RECONSTITUTION OF THE LIVER MICROSOMAL HYDROXYLASE SYSTEM INTO LIPOSOMES

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Received 23 March 1977

1. Introduction

The liver microsomal hydroxylase system participates in the metabolism of endogenous compounds such as steroids and fatty acids as well as in the detoxification of a variety of xenobiotics. Solubilisation and reconstitution of the hydroxylase system have been performed in several laboratories [1–5]. The technique for reconstitution has generally involved mixing of purified preparations of cytochrome *P*-450 and NADPH–cytochrome *P*-450 reductase plus phospholipid with substrate and NADPH [1–5]. The native environment of the hydroxylase system, however, is the lipid bilayer of the endoplasmic reticulum [6]. Complete reconstitution of the system will, therefore, not be obtained until the participating proteins have been incorporated into a membrane structure.

In the present paper a simple method is described for efficient incorporation of highly purified preparations of phenobarbital-induced cytochrome *P*-450 (*P*-450LM$_2$ [1]) and NADPH–cytochrome *P*-450 reductase into vesicles of phosphatidylcholine. The properties of the hydroxylase system in the vesicles are compared with those of preparations where the components of the system have simply been mixed together.

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2. Materials and methods

Egg yolk phosphatidylcholine, type III E, was obtained from Sigma and stored in batches of 20 mg in chloroform solution under nitrogen in sealed tubes at −20°C. Sodium cholate was obtained from Merck. [U-^{14}C]Phosphatidylcholine and sodium [2,4-^{3}H]-cholate were obtained from New England Nuclear. Rabbit liver cytochrome *P*-450 and NADPH–cytochrome *P*-450 reductase were prepared from phenobarbital-induced microsomes according to the method of Coon et al. [1]. The *P*-450 preparations (*P*-450LM$_2$ [1]) were homogeneous with SDS–polyacrylamide gel electrophoresis as criterium [7], had a specific content of *P*-450 of 10–15 nmol *P*-450/mg protein and contained less than 0.1 mg detergent/mg protein. NADPH–Cytochrome *P*-450 reductase had a specific activity of 1000 U/ mg protein. All spectrophotometric measurements were performed with a Cary model 118 spectrophotometer. *P*-450 was measured according to Omura and Sato [8]; protein according to Lowry et al. [9]; NADPH–cytochrome *P*-450 reductase as described by Masters et al. [10] and the activity expressed in units as defined by Lu et al. [11].

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2.1. *Incorporation of the liver microsomal hydroxylase system into liposomes*

Egg yolk phosphatidylcholine, 20 mg in chloroform, was taken to absolute dryness under a stream of nitrogen. The residue was suspended in 1 ml of 50 mM potassium phosphate buffer, pH 7.4, and the tube was shaken until all lipids had been dispersed. Sodium cholate was added at a concentration of 0.5% (w/w). When the solution was completely clear, 10 nmol *P*-450LM$_2$ and 1500 U NADPH-cytochrome *P*-450 reductase were added. The volume was adjusted to 3 ml with 50 mM phosphate buffer and the sample applied on a Sephadex G-50 column (2.2 × 60 cm) previously equilibrated with 10 mM Tris–HCl, pH 7.4, containing 0.1 M NaCl, 0.2 mM EDTA and 10^{-4} M DTE. The column was eluted with the same buffer and the void volume collected.

The liposomes obtained in the void volume were characterized by electron microscopical analysis of negatively stained specimens, chromatography on Sepharose 4 B, sucrose density-gradient centrifugation, and catalytic activity with regard to hydroxylation of androstenedione. The results obtained with the liposomes were compared with those obtained with non-vesicular preparations of the same amounts of *P*-450, *P*-450 reductase and phosphatidylcholine. These preparations were formed by incubating the three components in 2 ml 50 mM phosphate buffer, pH 7.4, at 37°C for 5 min followed by sonication in a water bath for 1 min.

2.2. *Electron microscopy*

For negative staining, the sample was diluted 1:1, 1:5 and 1:10. One droplet was placed on a carbon-coated copper grid and placed in a refrigerator for 30 min to obtain proper particle distribution on the grid. The droplet was touched with a torn edge of a filter paper, most of the liquid was removed and a droplet of 1% (w/v) phosphotungstic acid (neutralized with KOH) was immediately applied. After about 15 s the stain was removed with filter paper. After drying, the material was analyzed in a Jeol 100B electron microscope.

