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PURIFIED DETERGENT-SOLUBILIZED NADPH-CYTOCHROME C (P-450) REDUCTASE FROM CANDIDA TROPICALIS GROWN ON ALKANES

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SUMMARY

A microsomal NADPH-cytochrome c reductase from yeast was purified by column chromatography on Phenyl-Sepharose CL-4B, DEAE cellulose, hydroxylapatite and Sephadex G-150 in the presence of sodium cholate and Mulgofen BC-720, a non ionic detergent. On SDS-polyacrylamide gel electrophoresis, the purified enzyme gives a single band of 76,000 MW. FMN and FAD were present in approximately equal amounts. Contrary to the enzyme obtained by osmotic wash, the cholate - solubilized reductase is able to transfer the electrons from NADPH to cytochrome P-450 and may thus be considered as a NADPH-cytochrome c (P-450) reductase.

INTRODUCTION

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 (1). 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 side. In this yeast, these enzymatical systems are specifically induced by alkanes (2). It has been previously established that 70 % of the NADPH-cytochrome c reductase can be liberated by an osmotic wash of the microsomal membranes. This made it possible to purify and characterize a soluble form of this flavoprotein (3). The other component of the monooxygenase, cytochrome P-450, was partially purified and hydroxylates lauric acid in the presence of the detergent solubilized fraction of NADPH-cytochrome c reductase (4). This fact led us to study this reductase in order to compare its properties with those of the enzyme obtained after osmotic wash of the microsomal fraction and with other reductases present in other organisms.

MATERIALS AND METHODS

Purification procedure : The purification procedure for the reductase is summarized in Table 1. All steps were carried out at 4°C. 469 mg of microsomal protein were suspended and stirred during 15 minutes at 20°C.

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in a final volume of 31 ml in a 0.05 M Tris-HCl buffer (pH 7.5) containing 20 % glycerol, 5 mM mercaptoethanol, 1 mM EDTA and 1.2 % sodium cholate. The obtained solution was centrifuged at 220,000 x g during 120 min. The supernatant fraction was dialysed overnight against 50 vol. of a 0.01 M Tris-HCl buffer (pH 7.2) containing 20 % glycerol, 5 mM mercaptoethanol, 1 mM EDTA 1 uM FMN, 1 uM FAD and 0.1 % sodium cholate. The reductase was then purified first by hydrophobic chromatography (5) on Phenyl-Sepharose CL-4B (Pharmacia Chemicals) column (1.6 x 25 cm) equilibrated in a 0.01 M Tris-HCl buffer (pH 7.2) containing 10 % glycerol, 5 mM mercaptoethanol, 1 mM EDTA, 1 uM FMN, 1 uM FAD, 0.5 M NaCl, 0.05 % sodium cholate and 0.3 % Mulgofen BC-720 (buffer A). During this chromatography (fig 1A), 90 % of the NADPH-cytochrome c reductase was not fixed on the Phenyl-Sepharose CL-4B but was eluted with buffer A (5.5 ml fractions at a flow rate of 60 ml per h.). The fractions from Phenyl-Sepharose CL-4B which contained the NADPH-cytochrome c reductase were concentrated to 10 ml in an Amicon concentrator and dialysed three times during two hours against 30 vol. of buffer A without NaCl (buffer B). The dialysate was diluted three times in buffer B and applied to a DEAE cellulose (DE 52 Whatman) column (1.6 x 16 cm) previously equilibrated with buffer B. After washing with 60 ml of the same buffer, the reductase was eluted with 200 ml of a linear concentration gradient of NaCl from 0 to 0.5 M in the equilibration buffer. The flow rate was 40 ml per h and the eluate was collected in 6.5 ml fractions. The reductase rich fractions were pooled and concentrated to 2.5 ml in an Amicon concentrator.

