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Conference Paper

Intracellular Localization and Some Properties of Cytochrome P450 of *Rhizopus nigricans**

K. Breskvar and T. Hudnik-Plevnik

Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Ljubljana, Vrazov trg 2, Slovenia, Yugoslavia

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An attempt was made to localize in *Rhizopus nigricans* the enzyme cytochrome P450, which is required for the hydroxylation of progesterone. Cytochrome P450 was never found in the mitochondrial fraction but was detected in the fraction sedimenting at 105000 g. In *R. nigricans* this fraction does not seem to be identical with the rat liver microsomal fraction in respect to the enzyme activities characteristic for microsomes. In the presence of progesterone the subcellular fraction containing cytochrome P450 exhibits modified type II spectral change. Upon solubilization of this fraction with detergent Triton X-100 a significant amount of the enzyme is converted to its inactive form P420.

INTRODUCTION

The hydroxylation of progesterone (pregn-4-ene-3,20-dione) by the fungus *R. nigricans* is a well known reaction used also for industrial purposes¹. Very little is, however, known about the mechanism and the intracellular site of hydroxylation in these eucariotic cells. Hydroxylation of steroids has been studied extensively in various mammalian systems and the essential constituents of the reaction sequence have been established². We showed that in *R. nigricans*, like in higher organisms, the hydroxylation of progesterone is cytochrome P450 dependent; the reaction is *in vivo* and *in vitro* inhibited by carbon monoxide³. In the present paper we report data on the intracellular localization of cytochrome P450 in *R. nigricans* and on some properties of the subcellular fraction containing this enzyme.

EXPERIMENTAL

Rhizopus nigricans ATCC 6227b was grown by aeration in an Infors AG RC ITK rotary shaker at 28°C for 46 hr in the nutrient medium described in ref. 4. Progesterone was added at a concentration of 100 µg/ml 6 hr before the end of growth to induce the hydroxylases. The remaining progesterone and its hydroxylated products were removed from mycelia with cold saline as described by Shibahara *et al.*⁵. The cells were then suspended in 1 mM phosphate buffer, containing 0.2 mM EDTA, 0.04 mM glutathion⁶ and 0.25 M sucrose (1 g moist mycelia/ 5 ml buffer). The cells were disrupted in a Sorval Omni mixer at 16000 RPM at 0°C for 3 × 3 min⁶. Cell free extracts were obtained by differential centrifugation as described by Tamaoki⁷.

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Protein was assayed according to the method of Lowry *et al.*⁸. The reduced CO difference spectra were obtained by the method of Omura and Sato⁹.

NADPH cytochrome C reductase activity was assayed spectrophotometrically as described by Fleischer and Fleischer¹⁰, and NAD cytochrome C oxidase activity according to the method of Wharton and Tzagoloff¹¹.

RNA was extracted from the subcellular fractions according to Schneider¹² and the concentration estimated by the method of Mejbaum¹³.

All spectrophotometric measurements were performed on an automatically recording Gilford 2400-S spectrophotometer.

RESULTS AND DISCUSSION

R. nigricans hydroxylates progesterone preferentially at 11 α position. We showed earlier that the hydroxylating system in this organism is postmitochondrial supernatant³. This is in agreement with the findings of other authors, who studied steroid hydroxylation in cell free extracts of fungi^{5,14}. In order to get some information on the intracellular localization of cytochrome *P450*, different subcellular fractions of *R. nigricans* were tested for the presence of this enzyme. For this purpose we used the reduced CO difference spectra⁹. A typical spectrum obtained with the subcellular fraction sedimenting at 105 000 g is presented in Figure 1. The absorption maximum at 450 nm of a reduced CO complex confirms the presence of the cytochrome *P450* in this membrane fraction. The enzyme was never found in the mitochondrial fraction when tested by the same method. The result presented suggests, therefore, that in this organism cytochrome *P450* is not soluble but membrane bound.

