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Studies on the microsomal 11α-hydroxylation of progesterone in *Aspergillus ochraceus*: characterization of the hydroxylase system

K M MADYASTHA*, C R JAYANTHI and P MADYASTHA

Department of Organic Chemistry, Indian Institute of Science, Bangalore 560012, India

Abstract. From the induced vegetative cell cultures of *A. ochraceus*, a procedure for the preparation of cell-free extract with high 11α-hydroxylase activity was developed. To obtain optimal hydroxylase activity, EDTA (10 mM), glycerol (10%) and DTT (5 mM) were required in the grinding medium. Although the optimum pH for the grinding medium was 8·3, the hydroxylase has a pH optimum of 7·7. Microsomes (2 mg) isolated from the crude cell-free extract, hydroxylated progesterone in high yields (85–90% in 30 min) in the presence of NADPH and O_2. The apparent K_m for NADPH and progesterone were 0·052 mM and 0·625 mM respectively. The involvement of cytochrome P-450 system in the hydroxylation reaction was established by various inhibition studies. The hydroxylase activity was inhibited by metyrapone, carbon monoxide, SKF-525A and *p*-CMB. The presence of high levels of NADPH-cytochrome *c* reductase in the microsomal fraction and the strong inhibition of the hydroxylase system by cytochrome *c* indicated that the reductase could be one of the components of the hydroxylase system. Progesterone has the ability to induce the 11α-hydroxylase system significantly, whereas deoxycorticosterone and phenobarbital failed to bring about the induction. However, deoxycorticosterone acted as a good substrate for the 11α-hydroxylase system. The membrane-bound hydroxylase was solubilized using various ionic and non-ionic detergents. Solubilized membrane fraction contained considerable levels of cytochrome P-450 and NADPH-cytochrome *c* reductase, besides hydroxylase activity.

Keywords. 11α-Hydroxylase; progesterone; 11α-hydroxyprogesterone; *A. ochraceus*; cytochrome P-450; detergents; cell-free extract; microsomes; induction.

1. Introduction

Although cell-free preparations from mammalian tissues have been obtained which are capable of introducing hydroxyl groups at several positions in the steroid molecule (Hamberg *et al* 1974), our knowledge regarding the steroid hydroxylating enzymes from various filamentous fungi is still very limited. One of the problems normally encountered is the difficulty in retaining the enzymatic activity during the disruption of the rigid fungal cells. In many cases the lack of proper methods to prepare active cell-free extract from the vegetative mycelial cells has restricted further studies on the intracellular site and the nature of the enzymes involved in the steroid hydroxylations. However, there are reports on the hydroxylation of steroids by the cell-free extract prepared from different fungal systems (Zuidweg *et al* 1962; Chang and Sih 1965; Zuidweg 1968; Lin and Smith 1970; Shibahara *et al* 1970; Tan and Falardeu 1970; Abdel-Fattah and Badawi 1975; Breskvar and Hudnik-Plevnik 1977a, b; Breskvar and Hudnik-Plevnik 1981; Ghosh and Samanta 1981).

Peterson and Murray (1952) first reported the 11α-hydroxylation of progesterone by *R. nigricans*. Later it was shown that other fungal systems such as *A. ochraceus*

* To whom all correspondence should be addressed.

(Shibahara *et al* 1970) *A. niger* (Abdel-Fattah and Badawi 1975) and *R. arrhizus* (Breskvar and Hudnik-Plevnik 1981) could also bring about the 11α-hydroxylation of progesterone. However, it was Shibahara *et al* (1970) who reported for the first time the preparation of a cell-free system from *A. ochraceus* capable of converting progesterone to 11α-hydroxyprogesterone (11α-OHP). They used 24000 g supernatant of the cell-free extract as the source of the hydroxylating system and the reaction was found to be efficient when carried out at 4°C. Ghosh and Samanta (1981) have shown that the post-mitochondrial fraction of the cell-free extract prepared from a mutant strain of *A. ochraceus* hydroxylated progesterone to 11α-hydroxyprogesterone whereas the microsomal fraction failed to do so even after the addition of NADPH or NADH. The lack of hydroxylase activity in the microsomal fraction was attributed either to the distribution of the components of the hydroxylating system between membrane structure and cytosol or one of the components of the system is loosely bound to the membrane and hence easily removed during the preparation of the cell-free extract. In the present study we have standardized the optimum conditions for rupturing the vegetative mycelial cells to obtain crude homogenate with maximum hydroxylase activity. Our aim was also to establish the sub-cellular localization of the hydroxylase system.