3. Results

As evident from fig.1 chromatography on Sephadex G-50 of the cholate-treated solution of phosphatidylcholine, *P*-450 and *P*-450 reductase resulted in complete separation of cholate from the void volume containing all protein and radioactive phosphatidylcholine. The void volume and the non-vesicular preparation (prepared as described in Materials and methods) were chromatographed on separate Sepharose 4 B columns. As shown in fig.2, all phosphatidylcholine and protein were excluded from the Sepharose column in the case of the void volume from the Sephadex G-50 column. However, when the non-vesicular preparation was chromatographed, much of the protein was retarded on the column and separated from the lipid. Principally the same results were obtained when the two different types of preparations were ultracentrifuged in linear 5–20% sucrose-gradients for 7 h at 150 000 × *g* (cf. fig.3). With the Sephadex G-50 preparation, protein and lipid sedimented as a homogeneous zone (fig.3A). In the case of the non-vesicular preparation the lipid stayed at the top while the protein entered the gradient (fig.3B).

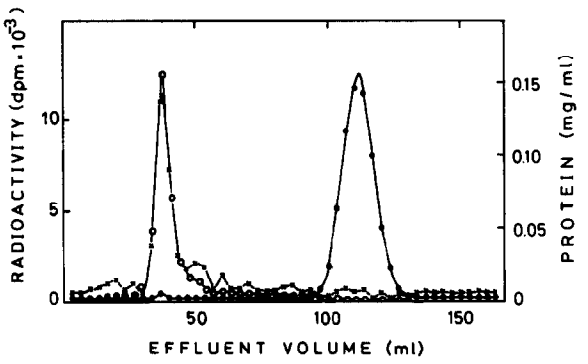

Fig.1. Chromatography of cholate-treated solution of 10 nmol *P*-450LM$_2$, 1500 U *P*-450 reductase and 20 mg phosphatidylcholine on Sephadex G-50. (-x-x-) Protein, (-●-●-) [^{3}H]cholate, (-o-o-) [^{14}C]phosphatidylcholine.

Electron microscopy of the void volume form the G-50 column revealed that protein and lipid existed in vesicular profiles with an average diameter of the monolamellar vesicles of about 600 Å (fig.4).

In conclusion, the results presented strongly indicate that all *P*-450 and *P*-450 reductase were incorporated into the phosphatidylcholine vesicles but that these proteins were not tightly associated with phosphatidylcholine in the non-vesicular preparations.

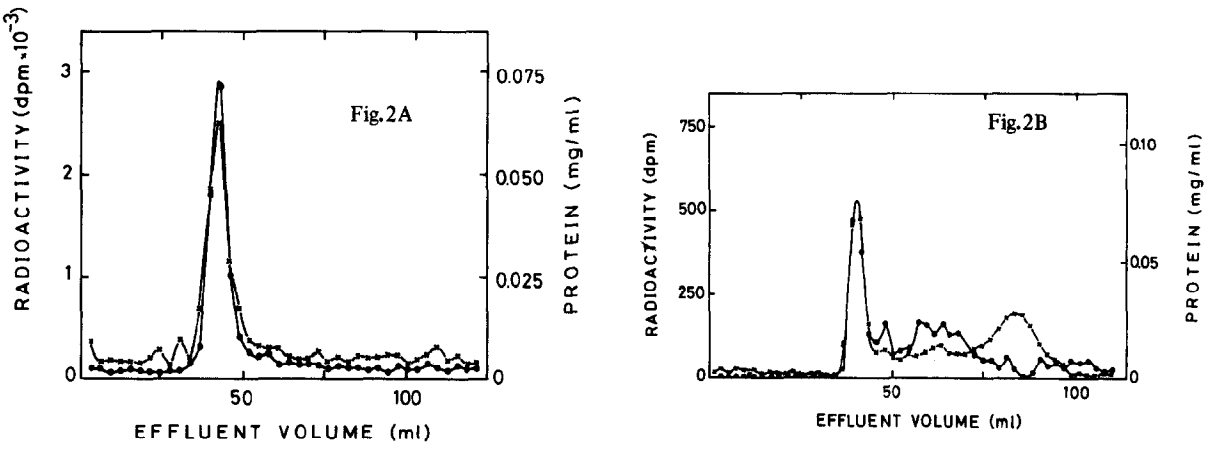

Fig.2. Chromatography on Sepharose 4-B columns (1.4 × 50 cm) of the void volume obtained from the Sephadex G-50 column (A) and the non-vesicular preparation prepared as described in Materials and methods section (B). The chromatographies were performed in 50 mM phosphate buffer, pH 7.4, containing 10^{-4} M DTE at a flow rate of 5 ml/h. (-x-x-) Protein, (-●-●-) [^{14}C]-phosphatidylcholine.

