PURIFICATION OF A SUBSTRATE COMPLEX OF CYTOCHROME P-450 FROM LIVER MICROSOMES OF 3-METHYLCHOLANTHRENE-TREATED RABBITS

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SUMMARY: Cytochrome P-450 was purified as a 3-methylcholanthrene complex from liver microsomes of 3-methylcholanthrene-treated rabbits to a specific content of 17 to 18 nmoles per mg of protein with a yield of about 10 %. The purified protein gave only a single protein band on sodium dodecylsulfate-urea-polyacrylamide gel electrophoresis, and its apparent molecular weight was estimated to be about 54,000, a value which is higher than that for cytochrome P-450 from phenobarbital-treated rabbits by about 4,000. The reconstituted system containing the purified cytochrome and NADPH-cytochrome c reductase was active in NADPH-dependent hydroxylation of benzo[α]pyrene.

Hepatic microsomal cytochrome P-450 is the key enzyme of the momooxygenase system catalyzing the oxidative metabolism of many xenobiotics as well as endogenous substrates. Evidence has accumulated that the liver can synthesize at least two different species of cytochrome P-450, i.e. PB- and MC-inducible ones, which differ from each other in many properties (1-4). Recently, three laboratories have reported the purification of these cytochromes to apparent homogeneity from PB- or MC-treated animals (3,5,6). By a modification of our previous method for the purification of cytochrome P-450 from PB-treated rabbits (5), we have now purified the cytochrome from MC-treated rabbits to a gel-electrophoretically homogeneous state and shown that it was in the form of a MC complex.

Purification of Cytochrome P-450. Male rabbits, about 2.5 kg, were injected intraperitoneally with MC (dissolved in olive oil) for 8 days at a daily dose of 25 mg per kg of body weight. Liver microsomes were prepared from the treated animals as described previously (7). All the subsequent manipulations were conducted at 0-4°. Potassium phosphate buffers, pH 7.25, containing 20 % (v/v) glycerol were used throughout; they will be referred to simply as 200 mM

Abbreviations: PB, phenobarbital; MC, 3-methylcholanthrene; DTT, dithiothreitol.

buffer, etc. Liver microsomes (1.9 g of protein) were suspended (to ~2 mg protein/ml) in 200 mM buffer containing 1 mM DTT, 1 mM EDTA, and 0.6 % (w/v) sodium cholate. The suspension was gently stirred for 30 min and then centrifuged at 77,000 x g for 2 h. The supernatant fraction ("solubilized supernatant") was applied to a column (2.7 x 30 cm) of ω-amino-n-octyl Sepharose 4B (prepared as described in ref. 7) which had been equilibrated with the same buffer. After washing the column with 3 times the column volume of 100 mM buffer containing 1 mM DTT, 1 mM EDTA, and 0.5 % cholate, elution was conducted with 100 mM buffer containing 1 mM DTT, 0.4 % cholate, and 0.08 % (w/v) Emulgen 913 (a polyoxyethylene nonylphenyl ether, Kao-Atlas Co., Tokyo), and then with the same buffer in which the Emulgen concentration was raised to 0.2 %. Thirty to 50 % of cytochrome P-450 was eluted with the latter buffer, and fractions from this elution having high specific contents of the cytochrome were pooled ("aminooctyl column eluate"). Fractions eluted with the former buffer showed absorption spectra rather similar to those of the cytochrome from PB-treated rabbits. The pooled fractions were diluted 3-fold with 20 % glycerol and adsorbed on a column (3.2 x 9 cm) of hydroxylapatite (for preparation, see ref. 8) equilibrated with 33 mM buffer. After washing the column stepwise with 35 mM, 100 mM, and 150 mM buffers all containing 0.2 % Emulgen 913, cytochrome P-450 was eluted with 150 mM buffer containing both 0.2 % Emulgen and 0.1 % cholate. The peak fractions of the eluate were combined ("hydroxylapatite eluate") and diluted 2-fold with 20 % glycerol containing 0.2 % Emulgen 913. The diluted solution was then applied to a CM-Sephadex C-50 column (1.6 x 6 cm, for a half of the pooled solution) equilibrated with 75 mM buffer containing 0.2 % Emulgen 913. The column was washed with 100 mM buffer and then with 150 mM buffer both containing 0.2 % Emulgen 913 till a slightly reddish color spread over the column. The cytochrome was then eluted out as a concentrated solution with 200 mM buffer containing 0.2 % Emulgen 913 ("CM-Sephadex eluate") Table I shows a summary of a typical purification experiment. This procedure yielded reproducibly cytochrome P-450 preparations having a specific content

TABLE I. Purification of cytochrome P-450 from liver microsomes of MC-treated rabbits. Cytochrome P-450 and protein were determined by the method of Omura and Sato (15) and the Lowry method (19), respectively.

