MULTIPLE FORMS OF CYTOCHROME P-450: RESOLUTION AND PURIFICATION OF RABBIT LIVER ARYL HYDROCARBON HYDROXYLASE

Eric F. Johnson and Ursula Muller-Eberhard

Department of Biochemistry
Scripps Clinic and Research Foundation
La Jolla, California 92037

Received March 11,1977

Summary: We describe the resolution and partial purification of two minor forms of cytochrome P-450 from liver microsomes of rabbits treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. Both forms have different electrophoretic mobilities when compared to the major form of cytochrome P-450 isolated from this source. The two cytochromes show different activities with several substrates. One form is very active in the hydroxylation of benzo(a)pyrene when reconstituted with highly purified NADPH-cytochrome P-450 reductase.

The functional diversity of the microsomal monooxygenases may be attributed to the existence of multiple forms of cytochrome P-450. Several laboratories, including our own, have recently characterized multiple forms of rabbit liver cytochrome P-450 by immunologic, spectroscopic, electrophoretic and functional criteria (1-4). In earlier work, we resolved the fractions, cytochromes P-450*ab* and P-450*c* from the liver microsomes of rabbits treated with the inducer TCDD[1] (1). Cytochrome P-450*c* has subsequently been purified to homogeneity (5). The present report details progress on the resolution of fraction P-450*ab* into two forms, *a* and *b*. Form *b* has been extensively purified and found to be as active as highly purified rat liver cytochrome P-448 in the hydroxylation of benzo(a)-pyrene (aryl hydrocarbon hydroxylase) (6,7). The turnover number determined for this cytochrome is much greater than that observed for other forms of purified rabbit liver cytochrome P-450. Cytochrome P-450*b* is a polypeptide of 60,000 daltons and exhibits an electrophoretic

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

ISSN 0006-291X

TABLE I

PURIFICATION OF CYTOCHROMES

Preparation	Vol (ml)	Protein (mg)	Cytochrome P-450 Content (nmole/mg protein)	Yield* (%)
Microsomes	31.2	600	3.6	100
Polyethylene glycol fractionation** (7-14%)	46.5	200	5.7	53
Hydroxylapatite chromatography	-	-	-	-
DEAE-cellulose chromatography				
5 mm Eluate (form a)	7.4	4.6	9.7	2
30-75 mm Eluate (form b)	8.2	4.4	12.0	2

*Based on total microsomal cytochrome P-450.

**Ref. 1.

mobility different from the major phenobarbital-inducible cytochrome P-450 or form *c*.

Purification of cytochrome P-450*a* and P-450*b*: New Zealand rabbits (3-4 kg) received 30 nmoles/kg i.p. of a 0.3 mM solution of TCDD in dioxane five days prior to sacrifice. All purification procedures were performed at 0-4°C. Results of one purification procedure are shown in Table I.

Cytochrome P-450*ab* was isolated from the livers of these animals by polyethylene glycol fractionation of cholate-solubilized microsomes and hydroxylapatite chromatography (1). The dilute material was then dialyzed against 5 mM potassium phosphate buffer, pH 7.4, containing

20% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.1% Nonidet P40[2]. The cytochrome was not characterized before the next purification step because of the presence of detergent and the high degree of dilution. Cytochrome P-450 and protein content are probably similar to that of cytochrome P-450*ab* (1) after treatment with calcium phosphate gel, which represented 6% of the total microsomal cytochrome P-450 content and contained 6 nmoles/mg protein.

The dialyzed material was chromatographed on a column (2.5 x 13 cm) containing DEAE-cellulose (Whatman DE-52) equilibrated with the dialysis buffer. After application of the sample, the column bed was washed with 125 ml of equilibration buffer. Cytochrome P-450*a* was eluted in the void volume and adsorbed onto 160 mg of calcium phosphate gel (Bio Rad). The suspension was centrifuged for 5 min. (4000 g) and the pellet was washed with 0.01 M potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. The cytochrome was recovered from the gel by elution with a small volume of 0.3 M potassium phosphate buffer, pH 7.4, containing 20% glycerol.

Cytochrome P-450*b* was retained by the DEAE-cellulose. The column bed was washed with 200 ml of the equilibration buffer containing 0.03 M potassium phosphate, and the cytochrome was eluted with 200 ml of the same buffer mixture containing 0.075 M potassium phosphate. This fraction was dialyzed overnight against the dialysis buffer and concentrated by treatment with 80 mg of calcium phosphate gel as described for cytochrome P-450*a*.