This solution was diluted ten times with a 0.01 M potassium phosphate buffer (pH 7) containing 10 % glycerol, 5 mM mercaptoethanol, 1 mM EDTA, 1 µM FMN, 1 µM FAD and 0.3 % Mulgofen BC-720 (buffer C). The diluted solution was then applied to an hydroxylapatite (Bio-Gel HTP Biorad-Rad Laboratories) column (0.8 x 13 cm) previously equilibrated with buffer C. After washing with 25 ml of buffer C, the column was eluted with a linear concentration gradient of buffer C (100 ml) containing from 10 mM to 200 mM potassium phosphate (pH 7). The flow rate was 10 ml per h. and the eluate was collected in 5 ml fractions. The peak reductase fractions were pooled, concentrated to 1 ml in an Amicon concentrator and applied to a Sephadex G-150 column (1.2 x 70 cm) equilibrated with a 0.15 M potassium phosphate buffer (pH 7) containing 1 mM mercaptoethanol, 1 mM EDTA, 1 µM FMN, 1 µM FAD and 0.3 % Mulgofen BC-720 (buffer D). The flow rate was 3.5 ml per h. and the fraction size was 5 ml. The reductase eluted from the column as a single peak and the fraction having the highest specific activity was concentrated to 0.5 ml. The purified reductase is stable at - 70°C for several months in buffer D containing 30 % glycerol.

Assays : The microsomal fraction was prepared as described elsewhere (1) from cells of Candida tropicalis strain CBS 6947 grown on alkanes. The reduction of cytochrome c, neotetrazolium chloride, menadione and dichloroindophenol was assayed according to described methods (3). The protein content was measured by the method of Lowry et al. (6) except on the material obtained after Sephadex G-150 where a fluorometric assay was used (7). FMN and FAD contents were determined by the method of Feader and Siegel (8). Sodium dodecyl sulfate - polyacrylamide gel electrophoresis was carried out using the method of Laemmli (9).

RESULTS AND DISCUSSION

The molecular weight of the pure enzyme is 76,000 as determined by electrophoresis (Fig. 2). It is thus different from the one obtained by

Table I - PURIFICATION OF CANDIDA TROPICALIS NADPH-CYTOCHROME C REDUCTASE

	Protein (mg)	Specific Activities (μmol x min^{-1} x mg $protein^{-1}$)	Total Activity (μmol x min^{-1})	Yield %
Microsomes	469	0.91	427	100
Solubilized microsomes	310	1.05	325	76
Phenyl-Sepharose	103	2.35	242	56
DE 52	17	9.2	156	36
Hydroxylapatite	3	32	96	22
Sephadex G-150	0.52	56	29	6.8

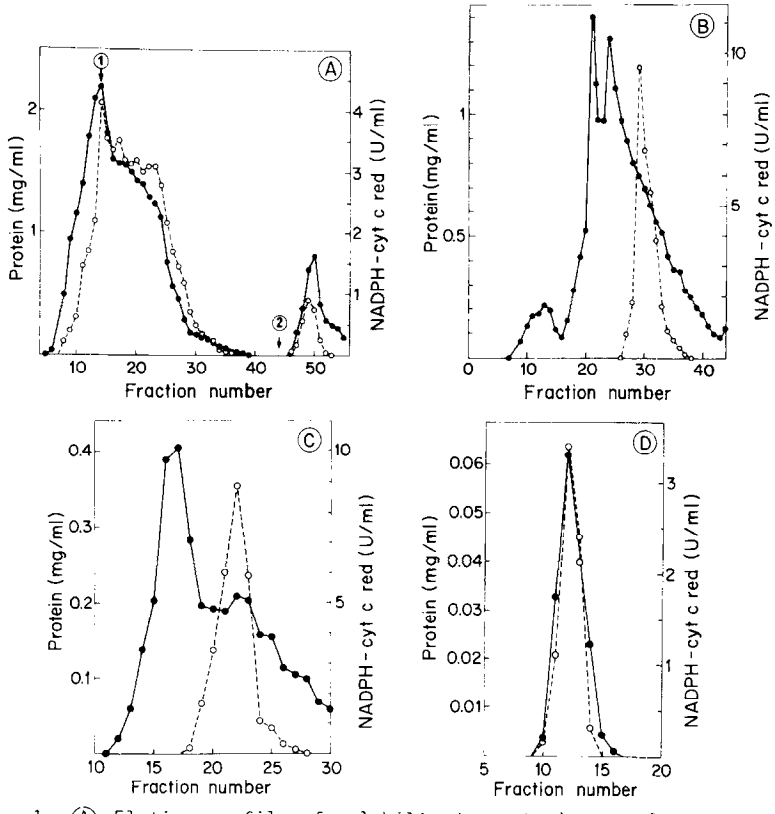

Figure 1 : (A) Elution profile of solubilized yeast microsomal fraction. NADPH-cytochrome c reductase (325 units) was applied to a Phenyl-Sepharose CL. 4B column. The elution was made with : (1) buffer A ; (2) buffer B.

