Biochimica et Biophysica Acta, 419 (1976) 425–434

BBA 77188

MICROSOMAL CYTOCHROMES OF *CANDIDA TROPICALIS* GROWN ON ALKANES

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(Received June 9th, 1975)
(Revised manuscript received October 2nd, 1975)

SUMMARY

A comparison of methods used in isolating microsomes and in measuring microsomal cytochrome *P*-450 demonstrated that separation following protoplast lysis gave the best results. By this latter technique a high amount of cytochrome *P*-450 (0.2–0.3 nmol/mg) was recovered but cytochrome *P*-420, considered as the denatured form, was absent.

The alkanes specifically induce cytochromes *P*-450 and b_5 localized on the microsomes. The denaturation in vivo of cytochrome *P*-450 into cytochrome *P*-420 even occurs during storage at 1 °C. This degradation is increased during preparation of subcellular fractions if no preventive measures are taken.

INTRODUCTION

Microorganisms, and yeasts in particular, metabolize alkanes via a series of well known enzymic steps [1–3]. In the degradation of alkanes the paraffinic hydrocarbon is first transformed into a primary alcohol; this reaction is catalyzed by a complex enzymatic system, the hydroxylase, which has an activity closely related to many different electron carrier systems [4]. In spite of the large numbers of publications on cytochrome *P*-450 of various origins and in view of the complexity of the hydroxylase system, there is still very little information concerning the actual mechanism involved. Neither the exact number of protein components active in this process nor the regulatory mechanisms resulting from it has yet been determined.

We have shown that in *Candida tropicalis* the enzyme system transforming the paraffin into the alcohol was specifically located within the microsomes and that this system is comprised of the cytochrome *P*-450 and the NADPH-cytochrome *c* reductase [5]. Duppell et al. [6] have recently demonstrated that in this organism the phosphatidylethanolamine was closely associated with these same two protein components. Their results allow a closer analogy between the hydroxylase of *C. tropicalis* and that of the mammalian hepatocyte, since the latter contains a ther-

mostable lipid fraction with a high level of phosphatidylcholine [7]. However, for the time being their results on the partial solubilization, the isolation of the principal components and the reconstitution of the hydroxylase do not allow any decision as to the exact nature of this enzyme system.

The hydrocarbons induce, in vivo, two groups of alcohol and aldehyde dehydrogenases, which are necessary for the complete transformation of the alkane into the corresponding fatty acid. One of these groups is of mitochondrial origin [3], whilst the other is microsomal in origin [8, 9]. Moreover it must be stressed that growth on alkanes also induces one or many compounds of heminic nature. These are characterized by two absorption peaks at 625 and 640 nm but neither their nature nor their physiological role is known. One may suppose that during growth on alkanes, in contrast to the classical growth substrates, the synthesis of these two heminic compounds would be linked with the formation of a more important quantity of hydrogen peroxide.

In *C. tropicalis* we have already shown [8] that only straight chain compounds with more than 14 carbon atoms are inducers of cytochrome *P*-450 and of the hydroxylase system and that these compounds are never free inducers.

In this paper we wish to describe an appropriate method of isolation of microsomes, compare it with previous methods and then apply the method to the study of the change of cytochromes *P*-450, *P*-420, and b_5, with respect to growth conditions and storage of whole cells.

MATERIALS AND METHODS

Organism. Two strains of *C. tropicalis* have been employed; the one currently used in industrial cultures is No. 100 in our collection, whilst the other, No. 101, was isolated from No 100 [2, 10].

Medium and Culture conditions. A minimal medium defined by Duvnjak et al. [10] was as follows: NH_4Cl, 2.5 g; KH_2PO_4, 7 g; Na_2HPO_4, 1.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g, NaCl, 0.1 g; distilled water to a final volume of 1 l. The pH was adjusted to 5.6. This mineral medium was supplemented with yeast extract (Difco), 100 mg/l, and oligo elements: $FeSO_4$, 5 mg; $ZnCl_2$, 0.5 mg; $CoCl_2$, 0.5 mg; $CaCl_2$, 0.5 mg; MoO_4Na_2, 0.5 mg; $CuSO_4$, 1 mg.