The recoveries of *P*-450LM$_2$ and *P*-450 reductase in the liposomes were 65–75% and 80–90%, respectively. The present method for preparing *P*-450-containing liposomes gives better yields when compared to other techniques tested involving sonication (resulting in denaturation of *P*-450) [12] or incubation of the proteins with phosphatidylcholine containing liposomes (resulting in no incorporation of *P*-450) [13] (data not shown). Dialysis of *P*-450, *P*-450 reductase and phosphatidylcholine vesicles after treatment with cholate [14] resulted in formation of oligolamellar vesicles (not shown in figure).

NADPH-Supported 6β-hydroxylation of androstenedione was assayed in the liposomal and in the

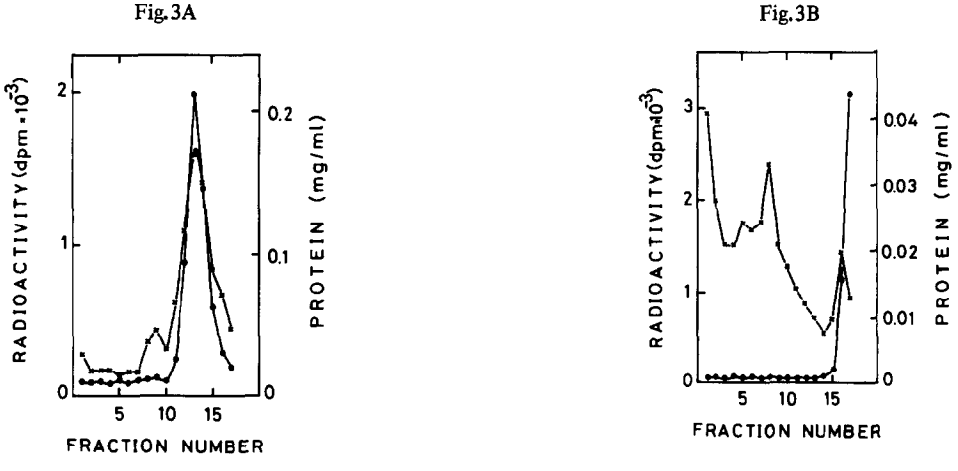

Fig.3. Sucrose-gradient centrifugation on linear (5–20% sucrose) 5 ml gradients prepared in 50 mM phosphate buffer, pH 7.4, of the void volume obtained from the Sephadex G-50 column (A) and the non-vesicular preparation (B). The samples in 200 μl buffer were layered on top of the gradient. Centrifugation was performed for 7 h at 150 000 × *g*. Fractions of 0.3 ml were collected from the bottom. (-x-x-) Protein, (-●-●-) [^{14}C]phosphatidylcholine.

