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INCORPORATION OF THE CYTOCHROME P-450 MONOOXYGENASE SYSTEM INTO LARGE UNILAMELLAR LIPOSOMES USING OCTYLGLUCOSIDE, ESPECIALLY FOR MEASUREMENTS OF PROTEIN DIFFUSION IN MEMBRANES

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Cytochrome P-450 and NADPH cytochrome P-450 reductase were incorporated into large unilamellar lipid vesicles (200-300 nm in diameter) removing octylglucoside from mixed micelles by dialysis. The large size of the protein-containing liposomes guarantees a negligibly small vesicle tumbling. Such large vesicles are better suited for studies of protein rotation in reconstituted membranes than vesicles prepared by use of bile salts. At present the octylglucoside reconstituted monooxygenase system seems to be the most appropriate model for studying protein-protein and protein-lipid interactions in liver microsomes due to the similarity with respect to the main structural and functional properties, including size.

Recently different techniques have been developed for measuring rotational mobility of proteins in membranes and in this way to monitor interactions with other proteins and membrane components. In this way protein dynamics can be analysed and informations about the structural organization of membrane constituents can be obtained (for a review see (1)). A reliable determination of the

Abbreviations: P-450 LM2, cytochrome P-450 isozyme induced in rabbit liver by phenobarbital (EC 1.14.14.1.); reductase, NADPH cytochrome P-450 reductase (EC 1.6.4.2.); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; octylglucoside, n-octyl-β-D-glucopyranoside; ML, microsomal lipid extract; τ_R, rotational correlation time.

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protein mobility in reconstituted lipid vesicles requires suppression of vesicle rotation. Several attempts have been made to solve the problem enhancing the local viscosity by addition of chemicals (2-5) or pelleting and aggregating the vesicles (6,7). The more appropriate procedure to circumvent undue perturbation of the system is to incorporate membrane proteins into lipid vesicles which are large enough in size so that their tumbling can not contribute to the observed diffusion rate.

From recent reconstitution experiments of biological membranes and pure lipid vesicles it became evident that the high rate of removal of octylglucoside yields much larger liposomes as compared to bile salts (8-12). On the other hand, only glycophorin, a somewhat atypical integral membrane protein, has been incorporated into well defined lipid bilayers using octylglucoside by Mimms et al. as yet (11).

In this paper we report the successful incorporation of typical membrane proteins - cytochrome P-450 and reductase - into large unilamellar phospholipid vesicles by removal of octylglucoside from mixed micelles of lipid, protein and the detergent. The proteo-liposomes formed have been thoroughly characterized with respect to structural and functional properties using physical and biochemical methods.

MATERIALS AND METHODS

P-450LM2 was obtained from liver microsomes of phenobarbital pretreated male rabbits according to Haugen and Coon (13) to a purity of about 14-18 nmol/mg of protein. The preparation was homogeneous on SDS slab gels. The concentration of P-450 was calculated from CO-difference spectra according to Omura and Sato (14). Reductase was purified from the same livers to an electrophoretically homogeneous fraction according to French and Coon (15) with a specific activity towards cytochrome c of about 39 units/mg of protein (16). Protein concentrations were estimated by the Lowry method using bovine serum as standard (17). Protein preparations were concentrated and dialyzed against 0.1 mol/l phosphate buffer, pH 7.4, with 20 % glycerol (v/v) (standard buffer) and then stored in liquid nitrogen until used.

Egg PC and egg PE were prepared as described by Singleton (18) and egg PA as described in (19). [^{14}C]PC was obtained by methy-

lation of PE (20) and [^{3}H]PE was made from partially tritiated egg PC by transphosphatidylation (19). The microsomal lipid extract was isolated according to (21). Octylglucoside was from Calbiochem (USA) and [^{14}C]octylglucoside (11.6 GBq/mmol) from New England Nuclear (USA). All lipid material was checked for purity by TLC. Other reagents were of analytical grade.

Preparation of liposomes. Reconstitution of the purified enzymes into lipid vesicles was achieved by a modification of the cholate dialysis method by Bösterling et al. (22). Different from (22) the detergent was added in solid form and the final volume was 2 ml. The mixed micelle system was dialyzed against 7 changes of standard buffer for 48 h at 4 °C followed by a gel filtration step on a Sepharose 4B column at 4 °C to separate non-incorporated protein and to suppress the amount of residual detergent.

After reconstitution nearly all lipid and 75-90 % of the proteins were recovered. No degradation of P-450 to cytochrome P-420 was found and the vesicles were stable at least three weeks at 4 °C.

Characterization of liposomes. Analysis of the reconstituted vesicles was carried out by Sepharose 4B gel chromatography. Fractions were analysed for their P-450 content with absorbance at 416 nm, reductase activity as described in (16), and radioactivity of [^{14}C]PC and [^{3}H]PE, respectively, by liquid scintillation counting.

For electron microscopy samples were jet-frozen from room temperature according to (23) with a Balzers Cryo-Jet QFD 101. Freeze-fracturing was performed in a Balzers BAF 400 D apparatus and the Pt/C replicas were examined in a JEOL JEM 100 B electron microscope.

