SOLUBILIZATION AND PARTIAL PURIFICATION OF CYTOCHROME P-450 FROM RAT LUNG MICROSOMES

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Summary

Cytochrome P-450 from rat lung microsomes has been solubilized and purified 8-fold by using affinity chromatography on an ω-amino-n-octyl derivative of Sepharose 4B. The purified fraction was free of cytochrome b_5 and NADPH-cytochrome c reductase and showed spectral characteristics similar to those of lung microsomal cytochrome P-450. When combined with NADPH-cytochrome c reductase partially purified from liver microsomes, the cytochrome P-450 fraction supported the hydroxylation of benzo(α)-pyrene and the activity was proportional to the content of the hemoprotein. No absolute requirement for phosphatidylcholine was found.

Introduction

Previous work in this (1) and other laboratories (2) has revealed characteristics of the cytochrome P-450 species present in rat lung microsomes which differ considerably from those of the liver hemoprotein. These include differences in spectral (1, 2, 3) and catalytic (4, 5) properties as well as in inducibility by phenobarbital pretreatment of the animals (1, 2). In an attempt to further elucidate the basis of these apparent differences, we have solubilized and partially purified rat lung cytochrome P-450. This seemed particularly pertinent since this pigment most probably is intimately associated with the metabolic activation of carcinogenic polycyclic hydrocarbons resulting in tumor formation.

Up to now lung cytochrome P-450 has resisted all attempts to purification primarily because of its low tissue concentration. However, the new procedure described by Sato et al. (6) involving the use of affinity chromatography has allowed us to obtain a cytochrome P-450 fraction with a reasonably good yield which was found suitable for further purification.

Materials and Methods

Whole lungs, obtained from male Sprague-Dawley rats (250 grs), were dissected free of bronchi, suspended in ice-cold 0.15 M phosphate buffer, pH 7.5, containing 10% glycerol (v/v), and homogenized in a glass-teflon Potter Elvehjem homogenizer. The homogenate, adjusted to a 20% suspension (w/v), was spun twice at 12,000 x g for 15 min, and the resulting supernatant chromatographed on a Sepharose 2B column (height 40 cm, width 5 cm), using the homogenization buffer as elutant. The microsomes, eluted in the void volume of the gel, were pooled, spun at 105,000 x g for 90 min and suspended in 0.1 M phosphate buffer, pH 7.25, 0.1 mM EDTA, 0.1 mM dithiothreitol (DTT) containing 30% glycerol, to a final concentration of around 2.5 mg protein per ml (appr. 0.2 nmole cytochrome P-450 per ml). Sodium cholate (35 mg per nmole cytochrome P-450) was added slowly and with constant stirring and the resulting suspension was then allowed to stand for 90 min before centrifugation at 105,000 x g for 90 min. The clear supernatant thus obtained was immediately applied to a column (15 x 2.5 cm) of ω-amino-n-octyl Sepharose 4B, prepared as described by March et al. (7), previously equilibrated with 0.1 M phosphate buffer, pH 7.25, 0.1 mM EDTA, 0.1 mM DTT, containing 30% glycerol (v/v) and 0.5% sodium cholate (w/v). The turbid material, eluted during the loading and subsequent washing of the column with the equilibration buffer (4 column volumes), contained cytochrome P-420 and low amounts of unadsorbed cytochromes P-450 and b_5 and NADPH-cytochrome c reductase (fraction: affinity column F_1). The tightly adsorbed cytochrome P-450 was then eluted with 0.01 M phosphate buffer, pH 7.25, 0.1 mM EDTA, 0.1 mM DTT containing 30% glycerol, 0.4% sodium cholate and 0.05% Triton X-100. The fractions containing high amounts of cytochrome P-450 were pooled and concentrated by ultrafiltration using Amincon centrifuge membranes (type 224-CF-50) (fraction: affinity column F_2). Finally, the column was washed (4 column volumes) with the same buffer but containing 1% Triton X-100. The protein peak thus obtained (fraction: affinity column F_3) was used as the source for further purification of NADPH-cytochrome c reductase. Fraction F_2, containing cytochrome P-450, was then ultrafiltrated in a Sephadex G-200 column, equilibrated and eluted with 0.01 M phosphate buffer, pH 7.0, 0.1 mM EDTA, 0.1 mM DTT, containing 20% glycerol and 0.05% Triton X-100. The fraction eluted in the void volume of the gel was then immediately applied to a DE-52 cellulose column, previously equilibrated with the same buffer as above. After washing the column with the equilibration buffer (4 column volumes) the adsorbed cytochrome P-450 was eluted by increasing the concentration of phosphate to 50 mM. The fractions containing cytochrome P-450 (fraction DE-52 cellulose fraction

