PURIFICATION OF THE MAJOR CYTOCHROME P-450 OF LIVER MICROSOMES FROM RABBITS TREATED WITH 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN (TCDD)

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Summary: Cytochrome P-450 was isolated in highly purified form from liver microsomes of adult male rabbits treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Preparations average 17.8 ± 0.8 nmoles cytochrome P-450 per mg protein and have an estimated molecular weight of 54,500. The visible absorption spectrum of the purified cytochrome displays absorption spectral maxima characteristic of high spin forms of cytochrome P-450. When reconstituted with highly purified NADPH-cytochrome P-450 reductase, this cytochrome catalyzes the hydroxylation of acetanilide and the O-deethylation of 7-ethoxyresorufin, two activities induced by TCDD.

The microsomal cytochrome P-450 monooxygenases oxidize a wide variety of xenobiotics. Resolution and characterization of the individual forms of cytochrome P-450 can be expected to aid our understanding of their participation in the metabolism of diverse substrates. In this report, we describe the purification of form *c*, one of three forms of cytochrome P-450 (1) isolated from liver microsomes of rabbits treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)[1], and discuss several properties of this protein.

Purification of cytochrome P-450*c*: Cytochrome P-450*c* is isolated from liver microsomes obtained from male New Zealand rabbits (3-4 kg) which have received 30 nmoles TCDD/kg (0.3 mM in dioxane) five days prior to sacrifice. The purification procedure is described in Table I. The isolation and solubilization of microsomes and the initial purification steps using polyethylene glycol fractionation and hydroxylapatite chromatography have been described for cytochrome P-450*c* (2). These and

[1] TCDD is an abbreviation for 2,3,7,8-tetrachlorodibenzo-p-dioxin, and SDS is an abbreviation for sodium dodecyl sulfate.

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TABLE I

PURIFICATION OF CYTOCHROME P-450*c*

Preparation	Vol (*ml*)	Protein (*mg*)	Cytochrome P-450 Content (*nmoles/mg protein*)	Yield * (%)
Microsomes	31.2	600	3.6	100
Polyethylene glycol fractionation **	46.5	200	5.7	53
Hydroxylapatite chromatography **	10.9	19.6	13.1	12
DEAE-cellulose chromatography	8.2	3.9	17.9	3

* Based on total cytochrome P-450 present.

** ref #2

subsequent purification procedures are carried out at 4°C, and the buffers contain 20% glycerol and potassium phosphate, pH 7.4, at the indicated molarity.

The partially purified cytochrome P-450*c* fraction obtained by hydroxylapatite chromatography precipitates when dialyzed against a 5 mM buffer, containing 0.1 mM DTE, 0.1 mM EDTA and 0.1% Nonidet P40[2], as noted earlier (2). The cytochrome is soluble at higher ionic strength, but this precludes further purification by ion-exchange chromatography. However, the precipitated cytochrome can be solubilized at a lower ionic strength in the presence of cholic acid and Nonidet P40 and then chromatographed on DEAE-cellulose. The precipitated cytochrome is isolated by centrifugation at 10,000 x g x 30

[2]Nonidet P40 is a nonionic detergent obtained from Accurate Chemical and Scientific Corp.

minutes, and the dark red pellet is suspended in *ca.* 10 ml of 5 mM buffer containing 1.0 mM DTE and 0.1 mM EDTA. The turbid suspension is added dropwise to the same buffer containing cholic acid and Nonidet P40. The final volume is 50 ml, and the final concentration of each detergent is 0.4%. The mixture is stirred for 5-10 minutes at $4^{o}C$ until the solution is clear.

The solubilized cytochrome is applied to DEAE-cellulose (2.5 x 5 cm column, Whatman DE-52) equilibrated with the dialysis buffer. A dark red band appears approximately one band width below the surface of the column bed. The column bed is washed with 2 column volumes of the equilibration buffer, and the cytochrome is slowly eluted after increasing the buffer strength to 10 mM. The eluted cytochrome is then adsorbed onto 80 mg of calcium phosphate gel (Bio-Rad). The gel is recovered by centrifugation (4000 g) for 5 minutes and is washed several times with 10 mM buffer. The cytochrome is eluted from the calcium phosphate gel into a small volume of 0.4 M buffer.