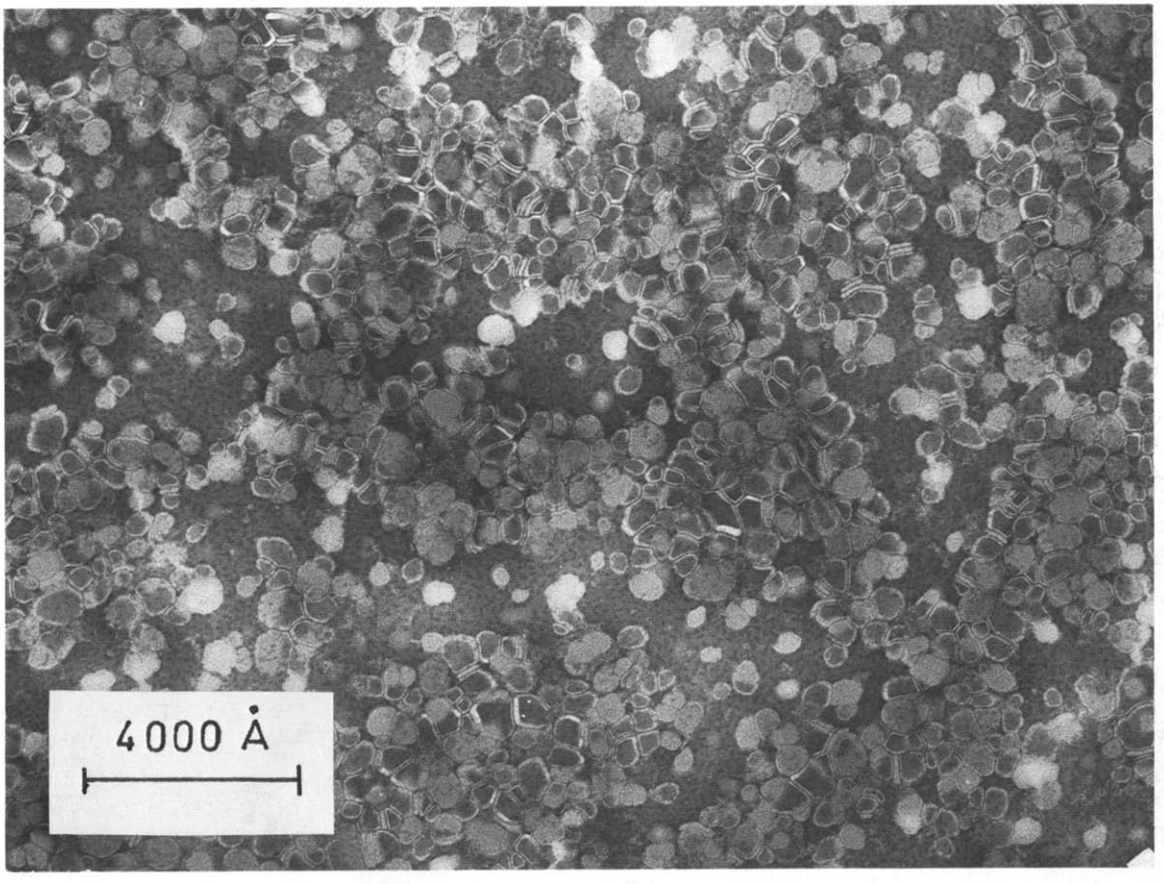

Fig.4. Electron micrograph of the liposomal preparation obtained in the void volume from the Sephadex G-50 chromatography. The liposomes were stained with 1% phosphotungstic acid. The fraction is composed of homogeneous vesicular particles with a mean diameter of 600 A delimited by a single membrane. A Joel 100B electron microscope was used at × 70 000.

Table 1
Apparent V_{max} of 6β-hydroxylation of androstenedione in the liposomal and non-vesicular preparations

Preparations	Apparent V_{max} (nmol product/nmol *P*-450/min)
Liposomes	1.3
Non-vesicular preparations	0.16

The incubation mixtures contained 1 nmol of *P*-450LM$_2$, 160 U *P*-450 reductase, 2 mg phosphatidylcholine and 0.5 mg NADPH in volume 2 ml 50 mM phosphate buffer, pH 7.4. In the incubations were started by addition of substrate in 25 μl of acetone and were performed for 20 min at 37°C. The incubations were analyzed as described elsewhere [15]. The apparent V_{max}-values were obtained by Lineweaver-Burk plots of results obtained at five different substrate concentrations.

non-vesicular preparations. The incubations were performed simultaneously with the same preparations of *P*-450LM$_2$ and *P*-450 reductase. As shown in table 1, the apparent V_{max} for 6β-hydroxylation was nearly ten-fold higher when the liposomal preparation was used instead of the non-vesicular one.

4. Discussion

In the present paper a simple method is described for introducing highly purified components of the liver microsomal hydroxylase system into monolamellar vesicles of phosphatidylcholine. Nearly complete incorporations of cytochrome *P*-450 and NADPH–cytochrome *P*-450 reductase were obtained. The catalytic activity, measured as 6β-hydroxylation of androstenedione, was about 10-times higher in the vesicles than in non-vesicular preparations with the same components. The reasons for this difference may be conformational changes of the enzymes after being introduced into the lipophilic milieu in the membrane or simply that interactions between *P*-450 and *P*-450 reductase are facilitated when these enzymes are incorporated into the membrane. Since reconstitution of the liver microsomal hydroxylase system hitherto has been performed by using non-vesicular preparations [1–5] it should be pointed out that optimal activity and perhaps also the physiological specificity of the system will not be obtained unless the components are embedded in a native or artificial membrane structure.

Acknowledgements

The skilful technical assistance of Miss Inger Johansson and Mr Kjell Jansson is gratefully acknowledged. This work was supported by grants from Magnus Bergvalls Stiftelse, Harald Jeanssons Stiftelse, Harald och Greta Jeanssons Stiftelse and from the Swedish Medical Research Council (Nos 2819 and 3492).

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