In the present paper we report that the microsomes isolated from the crude cell-free extract of *A. ochraceus* are the exclusive site for the 11α-hydroxylation reaction. The paper also deals with the characterization of the hydroxylase system and provides enough evidences for the involvement of cytochrome P-450 system in the hydroxylation reaction. Finally, the paper reports our attempts to solubilise this membrane-bound hydroxylase system using various ionic and non-ionic detergents and the presence of both cytochrome P-450 and NADPH-cytochrome *c* reductase in the detergent solubilised membrane fraction.

2. Material and methods

The following chemicals were obtained from the Sigma Chemical Co., St. Louis, MO, USA: progesterone, 11α-hydroxyprogesterone (11α-OHP), glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase, $NADP^+$, NADPH, NADH, Tris, dithiothreitol (DTT) *p*-chloromercuribenzoate (*p*-CMB), cytochrome *c* (type III), N-methylmaleimide, sodium cholate, Lubrol WX, Triton X-100, Tween-80, sodiumdeoxycholate and glucose oxidase. SKF-525A was from the Smith, Kline and French Labs., and metyrapone from the Aldrich Chemical Co.

Aspergillus ochraceus NRRL 405 was used in this investigation. The stock culture was maintained on Sabouraud agar slant, stored at 4°C and subcultured periodically.

2.1 *Shake culture growth of the organism*

Flasks containing 100 ml of modified Czapeck Dox (Prema and Bhattacharyya 1962) media were inoculated with conidial suspension from a 5-day old culture of *A. ochraceus* and were incubated at 29–30°C on a rotary shaker (220 rpm) for 36 hr. After this growth period 10–50 mg of progesterone in 0·2–0·5 ml of acetone were added to each flask and the fermentation was allowed to continue for 12 hr (induction period) before harvesting the mycelia.

2.2 *Preparation of cell-free extract*

The following operations were carried out between 0 and 4°C unless otherwise specified. Microsomes from crude cell-free extract were isolated as described earlier (Madyastha *et al* 1984). Briefly, washed mycelial mat was suspended in 0·1 M Tris-HCl buffer (pH 8·3) containing 0·25 M glucose, 1 mM KCl, 10 mM EDTA, 10% glycerol and 5 mM DTT (complete medium). The mycelial suspension was finely pulverized for 5 min with glass powder in a cold mortar and pestle. The ground mycelial suspension was filtered through cheese cloth. From the crude cell-free extract, microsomes were isolated by differential centrifugation. The washed microsomes were resuspended in 0·1 M Tris-HCl (pH 7·7) containing 0·25 M glucose, 1 mM KCl, 10mM EDTA, 20% glycerol and 0·5 mM DTT (medium A) at a concentration of about 10 mg protein per ml.

2.3 *Assay and incubation conditions*

The hydroxylase assay mixture contained 0·1 M Tris-HCl (pH 7·7), 0·33 μmoles progesterone in 20% Tween-80 in acetone (15 μl), 0·5 mM DTT, 0·75 μmoles G-6-P, 0·25 μmoles $NADP^+$, 0·2 units G-6-P dehydrogenase, 1 μM $MgCl_2$, 10 mM EDTA and microsomes (about 2 mg) in a total volume of 1·6 ml. The reaction was initiated by the addition of NADPH generating system and the incubation was carried out under aerobic conditions for 30 min at 30°C on a rotary shaker. The reaction was terminated by the addition of 1 ml of methylene chloride. The enzymatic product formed was extracted, isolated and estimated as described earlier (Jayanthi *et al* 1982)

Protein was estimated by the method of Lowry *et al* (1951). The NADPH-cytochrome *c* reductase activity was measured at 550 nm as previously reported (Madyastha and Coscia 1979) using a 557 Hitachi recording spectrophotometer. Cytochrome P-450 was estimated according to the method of Omura and Sato (1964).