(B) Column chromatography of NADPH-cytochrome c reductase on DE 52.

(C) Column chromatography on hydroxylapatite.

(D) Sephadex G-150 filtration.

(●---●) Protein ; (○-----○) NADPH-cytochrome c reductase

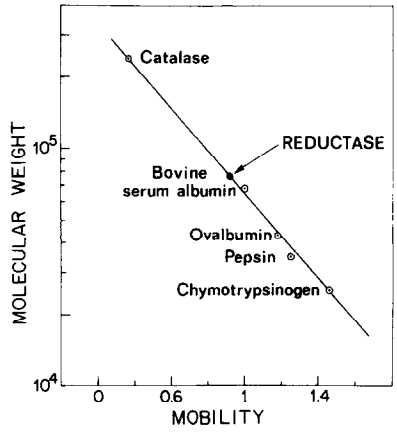

Figure 2 : Estimation of apparent molecular weight of NADPH-cytochrome c reductase by polyacrylamide gel electrophoresis on sodium dodecyl sulfate. The marker proteins used were : bovine pancreas chymotrypsinogen A (25000) ; pepsin (35000) ; ovalbumin (43000) ; bovine serum albumin (67000) ; catalase (240000).

osmotic wash or lipase or trypsin treatment (67,000). This difference obtained according to the method used for solubilization has been reported with all the organisms where this flavoprotein was studied (10-12) and is due to the loss of a part of the polypeptide chain which seems to remain in the microsomal membrane (13). Particularly with Saccharomyces cerevisiae, the reductase obtained by papain treatment has a molecular weight of 70,000 (12) whereas the one obtained by treatment with cholate has a molecular weight of 83,000 (14).

With Candida tropicalis, the enzyme purified after solubilization with cholate has properties similar to the one obtained by osmotic wash ; 1) The activity toward various electron acceptors is comparable (the specific activity is 42 units with dichloroindophenol, 28 units with menadione, 26 units ferricyanide and 19 units neotetrazolium chloride) ; 2) The optimal pH is the same (7.8) ; 3) It is inhibited by thiol inhibitors (chloromercuribenzoate and $HgCl_2$) and is influenced by ionic strength ; 4) It contains one mole of FMN and one mole of FAD per mole of reductase. It may be noted that if FMN and FAD are omitted during the course of the purification, the yield falls from 6.8 % to 4 %. This is due not only to the absence of the these co-factors but mostly to the dilution to which the enzyme is submitted during the purification.

Concerning this last point, Aoyama et al. (14) showed that with Saccharomyces cerevisiae a 5 minute incubation in the absence of FMN and FAD, of a much diluted solution of the enzyme results in a 80 % loss of the enzymatic activity. With Candida tropicalis in the presence or absence

of FMN and FAD there is a small loss of activity after a 5 minute incubation (8 %). However, after a 24 hour incubation a 42 % loss of activity is observed in the absence of FMN and FAD and a 26 % loss in the presence of FMN and FAD.

These results seem to indicate that the two prosthetic groups are more loosely bound to the apoprotein in Saccharomyces cerevisiae or in the higher plant Catharanthus roseus (15) than in Candida tropicalis where the enzyme would present similarities with the one present in the mammals (16). The detergent - solubilized preparation of reductase (0.4 - 0.6 units per system) retained its ability to transfer electrons from NADPH to cytochrome P-450 as shown by the recovery of hydroxylase activity toward lauric acid in a reconstituted system containing a partially purified cytochrome P-450 fraction (4). However, no activity was obtained when osmotic wash NADPH-cytochrome c reductase was used in the reconstitution system. These results confirm those already obtained with the other organisms studied and show that the loss of a part of the polypeptide chain leads to a loss of activity toward cytochrome P-450 (14-17).

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