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CYTOCHROME P-450 FROM LODDEROMYCES ELONGISPORUS: ITS PURIFICATION AND SOME PROPERTIES OF THE HIGHLY PURIFIED PROTEIN

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SUMMARY: An effective method, based on the chromatography on ω-aminooctyl Sepharose 4B, for the purification of the alkane-induced cytochrome P-450 is described. The purified cytochrome P-450 was homogeneous in SDS/polyacrylamide gel electrophoresis. In the oxidized state it showed a low spin type absorption spectrum. The reduced CO-complex is characterized by a Soret peak at 447 nm. The alkane hydroxylating enzyme system could be reconstituted combining purified cytochrome P-450 with partially purified NADPH-cytochrome P-450 reductase from the yeast microsomal fraction.

INTRODUCTION: The induction of cytochrome P-450 by long chain n-alkanes has been reported for several yeast strains which are able to grow on this unusual substrate (1-6). The published investigations suggest that the cytochrome P-450 acts as the terminal oxidase of a monooxygenase system catalyzing in vitro the hydroxylation of alkanes (1-4), fatty acids (1,2) and some drugs (1). In vivo it is presumably involved in the first step of alkane degradation.

Very recently, the purification of the NADPH-cyt c (P-450) reductase of Candida tropicalis was described (7). However, attempts to purify the terminal oxidase (1,4,8) have been little successful so far. Therefore, the molecular and functional properties of this monooxygenase system are still largely unknown.

Abbreviation: cytochrome, cyt;

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In this communication an effective procedure for the purification of the alkane-induced cyt P-450 from the yeast Lodderomyces elongisporus and some properties of the electrophoretically homogeneous hemoprotein are described. Its involvement in alkane hydroxylation could be proved by reconstitution with a partially purified NADPH-cyt P-450 reductase to the catalytic active system.

MATERIALS AND METHODS: The yeast strain Lodderomyces elongisporus EH "D" [+)] (from the Institute of Technical Chemistry, Leipzig) was grown on a mixture of n-alkanes (C_{11}-C_{19}) and cells were mechanically desintegrated as described previously (3). The cyt P-450 containing membrane fraction was obtained from the cellfree supernatant using a modified method of calcium-mediated sedimentation (9). The final concentration of calcium chloride was 20 mM in 50 mM Tris-HCl buffer pH 7,4 containing 1 mM EDTA and 0.5 mM DTE.

Cyt P-450 (10), cyt b_5 (11), NADPH-cyt c reductase (12), alkane hydroxylase activity (3), heme (13) and protein (14) were assayed according to described methods. The spectral studies were done with a SHIMADZU UV 300 dual wavelength spectrophotometer. Polyacrylamide gel electrophoresis was performed as described by LAEMMLI (15) in the presence of 0.1 % sodium dodecylsulfate using 10 % separating gels. ω-amino-octyl-Sepharose 4B was prepared by the method of Cuatrecasas (16).

The NADPH-cyt c reductase used for reconstitution experiments, was partially purified up to a specific content of 10 µmoles red. cyt c/mg protein x min after deoxycholate solubilization by chromatography on DEAE-Sephacel in 50 mM Tris-HCl-buffer pH 7.7 containing 0.3 % Präwozell W-ON 100, 0.1 % Na-cholate, 1 mM EDTA and 0.1 mM DTE.

In the course of the experiments buffer A (10 mM potassium phosphate pH 7.25, 20 % glycerol, 1 mM EDTA, 0.5 mM dithiothreitol) and buffer B (10 mM potassium phosphate pH 7.25, 35 % glycerol, 0.5 mM dithiothreitol, 0.6 % Na-cholate) were used.