DE-1) were pooled, concentrated by ultrafiltration and used as the purified preparation.

A NADPH-cytochrome c reductase fraction, free of cytochrome b_5, was obtained by DEAE Sephadex A-50 column chromatography of affinity fraction F_3. The reductase containing fraction was applied to a DEAE Sephadex A-50 column (10 x 1.5 cm), equilibrated with 0.1 M phosphate buffer, pH 7.25, containing 20% glycerol and 0.05% Triton X-100. The enzyme was eluted by increasing the phosphate concentration of the buffer to 0.2 M. The fractions containing high NADPH-cytochrome c reductase activity, were pooled, dialyzed 12 hours against 0.05 M phosphate buffer, pH 7.25, concentrated by ultrafiltration and used as such.

All the above operations were carried out at 0-4°C. Cytochromes P-450, P-420 and b_5 were determined as described by Omura and Sato (8) and NADPH cytochrome c reductase according to Dallner (9). Protein was measured according to Lowry et al. (10) using bovine serum albumin as standard.

The catalytic activity towards benzo(α)pyrene was followed as described by DePierre et al. (11) but using NADPH (final concentration 0.75 mM) instead of a NADPH generating system. The concentration of benzo(α)pyrene was 80 μM and the incubations were for 1 hr at 37°C.

Results

Table 1 shows the distribution of cytochrome P-450 and b_5 and of NADPH-cytochrome c reductase after solubilization of the

Table 1. Fractionation of rat lung microsomes.

Fraction	Protein, mg	Recovery, %	Cyt. P-450, nmoles	Recovery, %	Cyt. b_5, nmoles	Recovery, %	NADPH cyt.c red. units*	Recovery %
Original microsomes	236	100	18.9	100	22.6	100	24.20	100
Soluble fraction	136	57	17.7	94	13.1	58	16.90	70
Affinity column F_1	60	25	0.5	3	1.8	14	0.14	0.5
Affinity column F_2	29	12	5.8	33	0	0	0.11	0.4
Affinity column F_3	39	17	0	0	1.41	11	3.65	15

The denominations of the different fractions and details of the fractionation procedure are given in "Materials and Methods"
*1 unit = 1 μmol cytochrome c reduced/min.

microsomes by sodium cholate and subsequent chromatography using the ω-amino-n-octyl Sepharose 4B as an affinity or hydrophobic adsorbent. About 75% of cytochrome b_5 and NADPH-cytochrome c reductase was retained by the column even after elution with 1% Triton X-100. However, 15% of the reductase was eluted with 1% Triton X-100 (F_3) and could be freed of contaminating cytochrome b_5 by chromatography on DEAE Sephadex A-50. In contrast, the major portion of cytochrome P-450 was eluted in fraction F_2 (0.05% Triton). This was free of cytochrome b_5 and the contamination with NADPH-cytochrome c reductase was decreased more than 200 times. The F_2 fraction was further purified and freed of residual reductase by DE-52 cellulose column chromatography (Table 2). The final purified cytochrome P-450 fraction contained 0.66 nmole cytochrome P-450 per mg protein representing a purification factor of slightly above 8.

The spectral properties of the partially purified cytochrome P-450 fraction are shown in Figure 1. The absolute spectrum of the oxidized pigment had a Soret band at 418 nm which is similar to solubilized preparations obtained from liver microsomes (12).

Table 2. Fractionation of rat lung microsomal cytochrome P-450.

