A POSSIBLE ROLE OF CYTOCHROME P-450 IN HYDROXYLATION OF PROGESTERONE BY *RHIZOPUS NIGRICANS*

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SUMMARY

Hydroxylation of progesterone was performed with intact cells, cell homogenate and with different subcellular fractions. The hydroxylating system in *Rhizopus nigricans* was found in post-mitochondrial supernatant. The reaction was inhibited by carbon monoxide and the inhibition was reversed by irradiation with light at a predominant wavelength of 450 nm thus indicating the involvement of cytochrome P-450 in the hydroxylation reaction. Using the reduced CO difference spectra, we have located the enzyme cytochrome P-450 in the membrane fraction sedimenting at 105.000 g.

Various fungal cell-free extracts were shown to hydroxylate steroids (1-4). The cell-free extract of *R. nigricans* has been reported to hydroxylate progesterone[1] predominantly at 11α position (4); relatively little is, however, known about the intracellular site of hydroxylation and about the enzymes involved in the steroid hydroxylation reactions. Cytochrome P-450 is known to be an essential constituent of various hydroxylating systems in mammalian tissues (review ref. 5), in higher plants (6), yeast (7) and in bacteria (8). It was not reported thus far, whether the same mechanism operates also in hydroxylation of steroids in filamentous fungi; it was even suggested that the 9α-hydroxylation in *Nocardia restrictus* proceeds by a completely different mechanism (9). The present report provides, however, evidence that cytochrome P-450 is present in the fungus *R. nigricans* and

[1]Systematic nomenclature for steroids in text include: pregn-4-ene-3,20-dione for progesterone; 11α-hydroxypregn-4-ene-3, 20-dione for 11α-OH progesterone; 6β-hydroxypregn-4-ene-3,20-dione for 6β-OH progesterone.

is required for the 11α-hydroxylation of progesterone in this organism.

MATERIALS AND METHODS

Rhizopus nigricans ATCC 6227b was grown in the rotary shaker Infors AG RC 1TK at 28°C for 46 h in the nutrient medium described elsewhere (10). 6 h before the end of mycelial growth progesterone dissolved in dimethylformamide was added to the culture (final progesterone concentration 100 µg/ml) for induction of hydroxylases. At the end of the induction period the remaining progesterone and its hydroxylated products were removed by washing the cells with cold 0.5% saline (1). The cells were then resuspended in 1 mM phosphate buffer pH=5.5, containing 0.2 mM EDTA, 0.04 mM glutathion (4) and 0.25 M sucrose (1g moist mycelia per 5 ml of buffer). The cells were disrupted in a Sorval Omni mixer at 16.000 RPM for 3x3 min at 0°C (4). Cell-free extracts were obtained by differential centrifugation as described by Tamaoki (11).

Incubation conditions: 20 ml of cell suspension or an equivalent amount of the corresponding subcellular fraction were incubated with 3 mg of progesterone for 6 h at 28°C. The reaction products were extracted with chloroform and analyzed by gas chromatography. Identification of the products was performed by careful thin layer chromatography in several solvents using authentic steroids for comparison. The inhibition of the reaction was studied in the presence of the gas phase consisting of CO and air (3:1). In the control experiment pure nitrogen was used instead of CO.

Gas chromatography was performed on Varian 2100 gas chromatogram using glass U-tube columns (1.85 m x 0.2 mm) packed with 3% OV-17 and chromosorb HPW/AW-DUCS. Argon was used as carrier at a

Table 1 Light reversible CO inhibition of progesterone hydroxylation

Gas phase	The composition of $CHCl_3$ extracts (%)		
	unreacted progesterone	11α-OH progesterone	6β-OH progesterone
air	19	63	18
CO+air 3:1	100	-	-
N_2+air 3:1	25	55	20
CO+air 3:1 +light	33	53	14

20 ml of homogenate were incubated with 3 mg of progesterone for 6 h at 28°C. The steroids were extracted with $CHCl_3$ and the composition determined by gas chromatography.

flow rate of 30 ml/min. Flash heater temperature was 280°C, detector temperature 305°C and column temperature was 265°C. Peaks on the elution curves were identified by their characteristic retention times and the amount of each component was estimated by automatic area measurment. Steroid composition was expressed in terms of percentage of the total steroid found. The detector response to progesterone, 11α-OH progesterone and 6β-OH progesterone did not differ significantly and,therefore,allowed such estimation.

Protein was assayed by the method of Lowry et al. (12).

The reduced CO difference spectra were obtained according to the method of Omura and Sato (13).

All spectrophotometric measurments were performed on automatically recorded Gilford 2400-S spectrophotometer.