Cytochrome P-450 concentrations are determined by the method of Omura and Sato (3). Protein concentrations are estimated by the method of Lowry *et al* (4). To reduce interference in the determination of protein concentration from buffer components or residual detergent remaining after the calcium phosphate gel treatment, the samples (10-50 µl) are incubated for 15 min. with 500 µl 0.01% deoxycholate, and are then precipitated by addition of 500 µl 10% TCA (5). The precipitates are collected by centrifugation, dissolved, and assayed as described in the original procedure (4). Bovine serum albumin, treated in the same manner, serves as a standard. NADPH-cytochrome P-450 reductase is prepared from liver microsomes isolated from phenobarbital-treated rabbits as described by Yasukochi and Masters (6).

Properties of the purified cytochrome: The high degree of purity of the cytochrome preparations is demonstrated by SDS-polyacrylamide gel electrophoresis (Fig. 1). Using this method, a molecular weight of

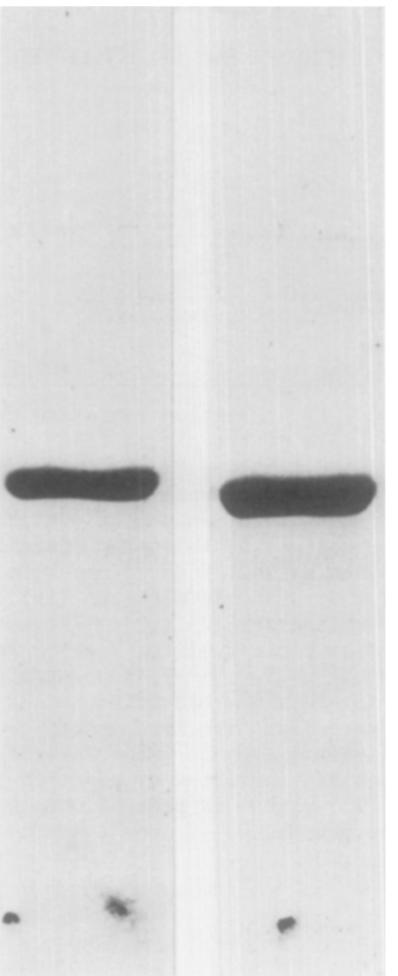

Figure 1: SDS-polyacrylamide slab gel (10 x 16 x 0.3 cm) electrophoresis of cytochrome P-450*c* performed according to the method of Laemmli (22). The gel strip on the right contains 9 µg and the gel on the left 18 µg of protein.

54,500 is estimated for the cytochrome. Assuming one mole of heme per mole of protein, a theoretical value of 18.3 nmoles of cytochrome P-450/mg protein is expected. The average cytochrome P-450 content of several preparations is 17.8 $\pm$ 0.8 nmole/mg protein. On this basis, we suggest that the cytochrome is essentially homogenous. The preparations contain no detectable NADPH-cytochrome *c* reductase activity.

TABLE II

SUBSTRATE OXIDATION BY THE RECONSTITUTED ENZYME SYSTEM

Substrate	Rate
Acetanilide	6.1
7-ethoxyresorufin	0.42
Benzo(a)pyrene	0.03
Benzphetamine	1.8

Reaction rates are expressed as moles of product formed per mole of cytochrome P-450 at 37°C (30° C for 7-ethoxyresorufin). Cytochrome P-450 content was varied from 0.0-0.3 nmoles, and the rates were estimated from the linear dependence of the rate on the amount of cytochrome P-450. Substrates are present at the following concentrations, and products are analyzed as indicated in the references: 1 mM benzphetamine (18); 80 μM benzo(a)pyrene (19); 1.5 μM 7-ethoxyresorufin (20); 4 mM acetanilide (21). The reaction mixture has a total volume of 1 ml (2 ml for 7-ethoxyresorufin) and contains 0.05 M Hepes buffer, pH 7.4; 30 μg dilauroyl-L-α-lecithin (100 μg for 7-ethoxyresorufin); and 0.3 or 0.6 units of NADPH-cytochrome *c* reductase with specific activities of 42-55 units/mg (1 unit = 1 μmole cytochrome *c* reduced per minute). Reactions are initiated by addition of 0.5 μmoles of NADPH. $MgCl_2$ (15 μmoles) is present when benzphetamine and benzo(a)pyrene are the substrates tested.

