A GEL-ELECTROPHORETICALLY HOMOGENEOUS PREPARATION OF CYTOCHROME P-450 FROM LIVER MICROSOMES OF PHENOBARBITAL-PRETREATED RABBITS

Yoshio Imai and Ryo Sato
Institute for Protein Research, Osaka University,
Suita, Osaka 565, Japan

Received July 12,1974

Summary: Cytochrome P-450 was purified from liver microsomes of phenobarbital-pretreated rabbits to a specific content of 16 to 17 nmoles per mg of protein with a yield of about 10 %. The purified cytochrome yielded only a single protein band on sodium dodecylsulfate-urea-polyacrylamide gel electrophoresis, and an apparent molecular weight of about 45,000 was estimated for the protein. The preparation was free of cytochrome b_5, NADH-cytochrome b_5 reductase, and NADPH-cytochrome c reductase activities. Aniline hydroxylase and ethylmorphine N-demethylase activities could be reconstituted upon mixing the purified cytochrome with an NADPH-cytochrome c reductase preparation (purified by a detergent method) and phosphatidyl choline.

Despite extensive efforts in several laboratories, hepatic microsomal cytochrome P-450 has not yet been purified to homogeneity. By using ω-amino-n-octyl Sepharose 4B as an affinity or hydrophobic adsorbent, we have recently developed a simple method permitting reproducible purification of cytochrome P-450 from liver microsomes of PB-pretreated rabbits to specific contents of 8 to 10 nmoles per mg of protein (1). Levin et al. (2) have also reported purification of the cytochrome to specific contents of 9 to 11 nmoles per mg of protein from liver microsomes of rats treated with either PB or 3-methylcholanthrene. These preparations were, however, still considerably heterogeneous. As an extension of our previous work (1), we have now established a procedure for purification of cytochrome P-450 to a gel-electrophoretically homogeneous state from liver microsomes of PB-pretreated rabbits. This communication reports the newly developed method and some properties of the purified cytochrome.

Purification of Cytochrome P-450. Male rabbits, weighing about 2.5 kg, were treated with PB and liver microsomes were prepared from the treated animals as described previously (1). All the subsequent manipulations were conducted at 0-4°. Potassium phosphate buffers, pH 7.25, containing 20 % (v/v) glycerol were employed throughout; they

Abbreviations: PB, phenobarbital; SDS, sodium dodecylsulfate.

will be referred to simply as 100 mM buffer, etc.

Liver microsomes (2.2 g of protein) were suspended (to ~4 mg of protein/ml) in 100 mM buffer containing 1 mM dithiothreitol, 1 mM EDTA, and 0.6 % (w/v) sodium cholate. The suspension was allowed to stand for 30 min and then centrifuged at 77,000 × g for 2 h. The supernatant fraction including the fluffy layer ("solubilized supernatant") was applied to a column (2.7 × 24 cm) of ω-amino-n-octyl Sepharose 4B (prepared as described in ref. 1) which had been equilibrated with the same buffer. After washing the column with 4 times the column volume of the same buffer (but containing 0.5 % cholate), tightly adsorbed cytochrome P-450 was eluted with 100 mM buffer containing 1 mM dithiothreitol, 0.4 % sodium cholate, and 0.08 % Emalgen 913 (a polyoxyethylene nonylphenyl ether, Kao-Atlas Co. Ltd., Tokyo), and fractions with high specific contents of cytochrome P-450 were pooled ("aminooctyl column eluate"). The pooled fractions were diluted 3-fold with 20 % glycerol and adsorbed on a column (3.2 × 9 cm) of hydroxylapatite (for preparation, see ref. 3) equilibrated with 33 mM buffer. After washing the column with 35 mM buffer containing 0.2 % Emalgen 913, elution was conducted stepwise with 80 mM buffer and then with 150 mM buffer both containing 0.2 % Emalgen 913. About 60 % of the cytochrome P-450 adsorbed was eluted with 80 mM buffer, and approximately 25 % with 150 mM buffer; the latter fraction had considerably lower specific contents. The peak fractions of 80 mM buffer eluate were combined ("hydroxylapatite eluate") and diluted 4-fold with 20 % glycerol containing 0.2 % Emalgen 913. The diluted solution was then applied to a CM-Sephadex C-50 column (2.2 × 13 cm) equilibrated with 20 mM buffer containing 0.2 % Emalgen 913. The column was washed with a small amount of the same buffer, and then cytochrome P-450 was eluted by increasing the buffer concentration to 100 mM; the cytochrome was thereby obtained as a highly concentrated solution ("1st CM-Sephadex eluate"). This solution was dialyzed against 50 vol. of 20 mM buffer for 48 h with one change of the outer fluid, and the dialyzed solution was adsorbed on a second CM-Sephadex C-50 column (1.2 × 16 cm) equilibrated with 20 mM buffer. The column was washed with 20 mM buffer until no absorption at 276 nm (due to Emalgen) was detectable in the eluate. Cytochrome P-450 was then eluted with 150 mM buffer ("2nd CM-Sephadex eluate") and used as the purified preparation. Most, if not all, of the Emalgen in the preparation was removed by this final step. Table I shows a summary of a typical purification experiment. This procedure reproducibly yielded cytochrome P-450 preparations