Batch cultures of 7 l were grown at 32 °C in a New Brunswick fermenter (capacity 14 l) with an aeration of 1 vols/vol per min and a stirring speed of 500 rev/min. For continuous cultures an airlift fermenter of 2 l capacity was used with aeration and dilution rates of 4 vols./vol per min and 0.25 h^{-1}, respectively.

Subcellular fractionation. Preparation of subcellular fractions, especially microsomes, has been previously described [9]. However, the composition of the buffer used in homogenization of the microsomes was modified as follows: KCl, 1.15 % w/v; EDTA, 10 mM; Tris buffer, 0.05 M (pH 7.4), and the resulting suspension of microsomes was stored under N_2 at −20 °C. Under such conditions the microsomal membranes are not altered and the cytochromes, especially the cytochrome *P*-450, are better protected from denaturation.

Purification of the subcellular fractions. Each of the particulate fractions obtained from a range of extraction procedures (treatment with the French press, disintegration with glass beads and protoplast lysis) was purified on a discontinuous

sucrose gradient according to the method of Gallo et al. [5, 8].

Measurement of enzymatic activities. The detection of antimycin-insensitive NADPH-cytochrome *c* reductase and cytochrome *c* oxidase activities has already been described [9]. The glucose-6-phosphatase was measured according to the method of Swanson [11] and the inorganic phosphate released determined by the technique of Martin and Doty [12]. The *p*-nitrophenylphosphatase was assayed at 410 nm using the method described by Schlesinger [13] in a 2 ml system containing 1.7 ml Tris/maleate buffer (0.05 M, pH 6.5) and 0.1 ml *p*-nitrophenylphosphate (0.1 M).

Protein determination. Protein content was measured after trichloroacetic acid precipitation by the method of Lowry et al. [14].

RESULTS

Analysis of microsomal cytochromes

Preparation of microsomes. Cells of *C. tropicalis* grown in a medium using as the sole carbon source tetradecane (2g/l), normal paraffins (10g/l) acetate (2g/l) or glucose (reference compound 2g/l) were harvested by centrifugation during the exponential phase. The cells were first washed in an isotonic NaCl solution (0.9 %) containing Tween 80 (0.05 % w/v) to remove most of the hydrocarbons adsorbed on the cell wall. Two subsequent washings with NaCl (0.9 %) eliminated most of the detergent. The cells were then converted into protoplasts with helicase according to a technique especially adapted for hydrocarbon-grown cells and described elsewhere in detail [9].

The method was improved by introducing 0.3 % w/v bovine serum albumin and ethylenediamine tetraacetic acid (EDTA, 2 mM) to all steps in the fractionation. These chelators protect the microsomal membranes and prevent denaturation of the cytochromes by inhibiting proteases and Ca^{2+}-dependent phospholipases [15].

Spectrophotometric examination. Detection and examination of the cytochrome *P*-450 have been carried out on the subcellular fractions obtained after protoplast lysis. An aliquot of the microsomal preparation (1.5 ml) containing between 1 mg (low turbidity) and 10 mg (high turbidity) of protein is introduced into a cell of 10 mm light path. The difference spectra were recorded with a double beam spectrophotometer adapted for light scattering material. The cytochrome b_5 level has been measured from the differential spectrum between 410 and 428 nm, using an extinction coefficient of 160 $mM^{-1} \cdot cm^{-1}$ [16].

The difference spectra of the carbon monoxide derivatives were used to assay cytochromes *P*-450 and *P*-420. Extinction coefficients of 91 $mM^{-1} \cdot cm^{-1}$ at 450–490 nm and 111 $mM^{-1} \cdot cm^{-1}$ at 420–490 nm were employed respectively in the estimation of the amount of cytochromes *P*-450 and *P*-420 [17].

The difference spectra of microsomes isolated from tetradecane-grown yeasts are shown in Fig. 1. Cytochrome b_5 was present (0.15 nmol/mg protein) (Curve 1) but no cytochrome *P*-420 could be detected.

However, microsomes from acetate-grown cells contain a small amount of cytochrome *P*-420 (2.7 pmol/mg protein) but again no *P*-450 is observed (Fig. 2, curve 2).