The activity of cytochrome P-450*c* in a reconstituted enzyme system utilizing several substrates is shown in Table II. The assay mixture contained dilauroyl-L-α-lecithin and highly purified rabbit liver NADPH-cytochrome P-450 reductase. Turnover numbers are determined under conditions where the reaction rate is linearly dependent on the amount of cytochrome. With acetanilide and 7-ethoxyresorufin as substrates, the turnover numbers for the reconstituted enzyme are several-fold greater than those found for microsomes. The hydroxylation of acetanilide (7) and the O-deethylation of 7-ethoxyresorufin (8) are induced by TCDD, 3-methylcholanthrene or β-naphthaflavone. These compounds do not induce the hydroxylation of benzo(a)pyrene (7,9,10)

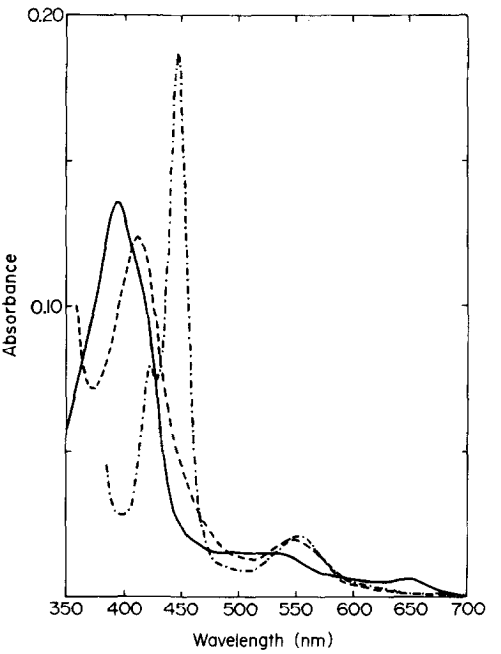

Figure 2: Absorption spectra of purified cytochrome P-450*c*, 1.5 μM in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol. The spectra shown are the oxidized form (————), the dithionite reduced form (— — — —), and the reduced-carbonyl complex (—·—·—).

or the N-demethylation of benzphetamine (9) in rabbit liver. The benzphetamine (11) and benzo(a)pyrene (1) reactions appear to be catalyzed more rapidly by other forms of rabbit liver cytochrome P-450.

Visible absorption spectra of the purified cytochrome are shown in Figure 2. The spectrum of the cytochrome displays a Soret maximum at 393 nm, poorly resolved *a* and *b* bands between 500-550 nm, and a weak absorption band at 645 nm. The Soret maximum appears to consist of a doublet. The second, unresolved component is centered at *ca.* 417 nm. In the presence of Nonidet P40, this is the principal component of the spectrum (not shown). The spectrum of the cytochrome reduced by dithionite is also shown in Figure 2. The Soret maximum is at 412 nm and a single visible absorption band is seen at 545 nm. In the presence

of carbon monoxide, these absorption bands are at 447 nm and 550 nm respectively.

The spectral properties of cytochrome P-450*c* including the changes observed when detergent is present and the n-octylamine difference spectrum described earlier (2) are similar to those reported by Haugen and Coon (12) for their cytochrome P-450 LM_4 isolated from rabbit liver, which contained predominantly high spin ferric heme. The spectrum of P-450*c* is also similar to those reported for highly purified cytochrome $P\text{-}450_{11\beta}$ from adrenocortical mitochondria (12), a substrate-cytochrome P-450 complex isolated from 3-methylcholanthrene-treated rabbits (14), and cytochrome P-450 isolated from *Pseudomonas putida* in the presence of substrate (15), all of which have been characterized as containing high spin heme iron.

Cytochrome P-450*c* was compared directly with cytochrome P-450 LM_4 (12) provided by Dr. M.J. Coon. This cytochrome was isolated from β-napthaflavone-induced rabbit liver microsomes. Antibody to cytochrome P-450*c* (2) shows a complete line of identity of form *c* with the cytochrome P-450 LM_4 when tested by double diffusion in agarose. The two cytochromes also have the same electrophoretic mobilities in SDS-polyacrylamide gels. Corroborating results were obtained in Dr. Coon's laboratory (16), where cytochrome P-448 isolated from 3-methylcholanthrene-induced rabbit liver microsomes (17) showed similar properties.

In summary, cytochrome P-450*c* appears to be identical to cytochrome P-450 LM_4 isolated from rabbits treated with β-napthaflavone as assessed by immunological, electrophoretic, and spectral criteria. β-napthaflavone and TCDD are inducers of acetanilide hydroxylation and 7-ethoxyresorufin O-deethylation in rabbit liver. When reconstituted with NADPH-cytochrome P-450 reductase, cytochrome P-450*c* catalyzed these two reactions.

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