2.4 *Solubilization of microsomal hydroxylase system*

Different ionic and non-ionic detergents used for the solubilization of the hydroxylase system are listed in table 7. Microsomal protein (≈ 30 mg) was suspended in medium A (6 ml) and to this 10% detergent solution was added dropwise with stirring to give a final detergent to protein ratio as mentioned in table 7. The mixture was stirred for an additional 30–40 min. It was then centrifuged at 100000 g for one hr and the clear buff coloured supernatant was used to estimate hydroxylase, cytochrome P-450 and NADPH-cytochrome *c* reductase activities. The detergent present in the solubilized material was removed either by ultrafiltration or by passing through Biobead SM-2.

3. Results

Our objectives in the present investigation were to find out the intracellular site and the nature of the enzyme system involved in the 11α-hydroxylation of progesterone in *A. ochraceus*. To achieve this, we first sought to explore the necessary conditions required to prepare active cell-free extract from progesterone induced mycelial cells. Different parameters such as pH, molarity and contents of the medium were tested during the preparation of the cell-free extract.

The effect of the pH of the grinding medium on the microsomal 11α-hydroxylase activity was studied (table 1). Different sets of cell-free extracts were prepared in

Table 1. Effect of pH of the medium during the preparation of cell-free extract.

pH of the grinding medium	11α-hydroxylase activity (%)
7·0	16·0
7·3	33·6
7·7	92·7
8·0	84·0
8·3	100·0
8·7	68·0

The same batch of mycelium was used to carry out these experiments. Grinding of the mycelium was carried out in a medium of the indicated pH. The contents of the Complete Medium were as described in §2. The cell-free extract was subjected to differential centrifugation to get microsomal fraction. Hydroxylase assays were conducted at pH 7·7 as described in §2 using 1·5 mg of microsomal protein and 100 μg of progesterone, 100% activity represents 169 nmol of 11α-OHP/h/mg protein.

Complete Medium having different pH values as described in §2 and microsomes were isolated from each set. Microsomes isolated at pH 8·3 had maximal hydroxylase activity (table 1). On the other hand, microsomes prepared at or below pH 7·0 showed only 15–20% of the optimal activity. Considerable loss ($\approx$ 35%) of the optimal activity was noticed when the pH of the grinding medium was maintained between 8·7 and 8·9. It was also observed that microsomes isolated in phosphate buffer (0·1 M, pH 7·8) proved to be nearly 40% less active than similar preparation obtained using Tris-HCl (0·1 M, pH 7·8) buffer.

Table 2 summarises the ideal conditions for the isolation of active cell-free extract. Presence of EDTA (10 mM), glycerol (10%, w/v) and DTT (5 mM) in Tris-HCl buffer (0·1 M, pH 8·3) was necessary for the preparation of active cell-free extract. Considerable (25–40%) hydroxylase activity was lost when any one of EDTA, glycerol or DTT was deleted from the grinding medium (table 2). When all these three components were deleted from the grinding medium, nearly 70% of the hydroxylase activity was lost. The optimal EDTA concentration required in the grinding as well as assay medium was found to be 10 mM. Although the presence of DTT in the grinding medium was essential, its addition to the hydroxylase assay medium did not seem to influence the activity to any significant extent.

3.1 *Identification of the metabolite*

Routine quantitative analyses of the enzymatic products formed were conducted by analysing the products formed by HPLC as described earlier (Jayanthi *et al* 1982). Formation of 11α-OHP in the assay mixture was established by performing a large scale incubation. The metabolite formed in 30 min was isolated and purified by TLC using

Table 2. Effect of different components in the grinding medium during isolation of 11α-hydroxylase system.

Components of the grinding medium	11α-hydroxylase activity (%)
Complete Medium	100
Complete Medium – (glucose, KCl, EDTA, glycerol, DTT)	26
Complete Medium – (EDTA, glycerol, DTT)	30
Complete Medium – (EDTA)	68
Complete Medium – (DTT)	61
Complete Medium – (glycerol)	73

The induced cells were ground in Tris-HCl (0·1 M, pH 8·3) containing 0·25 M glucose, 1 mM KCl, 10 mM EDTA, 10% glycerol and 5 mM DTT (Complete Medium). Cell-free extract (1100 g supernatant) was used as the source of 11α-hydroxylase system. One hundred percent activity represents 222 nmol of 11α-OHP/h/mg of protein. From the Complete Medium different components were deleted and the cell-free extract was prepared. Hydroxylase assays were conducted with 2 mg of protein as described in §2.

silica gel G plates with ethylacetate/hexane (85 : 15) as the solvent system. The purified compound was crystallized to constant m.p. 164–65°C (literature 166–67°C, Peterson and Murray 1952). The IR spectrum of the purified 11α-OHP was superimposable with that of the authentic compound.