Identification of microsomes. Whatever the growth substrate used (glucose or tetradecane), microsomal preparations show no significant glucose-6-phos-

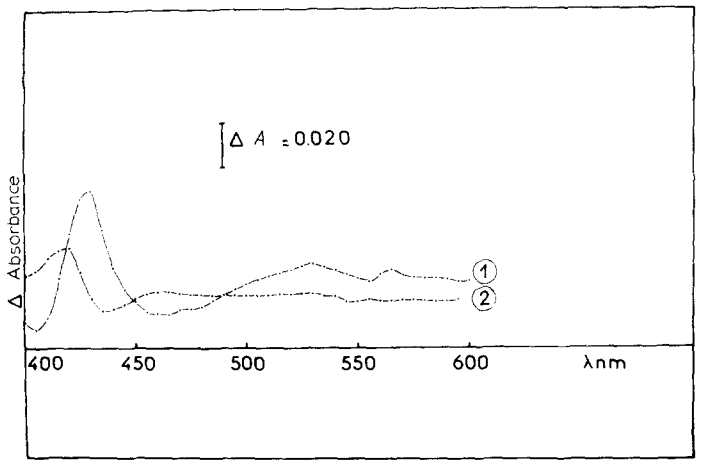

Fig. 1. Difference spectra of cytochromes b_5 and *P*-450 in microsomes of *C. tropicalis* grown on tetradecane. (1) Reduced versus oxidized (1 ml containing 1.8 mg protein, light path 10 mm, reduced with dithionite). (2) Reduced versus reduced; only the sample is bubbled with CO (1 ml containing 3.5 mg protein).

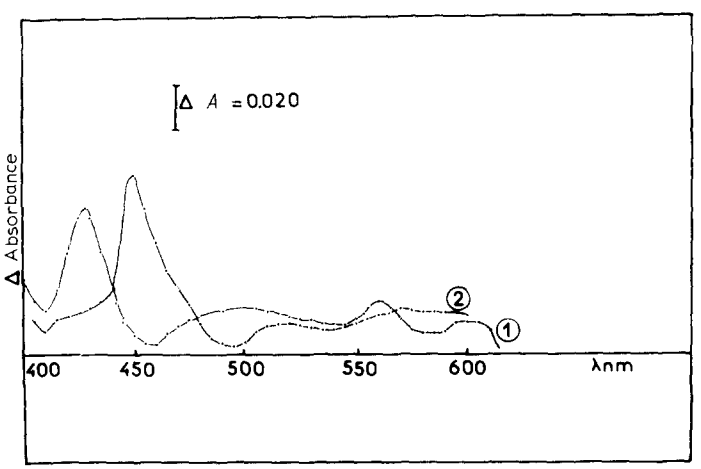

Fig. 2. Difference spectra of cytochromes b_5 and *P*-450 in microsomes of *C. tropicalis* grown on acetate (1 ml containing 10 mg protein, light path 10 mm). (1) Reduced versus oxidized (reduced with dithionite). (2) Reduced versus reduced; only the sample is bubbled with CO.

phatase activity, which is currently employed as a microsomal marker in eucaryotes.

However, a phosphatase activity of 150 nmol of liberated P_i/h per mg was detected. This was measured with respect to *P*-nitrophenylphosphate at a pH which is optimal for glucose-6-phosphatase activity and allows one to estimate the interference to the glucose-6-phosphatase assay by non-specific phosphatase activity. Therefore, it is likely that microsomes are slightly contaminated by the plasmalemma.

TABLE I

DISTRIBUTION OF ENZYMATIC MARKERS IN MICROSOMES ISOLATED BY DIFFERENTIAL CENTRIFUGATION AFTER LYSIS OF *CANDIDA TROPICALIS* PROTOPLASTS

Growth substrate	Cytochrome *P*-450*	NADPH-cytochrome *c* reductase ** antimycin-insensitive	*** Glucose-6-phosphatase	*** *p*-Nitrophenyl phosphatase	** Cytochrome *c* oxidase
Glucose	0.00	45	0	135	3
Tetradecane	0.150	235	0	155	3

* nmol · mg^{-1} protein.
** nmol cytochrome *c* reduced or oxidized/min per mg protein.
*** nmol liberated P_i/h per mg protein.