RESULTS AND DISCUSSION:

Purification of cyt P-450: The Ca^{2+}-sedimented microsomal fraction (8 g of protein) from n-alkane grown yeast was suspended to a protein concentration of 10 mg/ml in buffer A

[+)] In two previous communications (3,4) this yeast was signed as a strain of the species Candida guilliermondii. A taxonomic reexamination showed that it belongs to Lodderomyces elongisporus.

and solubilized by treatment with Na-cholate (final concentration: 0.8 %). After 30 minutes the cholate concentration was increased to 1.2 % and the resulting solution was immediately combined with a stirred suspension of ω-aminooctyl-Sepharose 4B equilibrated with buffer A containing 1.2 % Na-cholate (5 mg protein/ml suspension; about 26 mg protein/ml packed gel). After 60 minutes the loaded gel was separated from the not adsorbed membrane proteins by low speed centrifugation. Subsequently several washing steps followed: twice with 3 vol. buffer A containing 0.3 % Na-cholate, twice with 2 vol. buffer A containing 1.8 % Na-cholate and, finally twice with 2 vol. buffer B. The washed gel was packed in a chromatography column (diameter 3.5 cm) and equilibrated with buffer B. The cyt P-450 was eluted with 0.5 % Tween 20 in buffer B. This fraction was adsorbed to calcium phosphate gel, washed with 50 mM potassium phosphate buffer pH 7.25 containing 0.5 mM DTE and the cyt P-450 was eluted with 400 mM potassium phosphate buffer pH 7.25 containing 0.5 mM DTE. The results of this typical preparation are summarized in Tab. 1. The described procedure results in an about 50-fold purification of the cyt P-450 in 17 % yield with respect to the microsomal fraction.

The chromatography on ω-aminooctyl-Sepharose 4B is a modification of the procedure of IMAI and SATO (17) developed for the purification of liver microsomal cyt P-450. Differing from the original method the binding of the protein to the affinity adsorbent was carried out as a batch process using a high concentration of cholate and a low ionic strength. Under these conditions the hydrophobic binding of the NADPH-cyt c reductase and of other membrane proteins is largely reduced. For the specific elution of the cyt P-450 the weak

TABLE I Purification of the alkane-induced cyt P-450 from yeast cells (typical experiment)

step	cytochrome P-450 nmoles	cytochrome P-450 nmoles / mg protein	cytochrome P-450 yield %	NADPH-cyt c reductase μmoles cyt c / min x mg prot.	NADPH-cyt c reductase yield %
whole cells		0.06			
microsomal fraction	1970	0.27	100	0.78	100
ω-NH_2-octyl Sepharose 4B chromatography					
- not adsorbed	617	0.07	31	0.72	90
- eluate	623	n.d.	32	0.05	0
calcium phosphate gel eluate	335	10.6	17	0.02	0

non-ionic detergent Tween 20 turned out to be most suitable among a lot of checked detergents and of substrate analogous compounds.

Properties of the highly purified cyt P-450: The obtained cyt P-450 preparation was apparently homogeneous in SDS-PAGE (Fig.1). It was free of cyt b_5 and cyt P-420 and contained a neglectible low activity of NADPH-cyt c reductase (TABLE I). The absorption spectrum of the alkaline pyridine ferrohemochrome showed maxima at 555, 523 and 417 nm, indicating that protoheme is the prosthetic group of the isolated cyt P-450. One mole of cytochrome contains one mole of heme assuming millimolare extinction coefficients of 20.7 (for pyridine ferrohemochrome) and 91 (for CO-difference spectra). In contrast to these results showing a high purity of the cyt P-450 preparation, a specific content of only about 11 nmoles/mg protein could be estimated. This value is remarkably lower than the theoretical value of about 17, calculated from the molecular weight (see below).

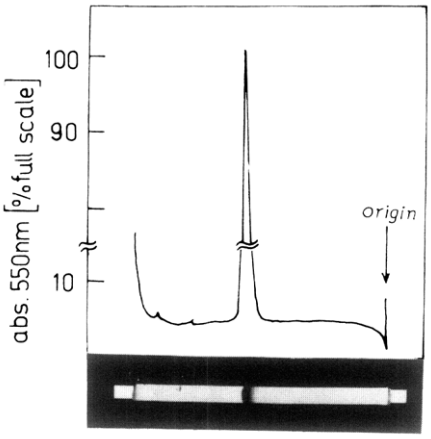

Figure 1 Polyacrylamide gel electrophoresis of purified cyt P-450 (20 µg of protein) in presence of sodium dodecylsulphate. Gels were stained with Coomassie blue and scanned at 550 nm.