3.2 *pH optimum*

Although pH 8·3 was ideal for preparing the active cell-free extract, the hydroxylase has a pH optimum of 7·7. Tris-HCl, Tris-maleate and potassium phosphate buffers in the pH range 6·5 to 8·8 were used to study the hydroxylation reaction. In all these buffer systems, the hydroxylase revealed the same pH optimum (7·7) and Tris-HCl (0·1 M) yielded highest activity.

3.3 *Intracellular site*

Differential centrifugation studies as reported earlier (Jayanthi *et al* 1982) clearly showed that most of the hydroxylase activity is associated with the microsomal fraction sedimenting at 105000 g. High NADPH-cytochrome *c* reductase activity was also observed in this fraction.

3.4 *Requirements for 11α-hydroxylation*

Hydroxylase activity was dependent upon NADPH and O_2 (table 3). NADPH could not be replaced by NADH. Addition of NADH along with NADPH did not produce any synergistic effect. The hydroxylase activity was inhibited to a significant level when assays were conducted in the absence of O_2. This suggests that the hydroxylase may be a typical monooxygenase. The effect of varying concentrations of NADPH under standard hydroxylase assay conditions was studied. Kinetic analysis revealed that the hydroxylase possessed an apparent K_m of 0·0525 mM for NADPH, calculated by the Lineweaver-Burk plots.

Table 3. Requirements of microsomal 11α-hydroxylation of progesterone.

Deletions	Additions	11α-OHP formed (nmoles/mg/h)
None (Complete)	—	249·0
NADPH generating system	—	18·0
NADPH generating system	NADPH (1 μmol)	214·0
NADPH generating system	NADH (1 μmol)	57·6
Complete minus O_2	—	51·5

Assays were conducted with 2 mg of microsomal protein and 100 μg of progesterone as described in §2. Septumcapped test tubes were used to conduct assays anaerobically. The tubes were deaerated with N_2 and the residual O_2 was removed by preincubating the assay mixture with glucose (0·5 mM) and glucose oxidase (200 units) for 10 min and after that the reaction was initiated by the addition of NADPH generating system.

3.5 *Effect of time, temperature and substrate concentration*

Microsomes prepared as described in §2 were found to convert 80–85% of the added progesterone to 11α-OHP in 30 min under standard assay conditions. The amount of 11α-OHP formed remained constant upto 180 min. On the basis of kinetic data, the product formation was linear upto a concentration of 100 μg of progesterone in the standard assay mixture. The apparent K_m for progesterone calculated by the Lineweaver-Burk plots was 0·625 mM. When the incubation period was extended to 90 min trace amounts of 6β,11α-dihydroxy progesterone was formed. The optimum temperature for the hydroxylase was found to be 30°C. If the hydroxylase assays were carried out at 25 or 35°C, considerable drop (30%) in the activity was noticed. In contrast to the earlier observations (Shibahara *et al* 1970), the hydroxylase assays conducted at 4°C showed very little activity.

3.6 *Effect of inhibitors*

When the microsomal 11α-hydroxylation reaction was carried out in the presence of metyrapone, SKF-525A, carbon monoxide, cytochrome *c*, *p*-CMB and N-methyl-maleimide, a significant inhibition was observed (table 4). Cyanide (1 mM) inhibited the hydroxylase system to the extent of 13% whereas there was no detectable inhibition at 0·1 mM level.

3.7 *Induction of 11α-hydroxylase system*

Microsomes prepared from uninduced cells did not contain any hydroxylase activity. However, progesterone added during the induction period (see §2) was shown to induce the hydroxylase system to a significant level (65%, table 5). As opposed to this deoxycorticosterone and phenobarbital failed to induce the hydroxylase system, whereas moderate induction was seen with androstenedione and testosterone (table 5).

3.8 *Substrate specificity of the hydroxylase system*

Preliminary studies carried out with progesterone-induced microsomes clearly indicated that the hydroxylase system can catalyze the 11α-hydroxylation of androstene-

Table 4. Effect of different inhibitors on the 11α-hydroxylase system.

