxylated products produced (nmol mg protein)
12,000 *g* supernatant (10 mg) (S-12)	1.29
12,000 *g* pellet (P-10, mitochondria)	No detectable activity
105,000 *g* supernatant (S-100)	No detectable activity
105,000 *g* pellet (5 mg) (P-100, microsomes)	11.6
Triton N-101 solubilized microsomes (1 mg)	15.2

Both microsomes and microsomal detergent-solubilized cytochrome *P*-450 preparations actively hydroxylated progesterone (Table 3) and retained the major site-specificities of whole cells and S-12 extracts making their use in cell-free hydroxysteroid production worth future consideration. Large quantities of highly active *P. blakesleeanus* microsomes could be quickly and routinely prepared from S-12 extracts by spinning them at about 30,000 *g* for 1–2 h.

Our preliminary work suggests that microsome and detergent-solublized microsome preparations have almost identical progesterone hydroxylation characteristics to those of S-12 extracts, but as expected on a mg-for-mg protein

basis they are both very much more active than S-12 extracts, having over 10 times their activity (Table 3). No other steroids have been tested to date and microsomal scale-up experiments have not yet been performed.

Acknowledgements—DNK and KES are grateful to the Wellcome Trust for providing a grant for this work to be carried out. Authority to use *P. blakesleeanus* was provided by the Ministry of Agriculture, Fisheries and Food, Licences PHF1019/41(60) and 1018/42(68).

REFERENCES

1. Capek A., Hanc O. and Tadra M.: In *Microbial Transformations of Steroids*. Akademia Press, Prague (1966).
2. Charney W. and Herzog H. L.: In *Microbial Transformations of Steroids: a Handbook*. Academic Press, New York (1967).
3. Smith L. L.: Microbiological reactions with steroids. In *Specialist Periodical Reports, Terpenoids and Steroids* (Edited by K. H. Overton). The Chemical Society, London, Vol. IV (1974) pp. 394–530.
4. Vezina C. and Rakhit S.: In *Handbook of Microbiology* (Edited by A. I. Laskin and H. Lechevalier). C.R.C. Press, Ohio, Vol. 4P (1974).
5. Smith K. E., Latif S. and Kirk D.N.: Microbial transformations of steroids–II. Transformations of progesterone, testosterone and androstendione by *Phycomyces blakesleeanus*. *J. Steroid Biochem.* **32** (1989) 445–451.
6. Breskvar K. and Hudnik-Plevnik T.: A possible role of cytochrome *P*-450 on hydroxylation of progesterone by *Rhizopus nigricans*. *Biochem. Biophys. Res. Commun.* **74** (1977) 1192–1198.
7. Breskvar K. and Hudnik-Plevnik T: Inducibility of cytochrome *P*-450 and of NADPH-cytochrome *c* reductase in progesterone-treated filamentous fungi *Rhizopus nigricans*. *J. Steroid Biochem.* **14** (1981) 395–399.
8. Elin A. E. and Kogan L. M.: A cell-free preparation of steroid 16α-hydroxylase from *Actinomyces roseochromogenes* ATCC 3348. *Dokl. Akad. Nauk. S.S.R.* **167** (1966) 1175–1176.
9. Ghosh D. and Samanta T. B.: 11α-Hydroxylation of progesterone by a cell-free preparation of *Aspergillus ochraceus TS*. *J. Steroid Biochem.* **14** (1981) 1063–1067.
10. Jayanthi C. R., Madyastha P. and Madyastha K. M.: Microsomal 11α-hydroxylation of progesterone in *Aspergillus ochraceus*: Part I: characterisation of the hydroxylase system. *Biochem. Biophys. Res. Commun.* **106** (1982) 1262–1268.
11. Madyastha K. M., Jayanthi C. R., Madyastha P. and Sumathi D.: Studies on the microsomal 11α-hydroxylation of progesterone in *Aspergillus ochraceus*: characterisation and solubilisation of the hydroxylase system. *Can. J. Biochem. Cell Biol.* **62** (1984) 100–107.
12. Samanta T. B. and Gosh D. K.: characterisation of progesterone 11α-hydroxylase of *Aspergillus ochraceus TS*: a cytochrome *P*-450 linked monooxygenase. *J. Steroid Biochem.* **28** (1987) 327–332.
13. Shibahara M., Moody J. A. and Smith L. L.: Microbial hydroxylation. V. 11α-Hydroxylation of progesterone by cell-free preparation of *Aspergillus ochraceus*. *Biochim. Biophys. Acta* **202** (1970) 172–179.
14. Tan L. and Falardeau P.: Interactions of steroids and fungi—III. 11α-Hydroxylation and degradation of progesterone 4-^{14}C by a cell-free preparation from *Aspergillus ochraceus*. *J. Steroid Biochem.* **1** (1970) 221–227.
15. Zuidweg M. H. J.: Hydroxylation of Reichstein's Compound S with cell-free preparation of *Curvularia lunata*. *Biochim. Biophys. Acta* **152** (1968) 144–158.
16. Imai M., Shimada H., Watanabe Y., Matsuchima-Hibiya Y., Makino R., Koga H., Horiuchi T. and Ishimura Y.: Uncoupling of cytochrome $P\text{-}450_{cam}$ monooxygenase reaction by a single mutation, threonine-252 to alanine or valine. A possible role of the hydroxyaminoacid in oxygen activation. *Proc. Natl Acad. Sci. U.S.A.* **86,** (1989) 7823–7827.
17. Cytochrome *P*-450: Biochemistry and biophysics. *Proc. 6th Int. Conf. of Cytochrome P-450* (Edited by I. Schuster). Taylor & Francis, London (1989).