Fraction	Protein	Cytochrome P-450		
		Total content	Specific content	Recovery
	(mg)	(nmole)	(nmole/mg)	(%)
Microsomes	1,920	7,210	3.75	100
Solubilized supernatant	1,510	6,690	4.42	93
Aminooctyl column eluate	167	2,130	12.8	30
Hydroxylapatite eluate	57.5	1,010	17.6	14
CM-Sephadex eluate	35.0	620	17.7	9

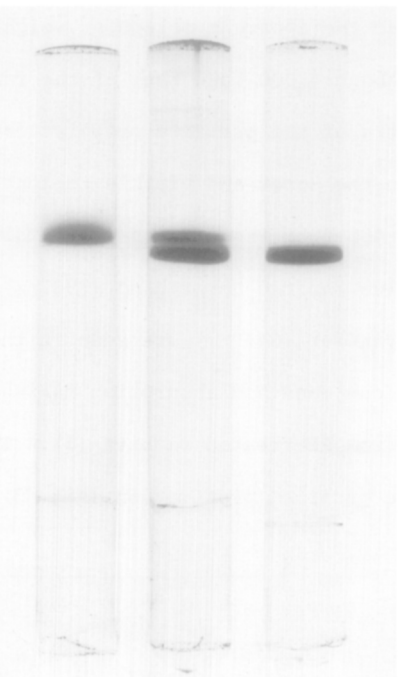

FIGURE 1. Sodium dodecylsulfate-urea-polyacrylamide gel electrophoresis of purified cytochrome P-450's. About 20 µg of each protein was subjected to electrophoresis (7.5 % gel) as described by Dunker and Rueckert (9). The gel was stained with Coomassie blue. Left: Cytochrome P-450 from MC-treated rabbits. Right: Cytochrome P-450 from PB-treated rabbits. Middle: Mixture of both cytochromes.

of 17-18 nmoles per mg of protein with an overall yield of about 10 %. The Emulgen in the preparation could be removed by washing thoroughly the purified cytochrome on a CM-Sephadex column with 100 mM buffer, followed by elution with 300 mM buffer.

Properties of Purified Cytochrome P-450. When the purified preparation was subjected to polyacrylamide gel electrophoresis in the presence of 1 % sodium dodecylsulfate, 2 M urea, and 0.1 % 2-mercaptoethanol by the method of Dunker and Rueckert (9), only a single protein band was observed (Fig. 1). By comparing its electrophoretic mobility with those of marker proteins (bovine serum albumin, yeast alcohol dehydrogenase, pig heart lactate dehydrogenase, trypsin, and cytochrome c), its apparent molecular weight was estimated to be about 54,000. Electrophoresis of a mixture of this protein and cytochrome P-450 purified from PB-treated rabbits (5) resulted in a clear separation of the two protein bands and the apparent molecular weight of the latter was found to be smaller by about 4,000 than that of the former.

The absorption spectra of the purified preparation after removal of Emulgen 913 (Fig. 2) resemble in the Soret and visible regions those of the substrate complex of cytochrome P-450 from Pseudomonas putida (10) or adrenocortical mitochondria (11). A blue-shifted Soret band (393 nm) and a peak attributable to a high-spin charge transfer (644 nm) are seen in the oxidized form. The reduced form and its CO compound had absorption maxima at wavelengths shorter by a few nm than those from PB-treated rabbits (5). The magnetic circular dichroism spectra of the purified preparation (Fig. 3) are again similar to

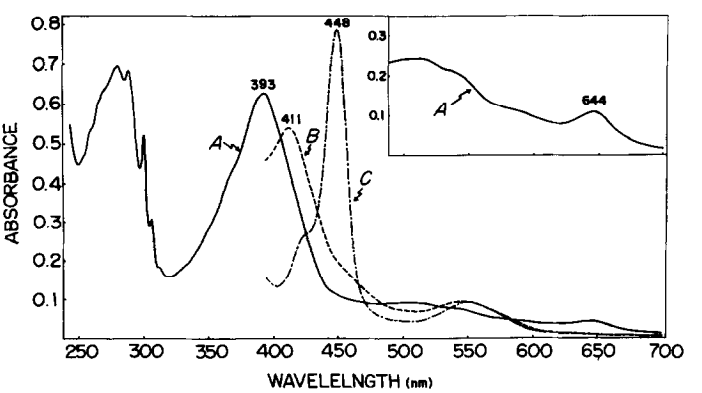

FIGURE 2. Absorption spectra of cytochrome P-450 from MC-treated rabbits (after removal of Emulgen 913) in 300 mM potassium phosphate buffer, pH 7.25, containing 20 % glycerol. Curve A: Oxidized form. (Cytochrome concentration was 2.5 fold for the insert.) Curve B: Dithionite-reduced form. Curve C: CO compound of the reduced form.