TABLE II

INFLUENCE OF THE GROWTH SUBSTRATE ON THE CYTOCHROME CONCENTRATION IN *C. TROPICALIS* MICROSOMES

Microsomes isolated from cells grown on	Cytochrome *P*-450 (nmol · mg^{-1} protein)	Cytochrome *P*-420 (nmol · mg^{-1} protein)	Cytochrome b_5 (nmol · mg^{-1} protein)
Tetradecane			
n° 1	0.122	0	0.150
n° 2	0.108	0	0.132
n° 3	0.115	0	0.122
n° 4	0.119	0	0.142
n° 5	0.106	0.010	0.117
n° 6	0.136	0	0.152
n° 7	0.188	0	0.158
n° 8	0.121	0	0.149
n° 9	0.192	0	0.156
n° 10	0.115	0	0.133
Tetradecane (microsomes purified on sucrose gradient)*	0.310	0.010	0.210
Glucose	0	0	0.062
Acetate	0	0.0027	0.058

* Microsomal fractions were layered on a discontinuous gradient of sucrose with concentration varying between 15 and 60 %. Purified microsomes are recovered at a density 1.08, after 3 h of centrifugation at 27 000 rev./min [8].

Nonetheless the presence of the antimycin-insensitive NADPH-cytochrome *c* reductase shows that we have in fact isolated some microsomes and that this activity is five times higher in preparations from cells grown on alkanes. This membranous fraction is free from cytochrome oxidase activity which is mitochondrial in origin (Table I).

In Table II are recorded the levels of microsomal cytochrome *P*-450 and cytochrome b_5 in ten experiments in which *C. tropicalis* was grown on alkanes. Purified microsomal membranes were further fractionated on a sucrose gradient as previously described [5] and the distribution of cytochrome *P*-450 in the gradient is also summarized in the same table.

The cytochrome *P*-450 concentration determined ranges between 0.11 and 0.20 nmol · mg^{-1} protein. Although the method used to detect cytochromes by difference spectra is very sensitive we have not been able to measure any cytochrome *P*-420 in any of the 10 experiments. However, if one considers the values obtained with purified microsomal membranes, the true amount of microsomal cytochrome *P*-450 would be of the order of 0.3 nmol · mg^{-1} protein. Cellular adaptation to hydrocarbons brings about a microsomal cytochrome b_5 concentration ranging between 0.10 and 0.16 nmol · mg^{-1} protein, which is 2–3 times higher than those obtained after growth on glucose or acetate.

Evaluation of cytochrome P-450 with respect to the method of preparing subcellular fractions

The technique used in the preparation of the membranous fractions influences the detection of the cytochrome *P*-450 in yeast cells.

(a) The technique described in this paper has permitted a determination of cytochrome *P*-450 per mg of microsomal protein of the order of 0.11 $\leqslant$ cytochrome *P*-450 $\leqslant$ 0.20 nmol.

(b) Other methods of subcellular fractionation can be used, such as treatment of the cells with the French press followed by differential centrifugation, or treatment of the cells by disintegration with glass beads [8].

In Table III we have summarized the amount of cytochrome *P*-450 present in the microsomes after each of these different preparations. Apparently the amount of cytochrome *P*-450 measured after these treatments is always 2–3 times lower than that obtained after protoplast lysis.

However, it must be noted that after purification on a sucrose gradient these microsomal membranes contain very similar levels of cytochrome *P*-450 (Table III).

TABLE III

INFLUENCE OF THE EXTRACTION PROCEDURE ON THE CYTOCHROME *P*-450 CONCENTRATION OF *C. TROPICALIS*

Extraction procedure	Fraction	Cytochrome P 450 (nmol · mg^{-1} protein)	
		Before sucrose gradient	After sucrose gradient*
French Press	crude particles	0.050	0.250
Disintegration with glass beads	microsomal	0.060	0.280
Protoplast lysis	microsomal	0.110	0.310

* Microsomal fraction purified according to the method already described [8].

Changes in microsomal cytochromes as a function of storage time

(a) In an initial series of experiments it was shown that cells of *C. tropicalis* grown on tetradecane in batch culture contain some cytochrome *P*-450 and cytochrome b_5, but no trace of cytochrome *P*-420. These are the results obtained when mild techniques of preparation are employed in order to preserve the membranes. Once isolated, these microsomes may be stored for 15 days at −20 °C under N_2 without any noticeable change to the cytochromes.