TABLE I. Purification of cytochrome P-450 from liver microsomes of PB-treated rabbits. The procedure is described in text. Cytochrome P-450 and protein were determined by the method of Omura and Sato (5) and the Lowry method (14), respectively.

Fraction	Protein	Cytochrome P-450		
		T.C.*	S.C.**	Recovery
	(mg)	(nmole)	(nmole/mg)	(%)
Microsomes	2,205	6,750	3.06	100
Solubilized supernatant	1,756	6,396	3.64	95
Aminooctyl column eluate	408	2,925	7.17	43
Hydroxylapatite eluate	68.1	1,004	14.7	15
1st CM-Sephadex eluate	39.4	648	16.5	10
2nd CM-Sephadex eluate	36.3	609	16.8	9

* Total content. ** Specific content (on a protein basis).

having specific contents of 16 to 17 nmoles per mg of protein with an overall yield of about 10 %.

Properties of Purified Cytochrome P-450. The purified preparation of cytochrome P-450 (oxidized form) was very stable in the presence of 20 % glycerol and could be stored at least for one month at -70° without any spectral changes. The dithionite-reduced form underwent slow conversion into the P-420 form even in the presence of glycerol, but this conversion was remarkably inhibited by including 0.2 % Emalgen 913 in the medium.

Fig. 1 shows the absorption spectra of purified cytochrome P-450 at the 1st CM-Sephadex eluate step. These spectra are essentially identical with those reported for a partially purified preparation from the same source (1). The fact that only a slight shoulder is seen at 423 nm in the spectrum of the CO compound indicates that the preparation was free of both cytochromes b_5 and P-420, as discussed previously (1). The ultraviolet region of the oxidized spectrum of this preparation was not measurable because of the intense absorption at 276 nm due to Emalgen 913. After removal of the detergent by the 2nd CM-Sephadex chromatography, the 276-nm peak was lowered to 45 % of the Soret peak, as shown in Fig. 2. At the same time, a fine structure became visible between 255 and 270 nm, suggesting a high phenylalanine content in the protein. Furthermore, upon alkaline denaturation, the peak at 276 nm disappeared and was replaced by a peak at

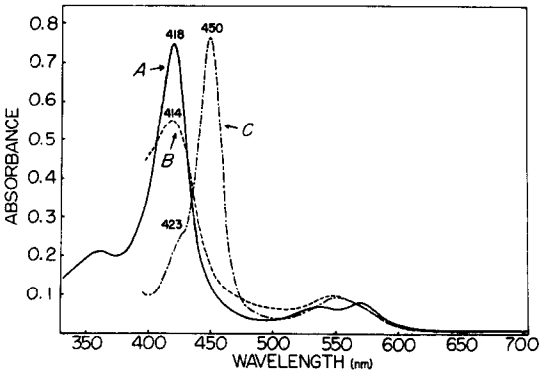

FIGURE 1. Absorption spectra of purified cytochrome P-450 (1st CM-Sephadex eluate) in 100 mM potassium phosphate buffer, pH 7.25, containing 20 % glycerol and 0.2 % Emalgen 913. Curve A: oxidized form. Curve B: dithionite-reduced form. Curve C: CO compound of reduced form.