Inhibitor used	% inhibition
None* (control)	0
Metyrapone (0·25 mM)	58
SKF-525 A (1 mM)	59
CO† (Bubbled for 2 min)	82
KCN (1 mM)	13
Cytochrome *c* 5 μM	69
10 μM	85
p chloromercuribenzoate (0·1 mM)	93
N-methylmaleimide (1 mM)	90

* One hundred percent activity (0% inhibition) represents 270 nmol of 11α-OHP formed per hr per mg protein. Inhibitors were preincubated with microsomes (2 mg) for 5 min at 30°C before the substrate (100 μg) and cofactors were added. For details of the incubation conditions, see §2.

† In the control experiment, instead of carbon monoxide nitrogen was bubbled for 2 min.

Table 5. Effect of different steroids and phenobarbital on the induction of hydroxylase system.

Compounds tested	11α-OHP formed (%)
Nil	0
Progesterone	65
Androstenedione	37
Testosterone	35
Deoxycorticosterone	0
Phenobarbital	0

The concentrations of the steroids used were 50 mg in 0·5 ml acetone, while that of phenobarbital was 50 mg in 0·5 ml water. Induction was carried out with the indicated amounts of compounds added to the growth medium as described in §2. After the induction period (12 hr), the mycelial mat was washed and used to determine its ability to hydroxylate progesterone. The experiment was carried out by incubating 500 mg of washed mycelium in 10 ml of 0·1 M phosphate buffer (pH 7·4) with progesterone (12·5 mg/0·13 ml 20% Tween-80 in acetone) for 2 hr at 30°C on a rotary shaker. The product formed was isolated and estimated as described earlier (Jayanthi *et al* 1982).

dione, testosterone, 17α-hydroxy progesterone, 16-dehydro progesterone, and deoxycorticosterone besides progesterone (table 6). However, estrone, estradiol, androsterone and androstanone were not accepted as substrates.

3.9 *Solubilization of microsomal* 11α-*hydroxylase*

A microsomal fraction (30 mg) was solubilized using different ionic and non-ionic

Table 6. Substrate specificity of the microsomal 11α-hydroxylase system.

Substrate	Hydroxylated product formed	% conversion
Androstenedione	11α-hydroxyandrostenedione	91
Testosterone	11α-hydroxy testosterone	75
17α-Hydroxyprogesterone	11α,17α-dihydroxyprogesterone	73
16 Dehydroprogesterone	11α-hydroxy, 16-dehydroprogesterone	76
Progesterone	11α-OHP	95
Deoxycorticosterone	11α-hydroxy deoxycorticosterone	95
Estrone	—	—
Estradiol	—	—
Androsterone	—	—
Androstanone	—	—

The assays were conducted as described in §2. The hydroxylated products formed were identified by comparing their HPLC profiles (retention time) with that of the authentic compounds. The % conversion of each substrate was determined from the peak height measurements as reported earlier (Jayanthi *et al* 1982).

detergents keeping the protein to detergent ratio as mentioned in table 7. The ability of different detergents to solubilize hydroxylase activity, cytochrome P-450 and NADPH-cytochrome *c* reductase is summarised in table 7. As seen in the table, Tween-80 was the most efficient detergent which solubilized 57% and 68% of the hydroxylase and NADPH-cytochrome *c* reductase activities respectively. The solubilized fraction also contained considerable levels of cytochrome P-450 (0·022 nmole/mg) as evidenced by the reduced CO-difference spectrum. The unsolubilized microsomal fraction (pellet) could still account for 30 and 32% of the microsomal hydroxylase and reductase activities respectively (table 7). Only 13% of the microsomal hydroxylase activity was lost during the solubilization of microsomes by Tween-80. The specific activity of the hydroxylase as well as the reductase was considerably higher than that of the microsomal fraction (table 7). Combination of Tween-80 and sodium cholate did not prove to be better than Tween-80 when used alone. When sodium deoxycholate, Triton X-100 and Lubrol WX were used to solubilize the microsomes, significant loss in the hydroxylase, cytochrome P-450, and reductase activities was observed. Earlier, we had reported the solubilization of microsomes using sodium cholate and it was observed that nearly 50% of the hydroxylase activity was lost during the solubilization process which could be due to slow inactivation of either cytochrome P-450 or one of the components of the hydroxylase system. However, solubilization using Tween-80 seems to prevent the inactivation of the hydroxylase system.