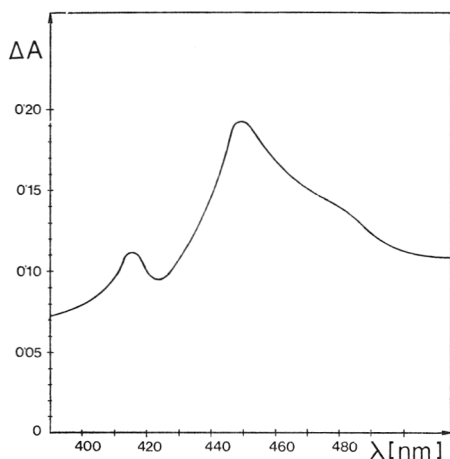


Figure 1. Reduced CO difference spectrum of the fraction sedimenting at 105 000 g from *R. nigricans* ATCC 6227b. The spectrum was obtained in the 0.05 M phosphate buffer pH = 7.3. The concentration of protein was 0.35 mg/ml.

In the presence of progesterone the fraction containing cytochrome *P450* from *R. nigricans* exhibits the modified type II spectral change, as defined by Schenkman *et al.*¹⁵. In Figure 2 we present such spectrum obtained with the fraction sedimenting at 105 000 g. Although this difference spectrum is not typical for the enzyme substrate interaction, which is characterized by a type I spectral change¹⁶, it nevertheless proves an interaction between cytochrome *P450* and progesterone. Upon solubilization of the fraction sedimenting at

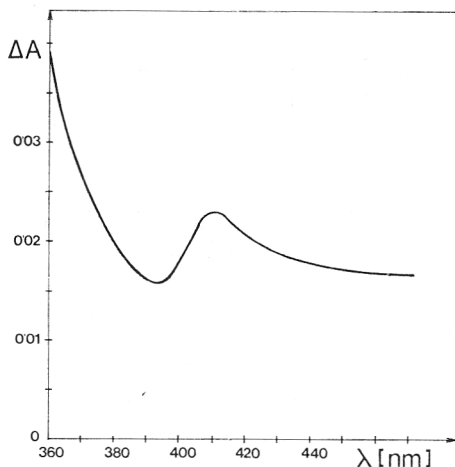


Figure 2. Progesterone induced spectral difference of the fraction sedimenting at 105 000 g from *R. nigricans* ATCC 6227b. The proteins were suspended in the 0.05 M phosphate buffer pH = 7.3 to 0.8 mg/ml and the difference spectrum was taken after addition of a few crystals of progesterone to one part of the suspension.

105 000 g with detergent Triton X-100, a significant amount of cytochrome P450 is converted into inactive P420 (Figure 3).

To learn more about the purity and intactness of the mitochondrial and microsomal fractions in the fungus, which were prepared according to the technique applied for mammalian tissues, we analyzed the corresponding fractions for the content of NAD cytochrome C oxidase (typical for mitochondria) and NADPH cytochrome C reductase (characteristic for microsomes)¹⁷. It is shown in Table I that rat liver mitochondria contain nearly the same amount of NAD cytochrome C oxidase as the mitochondria prepared from the fungus. The microsomal fraction of *R. nigricans* contains, however, about 60 times

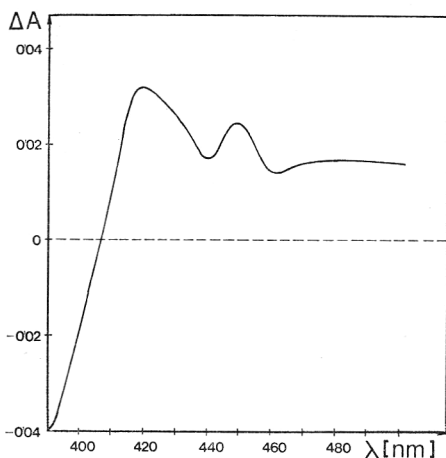


Figure 3. The effect of Triton X-100 on cytochrome P450 preparation from *R. nigricans* ATCC 6227b. Fraction sedimenting at 105 000 g from the fungus was solubilized with 1% detergent solution as described by Narasimhulu *et al.*¹⁸. The concentration of protein was 0.66 mg/ml.

less NADPH cytochrome C reductase than the corresponding fraction from the rat liver cells. A possible explanation for this observation is that the two microsomal fractions do not have the same function in both cell types. On the other hand, because of the chitin in the cell wall, the preparation of the homogenate from mycelia required a much more drastic treatment of the cells as compared to the liver tissue. A possible contamination with other membranes, therefore, has to be considered.