Table 2 Hydroxylation activity of subcellular fractions from *Rhizopus nigricans*

Sample	The composition of $CHCl_3$ extracts (%)		
	unreacted progesterone	11α-OH progesterone	6β-OH progesterone
homogenate	29	56	15
800 g supernatant	86	7	7
10.000 g supernatant	98	1	1
cytosol	100	-	-
105.000 g sediment	100	-	-

20 ml of homogenate or an equivalent amount of the corresponding subcellular fraction were incubated for 6 h with 3 mg of progesterone at 28°C. The reaction products were extracted with $CHCl_3$ and analyzed by gas chromatography.

RESULTS

R. nigricans hydroxylates progesterone mainly at 11α and to some extent at 6β positions. With the aim to find out whether hydroxylations of progesterone are mediated in filamentous fungi by cytochrome P-450 we studied the CO inhibition of these reactions and the reversibility of the inhibition by light (13). As shown in Table 1, CO in the gas mixture inhibited the hydroxylation of progesterone and the inhibition was reversed by irradiation with light at the predominant wavelength 450 nm. This inhibition is believed to be specific for cytochrome P-450 mediated reactions. The presence of nitrogen in the gas mixture under the same conditions affected the hydroxylation of progesterone only slightly.

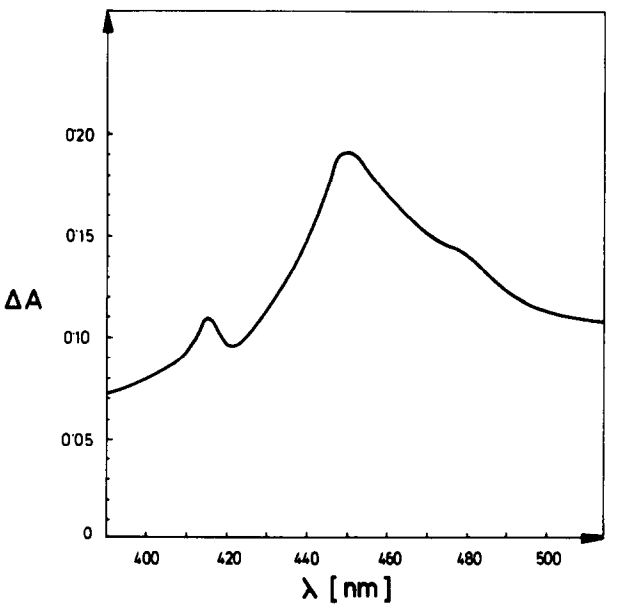

Fig. 1. The reduced CO difference spectrum of the fraction sedimenting at 105.000 g. The 105.000 g sediment was resuspended in 0.05 M phosphate buffer pH = 7.3 to a protein concentration of 0.35 mg/ml. The suspension was reduced with a few crystals of sodium dithionite and CO was bubbled through one part of the suspension for 60 sek. The spectrum was obtained 10 min after the addition of CO.

To get some idea on the intracellular localization of hydroxylation reactions in *R. nigricans* we prepared different subcellular fractions and tested them for the hydroxylating activity. The results on the transformation of progesterone with cell-free preparations from *R. nigricans* are presented in Table 2. The hydroxylating activity can be detected in the supernatant fraction after removing the intact cells, which escaped homogenization, nuclei and debris (800 g supernatant) and in the postmitochondrial supernatant (10.000 g supernatant). The hydroxylation was in both instances inhibited by CO. The results suggest that the site of hydroxylation in this organism is the postmitochondrial fraction. Neither cytosol nor the membrane fraction

sedimenting at 105.000 g alone exhibited the hydroxylating activity even in the presence of NADPH or NADH.

We tried to locate cytochrome P-450 in the subcellular fractions by measuring the reduced CO difference spectra. A typical spectrum obtained with the fraction sedimenting at 105.000 g is presented in Fig.1. The absorption maximum at 450 nm is typical for the CO complex of reduced cytochrome P-450 and confirms the presence of the enzyme in this subcellular fraction. The amount of cytochrome P-450, estimated according to the method of Omura and Sato (13), was about 2×10^{-9} moles per mg of protein in the fraction. Cytochrome P-450 was never detected in the mitochondrial fraction when tested by the same method.

DISCUSSION

The light reversible inhibition of progesterone hydroxylation by carbon monoxide indicates that in *R. nigricans* this reaction requires the enzyme cytochrome P-450. The presence of this enzyme in the fraction sedimenting at 105.000 g suggests strongly that at least one part of the hydroxylating system is bound to some membrane structure. The fraction sedimenting at 105.000 g does not hydroxylate progesterone even after addition of NADPH or NADH.This lack of activity can be explained if the hydroxylating system is distributed in the fungal cell between a membrane structure and the cytosol; it is also possible that one or more constituents of the system are bound very loosely to the membrane and, therefore, easily removed during the fractionation procedure.

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