This difference is probably due to the contamination of the cyt P-450 with residual detergents which influence the Lowry method or to the presence of some apoprotein.

By SDS-PAGE an apparent molecular weight of about 53 000 was estimated. The electrophoresis was carried out at different acryl amide concentrations using chymotrypsinogen A (25 000), ovalbumin (45 000), catalase (58 000) and bovine serum albumin (67 000) as marker proteins.

The oxidized form of the purified cyt P-450 exhibits a low-spin type absorption spectrum with a Soret peak at 417 nm and no absorption in the 650 nm region. The estimated spectral properties (TABLE II) essentially correspond to those reported for low-spin cyt P-450 species from other organisms (18). However, the Soret peak of the reduced CO complex at 447 nm is situated at a relative short wave length.

The purified cyt P-450 can be reduced enzymatically with NADPH in the presence of the partially purified NADPH-cyt c

TABLE II Spectral parameters of purified cyt P-450

State	Absorption maxima (nm)
oxidized	417, 535, 568
reduced	414 550
reduced CO-complex	447 555
reduced CO-difference	447

The cyt P-450 was dissolved in 200 mM potassium phosphate buffer pH 7.25 containing 20 % glycerol, 0.4 % Tween 20, 0.5 % Na-cholate and 0.5 mM DTE.

(P-450) reductase from the yeast microsomal fraction (Fig. 2). The extent of reduction strongly depends on the presence of substrate (hexadecane), which can be replaced by Tween 20, the detergent used for the specific elution of cyt P-450 from the affinity adsorbent.

TABLE III shows that the alkane hydroxylase activity can be reconstituted by mixing the purified cyt P-450 with the par-

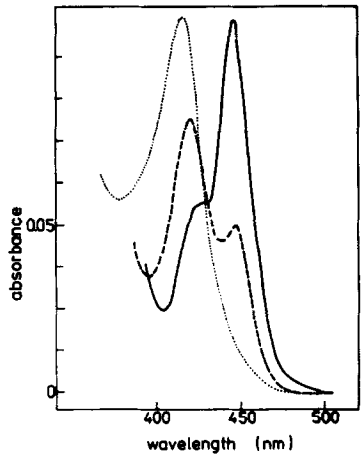

Figure 2 Enzymatic reduction of purified cyt P-450 with NADPH as judged by formation of the CO complex. The complete system (——) contained in a total volume of 2.5 ml in 200 mM potassium phosphate buffer pH 7.25: cyt P-450 (0.9 nmoles), NADPH-cyt P-450 reductase (1 /umole red. cyt c/min), hexadecane (5 /umoles) and NADPH (5 /umoles); (- - -) without hexadecane; (.....) without NADPH-cyt P-450 reductase. In the reference cuvettes the cyt P-450 was omitted.

TABLE III The reconstitution of the alkane monooxygenase system

Cytochrome P-450 nmoles	NADPH-cyt c reductase /umoles cyt c/min	Hydroxylase activity nmoles product/ min x nmol P-450
0.15	0.70	1.20
0.15	----	0.02 +)
----	0.70	---- +)

The hydroxylase activity was estimated with 100 nmoles /1-^{14}C/ hexadecane (3.10^5 DPM) and 2 /umoles NADPH in a total volume of 1 ml in 200 mM potassium phosphate buffer pH 7.25 containing 20 % glycerol.

+) no product formed

tially purified NADPH-cyt c reductase. The turnover number of the reconstituted system corresponds to the values estimated for the unresolved system of the microsomal fraction (4). Omission of one of the components leads to a loss of activity. In accordance with the results of the inhibition experiments and with the cofactor requirement (3) these investigations underline that cyt P-450 is the terminal oxidase of the membrane-bound alkane monooxygenase system of Lodderomyces elongisporus EH 15 D.

Concerning the electron transfer system the role of cyt b_5 which is still contained in the reductase preparation requires further investigations.

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