3.10 *Stability of the hydroxylase system*

The microsomal bound 11α-hydroxylase retained 90% of its activity for 4–5 weeks whereas Tween-80 solubilized microsomal fraction lost nearly 70% of its hydroxylase activity in 3–4 weeks when stored at −20°C. During this period nearly 30% of NADPH-cytochrome *c* reductase and 50% of cytochrome P-450 activities in the solubilized microsomal fraction were lost.

Table 7. Solubilization of microsomal 11α-hydroxylase system using different detergents.

Detergents used for solubilization	Detergent protein ratio	Protein fraction*	11α-hydroxylase		Reductase		Cyt. P-450 (in S.F.)
			Sp. activity (nmol/min/mg)	Activity in each fr. (%)	Sp. activity (nmol/min/mg)	Activity in each fr. (%)	Sp. activity (pmol mg)
—	—	Microsomes (30)	4·6	100	158·7	100	N.D.+
Sodium cholate	0·3 : 1·0	Pellet (21·6)	1·6	25·0	95·6	43·4	
		S.F. (8·3)	3·7	22·4	170·8	30·1	21·6
Triton X-100	0·3 : 1·0	Pellet (20·0)	0·39	5·6	54·8	23·0	
		S.F. (9·3)	1·68	11·40	194·1	38·3	23·4
Tween-80	0·6 : 1·0	Pellet (15·9)	2·6	29·5	94·4	31·7	
		S.F. (14·4)	5·4	56·5	224·7	68·0	21·6
Sodium deoxycholate	0·2 : 1·0	Pellet (19·6)	0·27	3·9	127·7	52·5	
		S.F. (10.6)	0·20	2·3	83·9	18·9	25·7
Sodium cholate—	0·3 : 1·0	Pellet (18.5)	0·73	9·7	87·4	34·0	
+	+						
Lubrol WX—	0·063 : 1·0	S.F. (9.6)	2·05	14·3	141·6	28·6	12·3
Triton X-100—	0·2 : 1·0	Pellet (19·4)	—	—	54·4	22·2	
+	+						
Sodium cholate—	0·2 : 1·0	S.F. (9·6)	0·5	3·6	251·9	49·5	27·1
Tween-80—	0·6 : 1·0	Pellet (15·0)	1·5	16·1	63·8	20·1	
+	+						
Sodium cholate—	0·2 : 1·0	S.F. (15·0)	4·13	44·9	193·2	60·9	12.0

Each experiment was carried out with 30 mg of microsomal protein. Solubilization was carried out keeping the detergent/protein ratio as mentioned in table 7. Hydroxylase, reductase and cyt. P-450 were estimated as described in §2. Sp. activity of reductase is expressed as nmol of cyt. *c* reduced per min per mg protein, utilizing the extinction coefficient for reduced minus oxidized cyt. *c* of $21 \times 10^3\ M^{-1}\ cm^{-1}$ (Williams and Kamin 1962).

* Values in parenthesis refer to the mg of protein present in each fraction; + N.D.—not determined; S.F.—solubilized fraction; hydroxylase was induced by adding 10 mg of progesterone during the induction period as described in §2.

4. Discussion

We have established that in *A. ochraceus* the 11α-hydroxylase activity is localized solely in the microsomal fraction contrary to earlier reports (Shibahara *et al* 1970; Ghosh and Samanta 1981). The requirement for NADPH and molecular O_2 classifies this enzyme system as a microsomal mixed function oxidase or monooxygenase. Earlier studies (Ghosh and Samanta 1981) have revealed that in *A. ochraceus* TS, the post-mitochondrial supernatant contained the 11α-hydroxylase activity and neither the microsomal pellet nor the post-microsomal supernatant (cytosolic) could individually carry out the hydroxylation reaction in the presence of NADPH and O_2. However, when the microsomal fraction was mixed with the cytosolic fraction measurable amounts of hydroxylase activity were observed (1·2 nmoles/h/mg). The present investigation has conclusively established the non-involvement of the cytosolic fraction in the 11α-hydroxylation reaction. Microsomes (2 mg) isolated following our method converted 80–85% of the added progesterone to 11α-OHP in 30 min at 30°C. At the end of 90 min only trace amounts of 6β,11α-dihydroxy compound were formed. Ghosh and Samanta (1981) have noticed earlier that the cell-free 11α-hydroxylase required 4 hours of induction period to get demonstrable amounts of 11α-OHP.