However, cytochrome *P*-420 is always present when less sophisticated techniques are used, whilst the same cells treated by our technique contain no trace of the denatured cytochrome.

(b) In a second series of experiments, *C. tropicalis* was grown on a mixture of paraffins in continuous cultures. We wished to assay the amount of cytochrome *P*-450 present and compare the microsomal concentration of this substance with the results obtained from batch cultures.

After 2 h the cells were harvested by centrifugation and kept in melting ice. They were then washed once with a solution of NaCl (0.9 %) and Tween 80 (0.05 % w/v) followed by two rinses in NaCl (0.9 %).

The pellet of cells was divided into 4 and stored at 0–1 °C. The microsomal cytochrome was then measured after various intervals in time to see if any change occurred in the cytochrome levels.

The microsomes isolated from lysates were analyzed by difference spectrophotometry. The results illustrated in Fig. 3 reveal that, even from the outset, the microsomes obtained from continous culture-grown cells always contain the three

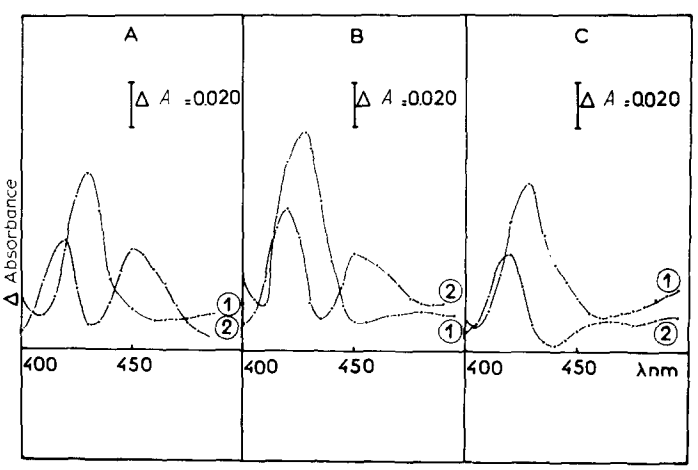

Fig. 3. Difference spectra of cytochromes b_5, *P*-450 and *P*-420 in microsomes of *C. tropicalis* grown on *n* paraffins in a chemostat (light path 10 mm, reduction with dithionite). (A) 2 h after harvest; 1, reduced versus oxidized (1 ml containing 3.5 mg protein); 2, reduced versus reduced, only the sample is bubbled with CO (1 ml containing 10 mg protein). (B) After 50 h storage at 1 °C; 1, reduced versus oxidized (1 ml containing 4 mg protein); 2, Reduced versus reduced, only the sample is bubbled with CO (1 ml containing 7 mg protein). (C) After 74 h storage at 1 °C (1 ml containing 3.4 mg protein); 1, reduced versus oxidized; 2, reduced versus reduced, only the sample is bubbled with CO.

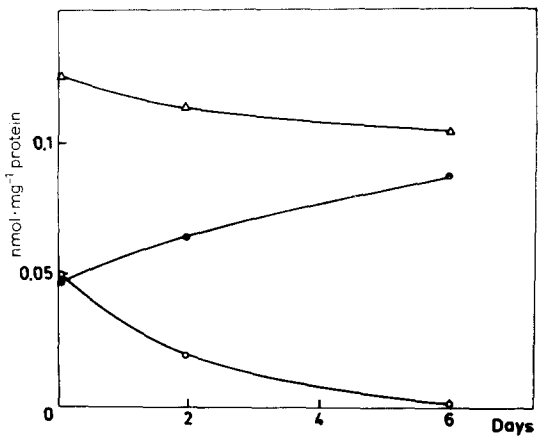

Fig. 4. Concentration changes of microsomal cytochromes of *C. tropicalis* grown in a chemostat. Cytochromes *P*-450, *P*-420 and b_5 were assayed from microsomes of *C. tropicalis* cells stored at 1 °C as a function of storage time. (○) Cytochrome *P*-450, (●) cytochrome *P*-420, (△) cytochrome b_5.

cytochromes: *P*-450 (0.05 nmol · mg^{-1} protein), *P*-420 (0.05 nmol · mg^{-1}) and b_5 (0.125 nmol · mg^{-1}) (Fig. 3A). After 2 days the same method was used to isolate the microsomal fraction from aged cells. The amount of cytochrome *P*-420 had increased whilst the cytochrome *P*-450 had decreased (Fig. 3B). After 6 days of storage it was evident that the cytochrome *P*-450 had entirely disappeared (Fig. 3C). The variations in the three microsomal cytochromes as a function of the time that the cells are stored are shown in Fig. 4.