Fractionation step	nmoles per mg protein	Recovery, %	Purification factor
Original microsomes	0.08	100	1.0
Solubilized microsomes	0.13	94	1.6
Affinity fraction F_2	0.20	31	2.5
Vo Sephadex G-200	0.24	21	3.0
DE-52 cellulose fraction DE-1	0.66	13	8.3

The denominations of the different fractions and details of the fractionation procedure are given in "Materials and Methods"

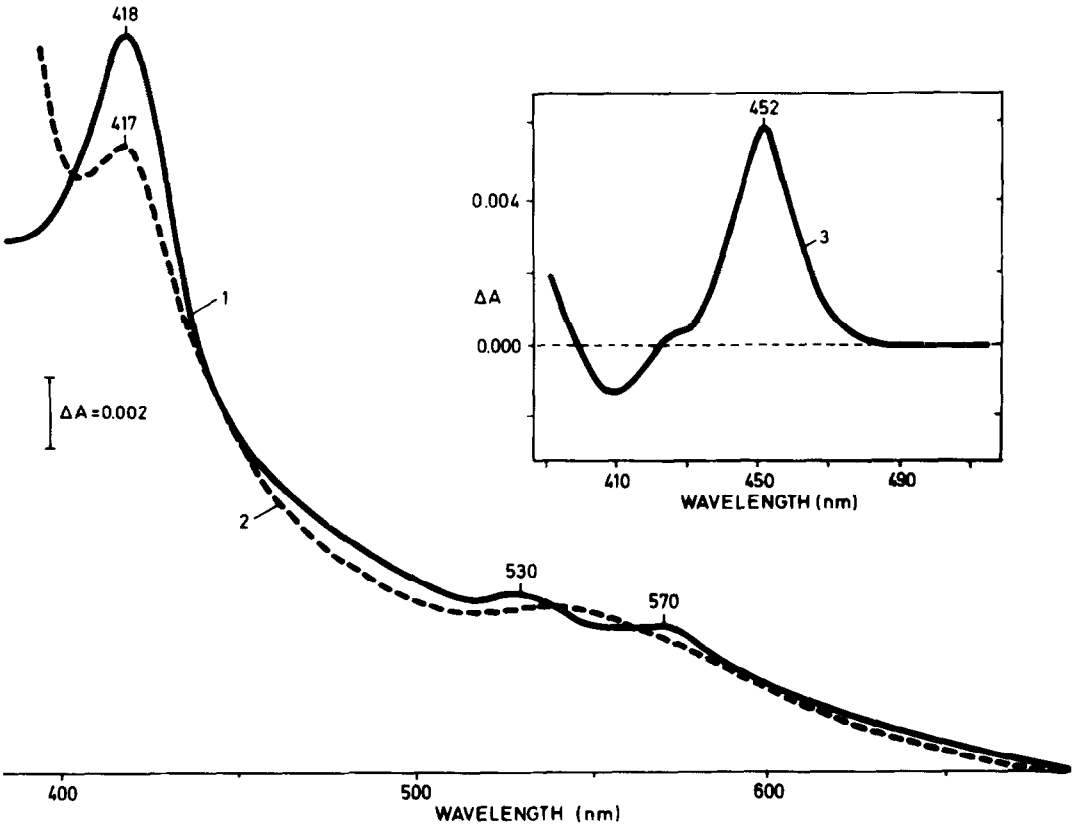

Fig. 1. Absorption spectra of partially purified lung cytochrome P-450: (1) Absolute (Fe^{3+}), (2) absolute (Fe^{2+}) and (3) difference ($Fe^{2+}\cdot CO$). The cytochrome P-450 concentration was 63 nM. The absolute spectras were obtained using 0.1 M phosphate buffer, pH 7.0, 0.1 mM EDTA, 0.1 mM DTT containing 20% glycerol (v/v) as reference.

Upon reduction the intensity was decreased and the peak position shifted one nm to the blue. The CO-difference spectrum showed an absorption maximum at 452 nm, essentially similar to the microsomal bound pigment (1) and identical to liver cytochrome P-450 purified to apparent homogeneity (13). The sholder at about 420 nm indicates the presence of small amounts of cytochrome P-420, which were most probably produced during the determination since we observed a fast denaturation of cytochrome P-450 in the presence of dithionite (cf. 13).