Steroid transformations by fungal spores or by vegetative cell mass are not significantly influenced by variations in pH of the medium (Vezina *et al* 1963). For example, hydroxylation of progesterone by *A. ochraceus* is not considerably influenced by pH in the range 4 to 8·5 (Vezina *et al* 1963). However, this does not seem to be applicable when the hydroxylation reaction is carried out at the cell-free level. In the case of *A. niger* 12Y, the 11α-hydroxylase activity in the cell-free extract decreased with increase of pH of the medium and was optimal at pH 3·4 (Abdel-Fattah and Badawi 1975).

Earlier investigators have used phosphate buffer for the preparation of microbial cell-free steroid hydroxylases (Zuidweg *et al* 1962; Chang and Sih 1965). The cell-free 11α-hydroxylase systems of *R. nigricans* and *A. ochraceus* were prepared by disrupting the cells in phosphate buffer at pH 5·5 and 7·1, respectively (Breskvar and Hudnik-Plevnik 1981; Shibahara *et al* 1970). However, our method of preparing cell-free extract from *A. ochraceus* is different from that reported earlier. We have observed that the grinding of the mycelium should be carried out at pH 8·3 in Tris-HCl medium to realize optimal hydroxylase activity in the cell-free extract, although the 11α-hydroxylase has a pH optimum of 7·7. Besides, the presence of EDTA (10 mM), DTT (5 mM) and glycerol (10%, w/v) in the grinding medium seems to be critical for isolating very active microsomes with good hydroxylase and NADPH-cytochrome *c* reductase activities. Deviation from these conditions resulted in a preparation with very low hydroxylase activity. We have noticed the requirement of 10 mM EDTA in the grinding medium as well as in the assay medium (table 2). Although the requirement of high levels of EDTA in the extraction medium is unusual particularly while isolating a hydroxylating system, such observations have been made earlier during the isolation of different hydroxylases (Zuidweg *et al* 1962; Zuidweg 1968; Madyastha *et al* 1976; Hosakawa and Stanier 1966). High levels of EDTA and the pH (8·3) of the medium could possibly have inhibited the proteolytic activity of the cell-free extract, thus preventing the inactivation of the hydroxylase system. In certain micro-organisms it is known that metal chelators inhibit proteolytic activity (Hazlewood and Edwards 1981). Mammalian cytochrome P-450 mediated mixed function oxidases are sensitive to

proteolytic enzymes, hence EDTA is generally used in the buffer during its isolation. However, there are conflicting reports regarding the role of EDTA during the isolation of 11α-hydroxylase from *A. niger* 12Y and *R. nigricans* (Abdel-Fattah and Badawi 1975; Breskvar and Hudnik-Plevnik 1981). We have noticed that the presence of both DTT and glycerol stabilizes the cell-free preparation (table 2). Cytochrome P-450 mediated systems are normally stabilized in the presence of glycerol and sulphydral agents such as DTT. Among the sulphydral reagents tested, DTT was found to be the most effective one.

The involvement of cytochrome P-450 in the 11α-hydroxylation reaction is based on various inhibition studies (table 4). Metyrapone, SKF-525A and CO are typical inhibitors of cytochrome P-450 mediated mixed function oxidases. The inhibition of the hydroxylase by cytochrome *c* and the presence of high levels of NADPH-cytochrome *c* reductase in the microsomal fraction suggest the participation of reductase in the 11α-hydroxylation reaction. This is supported by the fact that the microsomes obtained from uninduced cells of *A. ochraceus* do not carry out the 11α-hydroxylation and contain very low levels of NADPH-cytochrome *c* reductase. A possible role of cytochrome P-450 in the 11α-hydroxylation of progesterone by *R. nigricans* and *A. ochraceus* TS has been reported earlier (Breskvar and Hudnik-Plevnik 1977a, b; Ghosh and Samanta 1981; Breskvar and Hudnik-Plevnik 1981). In the case of *R. nigricans*, it was shown that the membrane fraction sedimenting at 105000 g contained both cytochrome P-450 and NADPH-cytochrome *c* reductase, whereas neither of them were found to be present in the membrane fraction of uninduced cells (Breskvar and Hudnik-Plevnik 1981). The hydroxylation reaction is also inhibited by *p*-CMB and N-methylmaleimide. It is known that one of the components of the cytochrome P-450 system viz. NADPH-cytochrome *c* (P-450) reductase is quite sensitive to these inhibitors (Madyastha and Coscia 1979). We have noticed that KCN at 1 mM concentration inhibits the hydroxylase reaction by 13% whereas at lower concentration (0·1 mM) there was no detectable inhibition (table 4). Earlier reports indicated that KCN activated steroid hydroxylation reactions in fungal systems (Zuidweg 1968; Ghosh and Samanta 1981). However, these studies were carried out with crude cell-free extract which is less defined than microsomes.