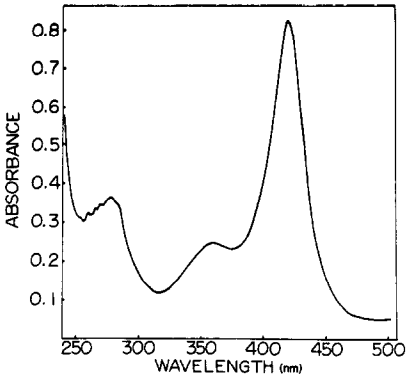

FIGURE 2. Absorption spectrum of the oxidized form of purified cytochrome P-450 after removal of Emalgen 913 (2nd CM-Sephadex eluate) in 150 mM potassium phosphate buffer, pH 7.25, containing 20 % glycerol.

about 290 nm (with a shoulder at about 285 nm). This suggested that the 276-nm peak of the final preparation was due to tyrosine and/or tryptophan residues rather than the Emalgen remaining in the preparation. When 10 mM aniline was added to the oxidized cytochrome, a spectral change was induced which was essentially the same as observed with intact microsomes (4). Based on the protoheme determination by the method of Omura and Sato (5), the millimolar extinction coefficient increment between 450 and 490 nm in the CO difference spectrum was estimated to be 92 in essential agreement with the value of 91 determined by an indirect method (5).

When the purified preparation (2nd CM-Sephadex eluate) was sub-

jected to polyacrylamide gel electrophoresis in the presence of 1 % SDS, 2 M urea, and 0.1 % 2-mercaptoethanol according to the method of Hinman and Philips (6), only a single protein band was observed, as shown in Fig. 3. By comparing its mobility in this electrophoresis

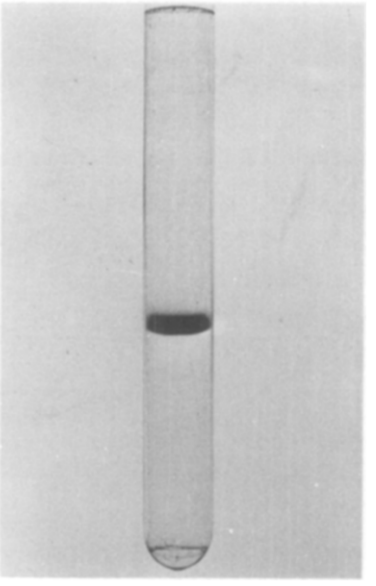

FIGURE 3. SDS-urea-polyacrylamide gel electrophoresis of purified cytochrome P-450 (2nd CM-Sephadex eluate). About 20 μg of protein was treated with SDS, urea, and 2-mercaptoethanol and subjected to electrophoresis on a 5 % gel disc, as described by Hinman and Philips (6). The gel was stained with Coomassie blue.

system with those of marker proteins (bovine serum albumin, yeast alcohol dehydrogenase, pig heart lactate dehydrogenase, detergent-solubilized rabbit liver cytochrome $\underline{b}_5$, and bovine cytochrome $\underline{c}$), an apparent molecular weight of about 45,000 was estimated for the cytochrome. This value was considerably lower than the minimal molecular weight calculated from the specific content of cytochrome P-450 in the purified preparation (about 60,000). Three possibilities may be cited to account for this discrepancy. First, a considerable amount of apocytochrome P-450 was present in the purified preparation. Secondly, the SDS-gel electrophoresis method gave a considerably smaller molecular weight because of the strongly hydrophobic nature of cytochrome P-450 (cf. ref. 7). Finally, the purified cytochrome was still con-

taminated by an impurity or impurities having the same or closely similar molecular weights as the cytochrome. These possibilities are now being examined in this laboratory.

As already reported, cytochrome $\underline{b}_5$ could be completely separated from cytochrome P-450 by chromatography on the aminooctyl Sepharose 4B column (1). In the present study, it was found that NADPH-cytochrome $\underline{c}$ reductase activity (measured as described in ref. 8) was also removed completely in the same chromatography step. On the other hand, NADH-cytochrome $\underline{b}_5$ reductase activity, as assayed by its NADH-ferricyanide reductase activity (9), was separated from the cytochrome in the hydroxylapatite chromatography step. The complete absence of the three activities in the final preparation could also be confirmed.