Thus it would seem that:

(1) There is a close relationship between the disappearance of cytochrome *P*-450 and the appearance of cytochrome *P*-420 which indicates a transformation from the functional form (*P*-450) into an inactive form with no hydroxylase activity.

(2) There is little change in the concentration of cytochrome b_5. Nevertheless, the presence of cytochrome *P*-420 in the cells right from the beginning of the experiment may be due to the fact that the cultures remained in melting ice for several hours before being recovered by centrifugation. Thus it may be concluded that the denaturation of cytochrome *P*-450 occurs soon after harvest and that it is very important to proceed with subcellular fractionation and spectrophotometric analysis with minimal delay in order to obtain an accurate and meaningful measurement of microsomal cytochrome *P*-450. When all these facts are taken into consideration it is obvious that we cannot compare measured cytochrome *P*-450 in continuous culture with that of batch cultures (Table II).

DISCUSSION

The experimental results reported above provide some information as to the changes occurring in microsomal cytochromes when *C. tropicalis* cells are grown on alkanes. These results indicate that the presence and determination of the cytochrome *P*-450, one of the constituents of the hydroxylase involved in the primary

oxidation of hydrocarbons, depend essentially on the method of preparation of the microsomes. As we have shown, best results are obtained after protoplasts lysis and separation of subcellular fractions by differential centrifugation. Thus it was estimated that microsomes of alkane-grown cells of *C. tropicalis* contain between 0.2 and 0.3 nmol cytochrome *P*-450 per mg protein. Only the native form of this cytochrome, characterized by an absorption maximum at 450 nm, is active in hydroxlylation.

The cytochrome *P*-450 can be readily converted to a cytochrome compound characterized by an absorption maximum at 420 nm. Since the preparation of microsomes is time-consuming, it is essential to protect this cytochrome throughout the procedure from denaturation by endogenous proteases or phospholipases. This denaturation also occurs in cells stored at 1 °C and when the cells are starved. Otherwise, growth conditions have little effect on the amount of microsomal cytochrome *P*-450 present, provided the precautions necessary are taken during the preparation of subcellular fractions.

The conversion of cytochrome *P*-420 into native cytochrome *P*-450 has not been realized in our case in spite of the utilization of reducing agents or polyols [18]. It is likely that in *C. tropicalis* the cytochrome *P*-450 contains hydrophobic regions which confer specificity to this cytochrome. During the preparation there is probably an alteration to these regions which in turn leads to the denaturation of the cytochrome *P*-450.

On the other hand the cytochrome *P*-450 can be considered as a specific microsomal marker because it is absent in plasmic membranes and external membranes of mitochondria.

No trace of the glucose-6-phosphatase, a microsomal marker currently employed, could be found in the case of *C. tropicalis*. According to Vignais et al. [19] this enzyme is present in *Candida utilis*, although Cartledge and Lloyd [20] were unable to show any activity in microsomes isolated from *Saccharomyces carlsbergensis*.

We have demonstrated that the cytochrome *P*-450 is absent in cells grown on classical substrates. Growth on alkanes, however, leads to a considerable increase at the level of NADPH-cytochrome *c* reductase and of the cytochrome b_5 usually present in the microsomes of this yeast. In other words, adaptation of yeast cells to hydrocarbons leads to certain modifications at the microsomal level.

ACKNOWLEDGEMENTS

We wish to thank Professor G. Ducet, Mrs M. Gilewicz and Miss V. Yahiel for the help provided in realizing the absorption spectra. The work was supported by a grant from Société Française des Pétroles BP. Thanks are also extended to Dr. M. M. Smith for her help with the manuscript.

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