Cytochrome P-450 concentrations were determined by the method of Omura and Sato (8). Protein concentrations were determined by the method of Lowry *et al* (9) after precipitation of the proteins in the presence of trichloroacetic acid and deoxycholic acid (10). NADPH-cytochrome

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

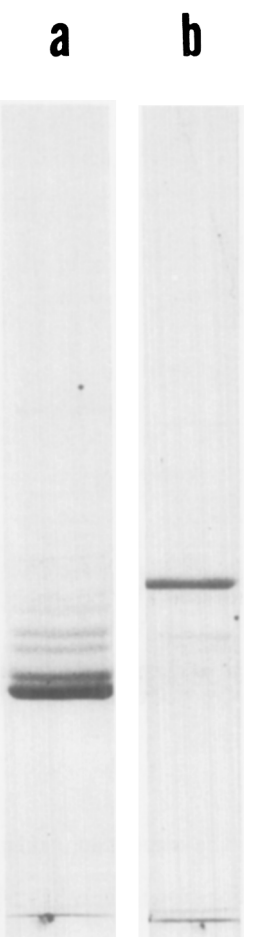

Figure 1: SDS-polyacrylamide slab gel (10 x 16 x 0.15 cm) electrophoresis was performed as described by Laemmli (21). Gel strip a contains cytochrome P-450*a*, 4 µg protein, and gel strip b contains cytochrome P-450*b*, 4 µg protein.

P-450 reductase was isolated from rabbit liver as described by Yasukochi and Masters (11).

Results and Discussion: Cytochrome P-450*a* and P-450*b* were purified to an average cytochrome P-450 content of 8.5 and 9.8 nmoles/mg protein with preparations as high as 9.7 and 12.0 nmoles/mg protein, respectively. Results of SDS-polyacrylamide gel electrophoresis of the cytochrome preparations are shown in Figure 1. Cytochrome P-450*b* consists of a single major peptide with a molecular weight of 60,000. Assuming one

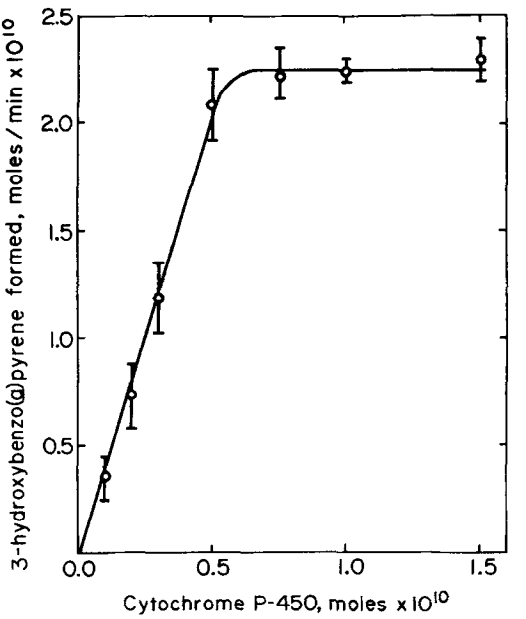

Figure 2: The dependence of benzo(a)pyrene hydroxylation on cytochrome P-450*b*. The assay conditions are the same as those described in the legend to Table II.

molecule of heme per peptide, the expected ratio of cytochrome to protein is 16.7 nmoles/mg. Therefore, our best preparations contain *ca.* 75% cytochrome P-450. Cytochrome P-450*a* consists of several peptides with different electrophoretic mobilities; the major peptide corresponds to a molecular weight of 48,000. In experiments not shown here, the mobilities of both cytochrome P-450*b* and the major peptide of cytochrome P-450*a* did not correspond to those of form *c* or the major phenobarbital-inducible form of cytochrome P-450.

The reduced carbonyl-difference spectra of cytochrome P-450*a* and P-450*b* display Soret maxima at 450.5 nm and 448 nm, respectively. Both cytochrome preparations are free of cytochrome b_5. Cytochrome P-450*b*, but not cytochrome P-450*a*, contains NADPH-cytochrome *c* reductase activity (0.004 units/nmole).[3]

[3] A unit of reductase activity is defined as the reduction of 1 μmole of cytochrome *c* per minute.