In most fungi the enzymes involved in steroid hydroxylation are known to be inducible (Chang and Sih 1965; Zuidweg 1968; Lin and Smith 1970). Earlier it was shown that progesterone induces 11α-hydroxylase system in *A. ochraceus* and *R. nigricans* (Shibahara *et al* 1970; Breskvar and Hudnik-Plevnik 1977a), whereas cells grown in the absence of progesterone either contained very little or no hydroxylase activity. Our attempts to find out whether or not 11α-hydroxylase activity in *A. ochraceus* are inducible by steroids related to progesterone, revealed that none of the steroids tested were as efficient as progesterone in inducing the activity (table 5). It is interesting to note that deoxycorticosterone, a very good substrate for the 11α-hydroxylase system (table 6), failed to induce the hydroxylase when it was added during the induction period. Although 11α-hydroxylation reaction in *A. ochraceus* is mediated by a cytochrome P-450 system (Jayanthi *et al* 1982), phenobarbital added to the growth medium 12 hr before the end of the mycelial growth, did not induce the microsomal cytochrome P-450 as well as the hydroxylase activity. The cytochrome P-450 in *Claviceps purpurea* has been shown to be inducible by phenobarbital (Ambike *et al* 1970). Such an effect has also been demonstrated for brewer's yeast (Wiseman and Lim 1975).

Microsomes isolated from progesterone-induced mycelia were found to catalyze the

11α-hydroxylation of androstenedione, testosterone, 17α-hydroxyprogesterone, 16-dehydroprogesterone, deoxycorticosterone besides progesterone, in very high yields, whereas estrone, estradiol, androsterone and androstanone were not accepted as substrates for the hydroxylase system (table 6). The products formed in each case were identified by comparative HPLC analyses carried out with authentic samples. It appears that one of the important structural features necessary for steroidal compounds to be accepted as substrate for the hydroxylase system is the Δ^4-3 keto functional group (4-ene-3-one group). Both C_{19} and C_{21} steroids with this functional group were readily hydroxylated at the 11α-position, whereas androsterone and androstanone which lack this structural feature failed to get hydroxylated (table 6). C_{19} steroids with an aromatic A-ring were also not accepted by the hydroxylase system. Hydroxylation of some of the steroids by the spores of *A. ochraceus* has been reported earlier (Vezina *et al* 1963).

Studies on the inhibition of microsomal 11α-hydroxylation of progesterone by various inhibitors (table 4) and the finding that both cytochrome *c* reductase and cytochrome P-450 are exclusively in the microsomes (Jayanthi *et al* 1982) suggested that these two enzymes are components of the monooxygenase system. To get further support for this assumption, the microsomal bound hydroxylase was solubilized using different ionic and non-ionic detergents. It was observed that Tween-80 was the most efficient detergent which solubilized nearly 57% of the original hydroxylase activity. The solubilized fraction also contained significant levels of cytochrome P-450 and NADPH-cytochrome *c* reductase. Sodium cholate proved to be the second best among the detergents tested. Significant loss in the hydroxylase activity as well as cytochrome P-450 and reductase were noticed when detergents such as sodium deoxycholate, Triton X-100 and Lubrol WX were used. Although 1% Triton X-100 was used by the earlier investigators (Breskvar and Hudnik-Plevnik 1981) to solubilize 11α-hydroxylase from *R. nigricans*, in our hands this detergent did not prove to be efficient in solubilizing the membrane bound hydroxylase system from *A. ochraceus* (table 7). Excepting Tween-80, the other detergents tested to solubilize the membrane-bound hydroxylase system resulted in a significant loss of not only the hydroxylase activity but also cytochrome P-450 and reductase activities. However, the effect was less pronounced in the case of sodium cholate.

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