The microsomal fractions were analysed also for the content of RNA and protein. These results are presented in Table II; one can see that the RNA/protein ratio in the total microsomal fraction of rat liver is much higher as compared to the same fraction from the fungus. The ratio 0.079 found in the

TABLE I
Enzyme activities of subcellular fractions

Subcellular fraction	NAD-cyt C oxidase	NADPH-cyt C reductase
rat liver mitochondria	4.8 ± 0.5	5.7 ± 0.8
rat liver microsomes	0.9 ± 0.2	88 ± 5
mitochondria from R. n.	4.9 ± 0.5	1.31 ± 0.14
105,000 g from R. n.	---	1.35 ± 0.15

The enzyme activities were measured in 0.05 M phosphate buffer pH = 7.4 at protein concentration 0.1-0.5 mg/ml. The activities are expressed in μ moles of cytochrome C oxidized or reduced per mg of protein per min.

TABLE II
RNA/protein ratios in the subcellular fractions sedimenting at 105,000g

Subcellular fraction	RNA/protein
rat liver microsomes	0.130
105,000g from R. n.	0.079

RNA was determined by the orcinol reaction (13) and the protein by the method of Lowry *et al.* (8).

microsomal fraction of *R. nigricans* is, however, close to the values found in some mammalian tissues for smooth microsomes⁷. One can conclude, therefore, that the fraction sedimenting at 105 000 g of the fungus corresponds most probably to smooth microsomal membranes. Our observation that cytochrome P450 is present in this subcellular fraction is in good agreement with what is known about the intracellular localization of this enzyme in higher organisms.

Further characterization of this membrane fraction is in progress.

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DISCUSSION

K. Ruckpaul:

It has been established that, in *Sacharomyces cerevisiae*, P450 is involved in the biosynthesis of Zymosterol from Lanosterol which is a intermediate in the biosynthesis of ergosterol. Thus, it seems plausible that you assume a similar biological function in your system. What I wish to ask is (1) by which substances can you induce P450 synthesis, and (2) did you find other substances which could be hydroxylated?

K. Breskvar:

(1) I have only tried progesterone as inducer for the hydroxylating activity. I suppose some other substances can induce progesterone hydroxylation, too, but I was not looking for them.

(2) No. I only used progesterone as a substrate for hydroxylation.

P. Debey:

Now tightly is your cyt *P450* bound to the membrane?

K. Breskvar:

I do not think that it is bound very tightly, because in some of my preparations of the fraction sedimenting at 105 000 g, I got the *P450* in the solution even without adding a detergent. However, in that case the enzyme is in its inactive, *P420* form,

H. Schleyer:

Does the *P450* solubilized in water (in the absence of detergent) bind CO?

K. Breskvar:

Yes. It exhibits a difference spectrum of a dithionite reduced sample with CO, its peak being at 420 nm.

IZVLEČEK**Intracelularna lokalizacija citokroma *P450* v glivi *Rhizopus nigricans* in nekaj njegovih lastnosti**

K. Breskvar in T. Hudnik-Plevnik

V glivi *Rhizopus nigricans* poteka hidroksilacija progesterona preko encima citokroma *P450*. Subcelularne frakcije glive smo testirali na prisotnost tega encima in ga lokalizirali na membranski frakciji, ki sedimentira pri 105.000 g. Po svojih encimskih lastnostih, karakterističnih za mikrosome, frakcija, ki sedimentira pri 105.000 g iz glive ni identična mikrosomski frakciji jeter podgane. V prisotnosti progesterona kaže membranska frakcija, ki vsebuje encim citokrom *P450* iz glive, diferenčni spekter modificiranega tipa II. Pri postopku solubilizacije z detergentom Tritonom X-100 se večji del encima pretvori v neaktivno obliko *P420*.

INŠTITUT ZA BIOKEMIJO
MEDICINSKA FAKULTETA
61000 LJUBLJANA, VRAZOV TRG 2
SLOVENIJA, JUGOSLAVIJA

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