As shown in Table II, the purified preparation of cytochrome P-450 (2nd CM-Sephadex eluate) could catalyze NADPH-dependent hydroxylation of aniline when mixed with an NADPH-cytochrome $\underline{c}$ reductase preparation and egg-yolk phosphatidyl choline. The reductase preparation employed was solubilized from rabbit liver microsomes with Emalgen 911 and purified to apparent homogeneity (10); it was completely free of cytochrome $\underline{b}_5$ (10). As can be seen, cytochrome P-450, the reductase, and NADPH were obligatorily required for reconstitution of the hydroxylase activity. Omission of phosphatidyl choline from the system resulted in only a slight decrease in the reconstituted activity. This is in contrast to the finding of Lu et al. (11) with partially purified preparations. The reason for this discrepancy is not known. The complete system also catalyzed N-demethylation of ethylmorphine (data not shown). The reconstituted aniline hydroxylase activity was about

TABLE II. Reconstitution of aniline hydroxylase activity. The complete system contained (in a final volume of 1.0 ml) 0.1 M Tris-acetate buffer, pH 7.5, 1.9 nmole of purified cytochrome P-450 (2nd CM-Sephadex eluate), 0.77 unit of purified NADPH-cytochrome $\underline{c}$ reductase (detergent-solubilized)(10), 0.1 mg of egg-yolk phosphatidyl choline, 1 mM NADPH, 1 mM $MgCl_2$, and 10 mM aniline. The mixture was incubated aerobically at 37° for 30 min and p-aminophenol formed was determined as described by Imai et al. (15).

System	Complete	-P-450	-Reductase	-NADPH	-PC*
p-Aminophenol formed (nmole/nmole of P-450/min)	0.28	0.04	0.04	0.04	0.23

one third that of starting liver microsomes on the basis of cytochrome P-450 content. However, it is expected that this activity will be further improved by refinement of the reconstitution conditions. Since the purified preparations of both cytochrome P-450 and the reductase used for the reconstitution were completely devoid of cytochrome b_5, it could be concluded that cytochrome b_5 is not obligatorily required for aniline hydroxylation and ethylmorphine N-demethylation at least in the reconstituted system, in confirmation of the finding of Levin *et al.* (2) with partially purified preparations. However, this does not necessarily exclude the possibility of cytochrome b_5 involvement in the NADH synergism of the monooxygenase system in intact liver microsomes (12,13).

We wish to thank Mr. H. Satake for a gift of a purified preparation of detergent-solubilized NADPH-cytochrome *c* reductase.

REFERENCES

1. Imai, Y., and Sato, R. (1974) J. Biochem. 75, 689-697.
2. Levin, W., Ryan, D., West, S., and Lu, A.Y.H. (1974) J. Biol. Chem. 249, 1747-1754.
3. Levin, Ö. (1962) Methods Enzymol. 5, 27-32.
4. Imai, Y., and Sato, R. (1967) J. Biochem. 62, 239-249.
5. Omura, T., and Sato, R. (1964) J. Biol. Chem. 239, 2370-2378.
6. Hinman, N. D., and Philips, A. H. (1970) Science 170, 1222-1223.
7. Helenius, A., and Simons, K. (1972) J. Biol. Chem. 247, 3656-3661.
8. Phillips, A.H., and Langdon, R. G. (1962) J. Biol. Chem. 237, 2652-2660.
9. Takesue, S., and Omura, T. (1970) J. Biochem. 67, 259-266.
10. Satake, H., Imai, Y., and Sato, R. (1972) Seikagaku 44, 765.
11. Lu, A.Y.H., Jacobson, M., Levin, W., West, S.B., and Kuntzman, R. (1972) Arch. Biochem. Biophys. 153, 294-297.
12. Hildebrandt, A., and Estabrook, R. W. (1971) Arch. Biochem. Biophys. 143, 66-79.
13. Mannering, G.J., Kuwahara, S., and Omura, T. (1974) Biochem. Biophys. Res. Commun. 57, 476-481.
14. Lowry, O.H., Rosenbrough, N. J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
15. Imai, Y., Ito, A., and Sato, R. (1966) J. Biochem. 60, 417-428.