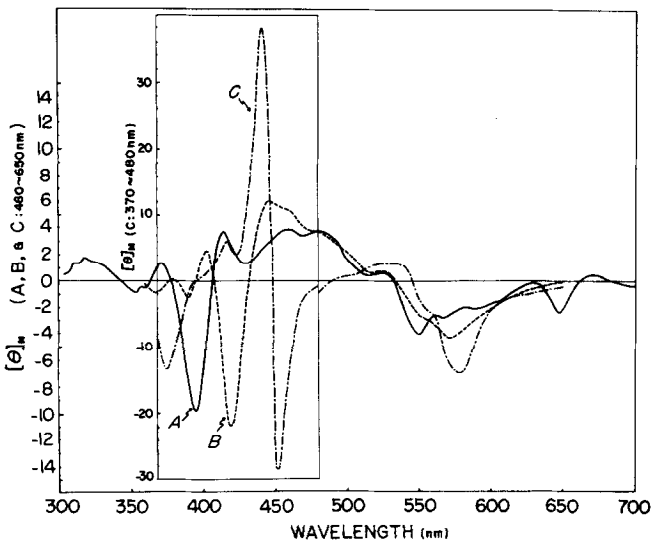

FIGURE 3. Magnetic circular dichroism spectra of purified cytochrome P-450 in 200 mM potassium phosphate buffer, pH 7.25, containing 20 % glycerol and 0.2 % Emulgen 913. The spectra were measured at 0° in collaboration with Mr. Toru Shimizu of Tohoku University. Curve A: Oxidized form. Curve B: Dithionite-reduced form. Curve C: CO compound of the reduced form.

those of the camphor complex of P. putida cytochrome P-450, the oxidized form of which is in the high-spin state (12). The spectrum for the oxidized form differs clearly from that reported for purified cytochrome P-450 from PB-treated rabbits, which is essentially in the low-spin state (13).

The absorption spectrum of the purified cytochrome (Fig. 2) is unique in the ultraviolet region in that it has sharp peaks superimposed on the absorption due to aromatic amino acid residues, suggesting the presence of MC or its metabolites in the preparation. Furthermore, the n-heptane extract of the preparation showed an absorption spectrum identical with that of MC in n-heptane. Quantitative spectral analysis indicated that the molar ratio of MC and cytochrome P-450 was about 1:1. It was concluded that the purified cytochrome was in the form of a MC complex and that its oxidized form was in the high-spin state.

Dilution of the detergent-free preparation of the oxidized cytochrome with a large amount of 20 % glycerol containing 0.2 % Emulgen 913 caused the lower-

Table II. Reconstitution of benzo[α]pyrene hydroxylase activity. The complete system (1.0 ml) contained 0.1 M potassium phosphate buffer, pH 7.25, 0.33 nmole of purified cytochrome P-450, 0.2 unit of purified NADPH-cytochrome c reductase (detergent-solubilized), 0.005 % Emulgen 913, 0.4 mM NADPH, 3 mM $MgCl_2$, and 80 μM benzo[α]pyrene (in 40 μl of acetone). The reaction was run at 37° for 5 min and hydroxylated products were determined as described by Lu et al. (18,20).

	Complete	- P-450	- Reductase	-NADPH
Hydroxylated products (pmole/nmole of P-450/min)	14.3	0	0.4	0.6

ing of the Soret peak at 393 nm and the appearance of a peak around 416 nm, indicating the partial conversion to a low-spin form. This may be due to partial release of MC from the substrate-binding site to produce the substrate-free form or a form in which MC is replaced by Emulgen 913. Ryan et al. (14) have separated two forms of cytochrome P-450 from MC-treated rat liver microsomes; one has Soret absorption maxima at 414 and 395 nm together with a high-spin charge transfer band at 642 nm, while the other has only one Soret peak at 417 nm (in the oxidized form). Since addition of MC to the latter form converted its spectrum to the former type, they have suggested that the high-spin form is a MC complex of the cytochrome.

After removal of the detergent, the purified preparation was rather stable in the presence of glycerol; the CO compound hardly underwent conversion to the P-420 form at room temperature at least for 1 h. This may be due to the stabilizing effect of bound MC.

The purified preparation catalyzed NADPH-dependent hydroxylation of benzo-[α]pyrene when mixed with a purified preparation of detergent-solubilized NADPH-cytochrome c reductase (16) and Emulgen 913 instead of phosphatidylcholine (17). As shown in Table II, the cytochrome, the reductase, and NADPH were obligatorily required for the reconstitution of the hydroxylase activity. The reconstituted activity was about same that of the starting microsomes on the basis of cytochrome P-450 content. This activity may be further improved by refinement of the reconstitution conditions. The reconstituted system was,

however, rather inactive in demethylation of benzphetamine; the turnover number based on the cytochrome P-450 content was about one tenth that the corresponding system containing cytochrome P-450 from PB-treated rabbits. These substrate specificities of the two cytochromes are consistent with those reported previously (3,18).

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