PARTIAL PURIFICATION OF CYTOCHROME *P*-450 OF *CANDIDA TROPICALIS* AND RECONSTITUTION OF HYDROXYLASE ACTIVITY

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1. Introduction

The various animal cytochromes *P*-450 have been widely studied and purified [1–5] but very few results have been obtained in this field concerning the cytochromes *P*-450 synthesized by yeasts [6–8].

The microsomal membranes of *Candida tropicalis* grown on hydrocarbons catalyse the transformation of lauric acid to ω-hydroxydodecanoic acid and then to 1,12-dodecandioic acid [9]. This transformation is due to a monooxygenase comprising a cytochrome *P*-450 and a NADPH-cytochrome *c* reductase on one side and to alcohol- and aldehyde-dehydrogenases on the other. In this yeast, these enzymatical systems are specifically induced by alkanes [10]. We have established that it is possible to liberate 70% of the NADPH-cytochrome *c* reductase by an osmotic wash of the microsomal membrane [11]. This made it possible to purify and characterize a soluble form of this flavoprotein [11].

This paper describes a method for the isolation and partial purification of cytochrome *P*-450, the other proteic component of the monooxygenase induced by the alkanes, in *Candida tropicalis*. This method based on the solubilization of the microsomal membranes by a detergent is different from that in [12] where a solubilization of the cytochrome *P*-450 from a very particular strain of *C. tropicalis* was obtained. Effectively, the cytochrome *P*-450 of this strain was liberated from the microsomal membrane by French press treatment of the cells of this strain in presence of 30% glycerol. This possibility was not met again in any other strain of *Candida tropicalis*.

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2. Materials and methods

Candida tropicalis strain CBS 6947 was described in [10]. It was grown on tetradecane and the microsomal fraction was prepared as in [9].

Proteins [13], cytochrome *P*-450 [14], cytochrome b_5 [15], NADPH-cytochrome *c* reductase [11] and alcohol dehydrogenase [16] were assayed according to described methods.

The hydroxylase activity was assayed as in [17] with a system generating NADPH.

3. Results and discussion

Cytochrome *P*-450 was purified from microsomal membranes containing 0.080–0.130 nmol cytochrome *P*-450/mg protein and having a hydroxylase activity between 0.2 and 0.3 units. The solubilization was made by addition of 1.6% sodium cholate (detergent/protein ratio 0.8). Detergents such as Triton X-100 or sodium deoxycholate lead to a transformation of cytochrome *P*-450 to the denatured form, cytochrome *P*-420. After a 20 min incubation in presence of cholate the material is centrifuged for 2 h at 150 000 × *g*. The supernatant obtained, which contains 72% of cytochrome *P*-450 and 65% of the initial proteins has no hydroxylase activity. This supernatant was dialysed overnight against 50 vol. of a 0.01 M Tris–HCl buffer (pH 7.2) containing 20% glycerol, 0.1%

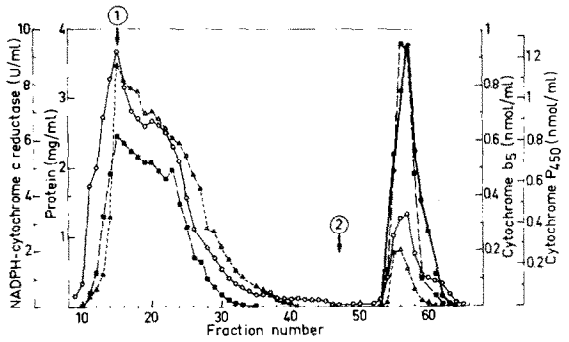

Fig.1. Elution profile of yeast solubilized microsomal fraction. Cytochrome *P*-450 (68.7 nmol) was applied to a phenyl–Sepharose CL-4B column (1.6 cm diam., 30 cm height) and elution was performed with: (1) buffer B (6.5 ml fractions, no. 15–46); (2) buffer C (9.5 ml fractions no. 47–65). The flow rate was 60 ml/h. (○——○) Protein; (●——●) cytochrome *P*-450; (■– – –■) cytochrome b_5; (△- - -△) NADPH-cytochrome *c* reductase.

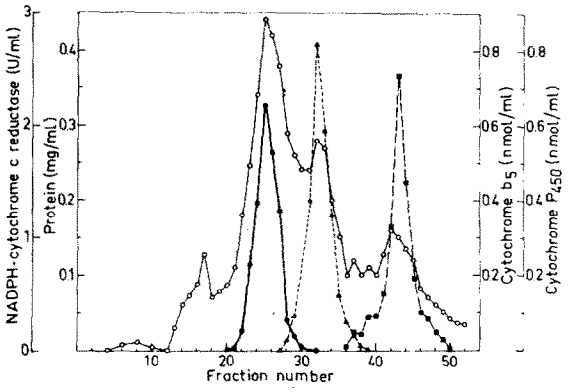

Fig.2. Column chromatography of cytochrome *P*-450 on DEAE-cellulose. The charge consisting of 43.3 nmol cytochrome *P*-450 obtained from phenyl–Sepharose was applied to a column (2.6 cm diam., 10 cm height) equilibrated with buffer C. The elution was realized with an increasing linear gradient of NaCl from 0–0.6 M in the same buffer. The flow rate was 20 ml/h, and the eluate was collected in 3 ml fractions. (○——○) Protein; (●——●) cytochrome *P*-450; (■– – –■) cytochrome b_5; (△- - -△) NADPH-cytochrome *c* reductase.

cholate, 0.01 M mercaptoethanol and 1 mM EDTA (buffer A). In this fraction, restored hydroxylase activity (0.08 units) is found. Cytochrome *P*-450 was then purified first by hydrophobic chromatography on phenyl–Sepharose CL-4B (Pharmacia chemicals) [19] equilibrated in a Tris–HCl buffer (pH 7.2) containing 10% glycerol, 0.1% sodium cholate, 0.3% Mulgofen BC-720 (Gaf, France), 0.5 M NaCl, 1 mM mercaptoethanol and 1 mM EDTA (buffer B).