As shown in Table 3, the partially purified lung cytochrome P-450 fraction when combined with NADPH-cytochrome c reductase partially purified from rat liver microsomes, catalyzed the hydroxylation of benzo(α)pyrene. The activity was not dependent on the addition of exogenous phosphatidylcholine. The NADPH-cytochrome c reductase purified from rat lung microsomes could not replace the liver enzyme preparation in supporting the reaction.

The rate of benzo(α)pyrene hydroxylation catalyzed by the reconstituted system was directly proportional to the concentration of cytochrome P-450 added in the range studied (Table 4). On the other hand, moderate variation in the concentration of the reductase produced no significant changes in the rate of the reaction.

Comments

Low tissue concentration and contaminating hemoglobin have interfered with previous attempts to study the properties of lung microsomal cytochrome P-450 and made the solubilization and purification of this pigment impossible. By gel filtration we have

Table 3. Benzo(α)pyrene hydroxylation in a reconstituted system containing partially purified rat lung cytochrome P-450.

	nmoles BP metabolized /hr/mg protein	nmoles BP metabolized /hr/nmole P-450
Cytochrome P-450[a/]+pc[b/]	4.2	2.8
Reductase[c/]+pc	1.6	0
Cytochrome P-450+reductase	139.3	92.0
Cytochrome P-450+reductase+pc	142.4	94.0[d/]

a/ Cytochrome P-450: 0.1 nmole/ml incubation mixture. b/ Synthetic phosphatidylcholine: 0.1 mg/ml incubation mixture. c/ NADPH-cytochrome c reductase from rat liver: 20 units/ml incubation mixture. d/ Benzo(α)pyrene hydroxylase activity in the lung microsomes was: 150 nmoles BP metabolized/hr/nmole P-450.

Table 4. Effects of varying concentrations of lung cytochrome P-450 and liver NADPH-cytochrome c reductase on benzo(α)pyrene hydroxylation in a reconstituted system

nmoles cyt. P-450	NADPH-cyt. c red. units	nmoles BP metabolized/hr	nmoles BP metabolized/hr/nmole P-450
0.0215	5	1.74	80.7
0.0429	5	3.47	79.8
0.0640	5	4.89	76.3
0.0858	5	6.72	78.0
0.0358	2.5	2.60	73.9
0.0358	5	2.47	69.0
0.0358	8	2.43	68.0
0.0358	12	2.80	81.0

isolated a microsomal fraction essentially free of contaminating hemoglobin and with the highest concentration of cytochrome P-450 ever reported (1). This and the introduction of affinity chromatography, which minimizes loss of cytochrome P-450 during purification, have made possible the isolation of a partially purified cytochrome P-450 fraction with a reasonable yield and a purification factor of more than 8. Since this fraction was obtained free of cytochrome b_5 and NADPH-cytochrome c reductase, it was suitable for both spectral and catalytic characterization.

The absolute spectra obtained with the partially purified cytochrome P-450 fraction were essentially similar to those previously reported for partially purified liver cytochrome P-450, whereas the CO-difference spectrum retained the peak position at 452 nm typical of the membrane bound pigment (1) but different from that of liver cytochrome P-450 (8).

Polycyclic hydrocarbons are the only substrates actively hydroxylated by rat lung microsomes (2, 14) and benzo(α)pyrene

was therefore used as a model substrate in the present study. When calculated per nmole of cytochrome P-450, the optimal activity of the recombined system was about 60% of the microsomal activity. Under the conditions employed, cytochrome P-450 appeared to be the rate-limiting component. The system was active without the addition of exogenous phosphatidylcholine indicating that (a) enough phospholipid was present as a contaminant in either the cytochrome P-450 or reductase fraction, (b) Triton X-100 was replacing the phospholipid (15) or (c) that a conformational factor was not required. The fact that the partially purified NADPH-cytochrome c reductase from lung microsomes, in the presence of the cytochrome P-450 fraction, could not support benzo-(α)pyrene hydroxylation is still unclear. One possible explanation is, however, that the concentration of Triton X-100 in this fraction was high enough to inhibit the reaction (15).

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