Cytochrome P-450*b* hydroxylates benzo(a)pyrene when reconstituted with NADPH-cytochrome *c* reductase as shown in Figure 2. Phenolic metabolites were assayed as described by Nebert and Gelboin (12) and expressed as equivalent to 3-hydroxybenzo(a)pyrene. A turnover number of 4.2 moles/min/mole cytochrome P-450*b* is estimated from the linear dependence of the rate on cytochrome P-450*b* concentration. This value is comparable to values reported by others for purified rat liver cytochrome P-448 (6,7). Others have shown that aryl hydrocarbon hydroxylase activity is not induced by TCDD in adult male rabbit liver (10). The major form of cytochrome P-450, form *c*, isolated from this tissue, has very low benzo(a)pyrene hydroxylase activity (0.03 moles/min/mole cytochrome P-450*c*) (5). Cytochrome P-450*b* appears to be present in smaller quantities than cytochrome P-450*c*, which may explain the low level of total microsomal aryl hydrocarbon hydroxylase activity.

We have not as yet determined the other known products of benzo(a)pyrene metabolism with the preparations described here. Additional forms of cytochrome P-450 may be active in the oxidation of benzo(a)pyrene or its metabolites to non-phenolic compounds. Wiebel *et al* reported qualitative and quantitative differences in benzo(a)pyrene metabolism using several forms of rabbit liver cytochrome P-450 (11). The turnover number for cytochrome P-450*b* is 4-5 times higher than a mixture of cytochromes ($LM_{1,7}$) isolated from livers of phenobarbital-treated animals (14) and is much higher than the highly purified forms LM_2 and LM_4 (14). Other forms of purified rabbit liver cytochrome P-450 also show very low activities toward benzo(a)pyrene (15,16).

The activities of cytochrome P-450*a* and P-450*b* are given in Table II for a reconstituted enzyme system with benzo(a)pyrene and three other substrates. The values obtained with cytochrome P-450*c*,

TABLE II

RECONSTITUTED ENZYME ACTIVITIES

Substrate	Cytochrome P-450*a*	Cytochrome P-450*b*	Cytochrome P-450*c*[*]
Benzphetamine	12	4.0	1.80
Benzo(a)pyrene	0.42	4.1	0.03
7-ethoxyresorufin	0.04	0.4	0.42
Acetanilide	1.20	1.3	6.00

[*] Reference 5.

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 cytochrome P-450 concentration. Substrates are present at the following concentrations, and the products are analyzed as indicated in the references: 1 mM benzphetamine (18); 80 μM benzo(a)pyrene (12); 1.5 μM 7-ethoxyresorufin (19); 4 mM acetanilide (20). The reaction mixture 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 a specific activity of 42-55 units/mg. Reactions are initiated by addition of 0.5 μmoles of NADPH. $MgCl_2$ (15 μmoles) is present when benzphetamine and benzo(a)pyrene are substrates. The total volume is 1 ml (2 ml for 7-ethoxyresorufin).

the major form of the cytochrome isolated from TCDD-induced rabbit liver microsomes, are included for comparison (5).

In general, substrates, such as benzphetamine, which are rapidly metabolized by the microsomal enzymes show higher rates with all three cytochromes than substrates, such as 7-ethoxyresorufin, which are metabolized at lower rates by microsomes. However, with each substrate, the purified cytochrome displaying the highest activity exhibits a higher turnover number than that observed using microsomal preparations. Cytochromes showing lower activities tend to have turnover numbers lower than those observed with microsomes.

Thus, although form *b* metabolized benzphetamine and benzo(a)-pyrene at comparable rates, the turnover number of benzo(a)pyrene shows a large increase over that determined for microsomes. This is not the case with benzphetamine which is metabolized more rapidly by the major phenobarbital-inducible form of the cytochrome (12) and form *a*.

Acknowledgement: This research was supported by the National Institutes of Health Grants #HD-04445 and #CA-17735 (Dr. U. Muller-Eberhard) and by a California Division, American Cancer Society Junior Fellowship Number J-301 (Dr. E.F. Johnson).

TCDD was kindly supplied by the Dow Chemical Co., Midland, Michigan; 3-hydroxybenzo(a)pyrene was a gift of Dr. H.V. Gelboin of the National Cancer Institute; 7-ethoxyresorufin and resorufin were gifts from Dr. R. Prough of the University of Texas Health Science Center at Dallas, and benzphetamine was provided by Dr. W. O'Connell of the Upjohn Co., Kalamazoo, Michigan.

We wish to thank G. Schwab and M. Zounes for their excellent assistance.

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