During this chromatography (fig.1), NADPH-cytochrome *c* reductase, which is not fixed on the phenyl–Sepharose (90%), is first eluted with the buffer B. A fraction is then eluted with buffer C (buffer B without NaCl) containing 100% of cytochrome *P*-450, 40% of cytochrome b_5 and 10% of the reductase which has an hydroxylase activity similar to that found in the dyalysate (0.12 unit). This fraction is then chromatographed on a DEAE-cellulose (Serva) column equilibrated in buffer C supplemented with sodium cholate (0.05%). The separation of cytochromes *P*-450, b_5 and NADPH cytochrome *c* reductase was made with a 0–0.6 M NaCl gradient (fig.2). Cytochrome *P*-450 was eluted at 0.15 M NaCl. The specific activity of this fraction is 1.18 nmol.mg protein^{-1} and corresponds to a 13-fold purification with a yield of 6.8% (table 1). All purification steps were performed at 4°C. It must be noted that the nature of the detergent used during the purification is very important. Effectively, cytochrome *P*-450 is denatured to cytochrome *P*-420 in presence of deter-

Table 1
Cytochrome *P*-450 purification after cholate solubilization of the microsomal fraction obtained from cells grown on *n*-tetradecane

Prep.	Proteins (mg)	Spec. act. (nmol.mg prot.$^{-1}$)	Total act. (nmol)	Yield (%)	Purifn. (– fold)
Microsomes	1060	0.09	95.4	100	
Solubilized microsomes	689	0.099	68.2	71	1.1
Phenyl–Sepharose	49	0.883	43.3	45	9.8
DEAE-cellulose	5.5	1.18	6.5	6.8	13.0

Table 2
Reconstitution of the hydroxylase activity in the presence of partially purified cytochrome *P*-450

Cytochrome *P*-450 (nmol)	NADPH-cytochrome *c* reductase (μmol.min^{-1})	Hydroxylase act. (nmol.min^{-1} .mg prot.$^{-1}$)
0.03	0	0.06
0	0.4	0
0.03	0.4	1.25

The reaction mixture was incubated at 30°C and contained in 1 ml Tris buffer (0.1 M, pH 7.6): cytochrome *P*-450 (25 μg protein); cholate-solubilized purified NADPH-cytochrome *c* reductase (8 μg protein); NADP (1.5 mM); glucose-6-phosphate (6.7 mM); glucose-6-phosphate dehydrogenase (4 units); $MnCl_2$ (7 μM); $MgCl_2$ (1.7 mM); [^{14}C]dodecanoic acid (2 nmol, 54 mCi/mmol); dodecanoic acid (1 μmol)

gents such as Emulgen 911 or Renex 690. Only mulgofen BC-720 (10 molecules of ethylene oxide on a tridecylic alcohol) did not cause any denaturation of cytochrome *P*-450.

The results of table 2 show that the constituents separated by DEAE-cellulose have very little hydroxylase activity but a mixture of the cytochrome *P*-450 fraction and cholate-purified NADPH-cytochrome *c* reductase (unpublished data) permits a reconstitution of the hydroxylase activity with a specific activity of 1.25 units. It should be stressed that the alcohol-dehydrogenase is not found in any of the purified fractions. This results in the formation of the ω-hydroxyacid to the exclusion of the diacid during the hydroxylation of lauric acid by the mixture of DEAE-purified cytochrome *P*-450 and pure NADPH-cytochrome *c* reductase as revealed by the thin-layer chromatography technique [17]. The addition of lipids in the form of a Folch extract does not increase the hydroxylase activity. This could be due to the fact that the neutral detergent has the same effect as lipids as suggested [20]. It may also be explained by the presence of residual lipids in the fraction containing the partially purified cytochrome *P*-450, sufficient to facilitate the electron transport [4].

After phenyl–Sepharose, the fraction where total cytochrome *P*-450 is localized still contains 60% of the hydroxylase activity but only 10% of the NADPH-cytochrome *c* reductase eluted from this phenyl–Sepharose. It should be noted that when the microsomal fraction is treated by an osmotic shock, total cytochrome *P*-450 and 25% of NADPH-cytochrome *c* reductase remain associated to the microsomal membrane but the whole of the hydroxylase activity is maintained [11]. These results seem to indicate that in *C. tropicalis*, as in the mammals [21,22], there is a very close association between cytochrome *P*-450 and a fraction of NADPH-cytochrome *c* reductase.

We have shown [11] that the growth of *C. tropicalis* on alkanes leads to an increase of the NADPH-cytochrome *c* reductase activity and total induction of cytochrome *P*-450. The close association between many enzymes involved in the same metabolic pathway (transformation of alkanes into fatty acids) may be explained by a 'coordinated' synthesis of these enzymes and of the membranes in which these enzymes are integrated.

Finally, it must be stressed that the growth on alkanes does not lead to an increase in the levels, either of cytochrome b_5 or NADH-cytochrome *c* reductase [11] and that the hydroxylase activity is reconstituted in the total absence of cytochrome b_5. This indicates that cytochrome b_5 is not an essential component of the hydroxylation of lauric acid by *Candida tropicalis*.

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