Dynamic light scattering measurements were performed at 20 °C and at a scattering angle of 90 °C using a He-Ne Laser (λ = 633 nm). Mean hydrodynamic radii and homogeneity parameters of the vesicle preparations were determined as described in (24).

Monooxygenatic activity was checked by determination of the rate of benzphetamine N-demethylation by measuring formaldehyde formation according to Nash (25).

RESULTS AND DISCUSSION

Fig. 1 shows the elution profile of the monooxygenase system reconstituted into ML vesicles at a lipid/protein ratio of 5 (w/w). At a molar detergent/lipid ratio of 10 almost all lipid, 75 % of P-450, and 82 % of reductase coelute in a relatively single narrow peak at the void volume (V_o). The rest elutes approximately at the same position as P-450 and reductase in aqueous solution without lipid and detergent (P and R in Fig.1). Applying higher lipid/protein ratios a yet higher percentage of the proteins can be reconstituted.

Using the first peak fraction the N-demethylation of benzphetamine was determined with an average turnover rate of about (13 ± 3) nmol CH_2O/min/nmol of P-450 at a lipid/protein ratio of

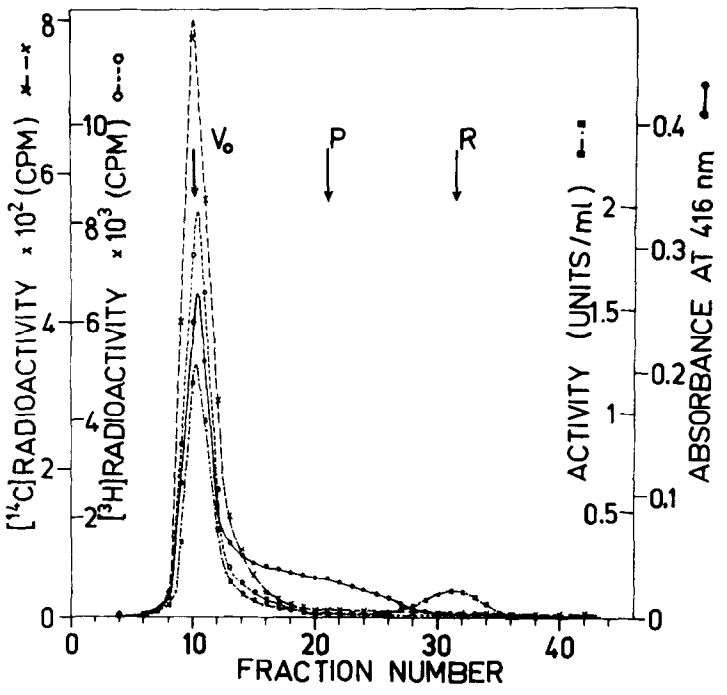

Fig. 1 Elution profile in the reconstitution of P-450LM2 and reductase into ML vesicles on Sepharose 4B. Vesicle preparation: Lipid:3.3 mg/ml; lipid/protein = 5:1 (w/w); P-450/reductase = 5:1 (mol/mol); octylglucoside/lipid = 10:1 (mol/mol). Fractions were analysed for P-450 content (●——●), reductase activity (■-.-.-■), and for ⟨^{14}C⟩PC (x- - -x) and ⟨^{3}H⟩PE radioactivity (o- - -o) as described under "Materials and Methods".

5 (w/w). Enzymatic activity and the coelution of lipids and proteins suggest that both proteins are present in the same vesicle.

An optimal molar detergent/lipid ratio for reconstitution of vesicles with a lipid/protein ratio of 5 (w/w) amounts up to about 10 which is at least 2-fold higher than the concentration of cholate in reconstitution experiments (22,2). For a detergent/lipid ratio < 7.5 the vesicles were smaller than 300 nm (see Table 1) and a significantly lower portion of P-450 and lipids (only 40-60 %) was found in vesicular form, whereas for a detergent/lipid ratio > 15 the vesicles are slightly smaller and the amount of active P-450 is reduced (recovery < 70 %). The amount of P-450 incorporated into PC/PE/PA vesicles at a lipid

Table 1

Properties of octylglucoside reconstituted pure lipid vesicles and proteo-liposomes

Vesicle system (Lipid:Protein) a)	Lipid conc. (mg/ml)	D/L f)	$D_T \times 10^8$ (cm^2/s) b), c)	Lipos. diameter (nm) b)	Q value d)	τ_R (μs) e)
PC/PE/PA	10	10	0,75	304	0.25	7000
PC/PE/PA:P-450,R	10	10	0.75	306	0.22	7000
PC/PE/PA:P-450,R	10	5	1.09	218	0.19	2500
PC/PE/PA:P-450,R	3.3	10	1.61	143	0.11	580
PC/PE/PA:P-450,R	2.5	10	1.78	129	0.08	580
ML	5	10	1.45	158	0.13	1000
ML:P-450,R	5	10	1.43	161	0.13	1000
PC	10	10	1.11	206	0.22	2000
PC:P-450	2.5	10	2.00	115	0.16	380
PC/PE/PA:P-450,R (cholate reconstituted) g)	7.5 - 10	4 -5	-	60	-	52

a) Protein content: 5-20 % (w/w); vesicle size was found nearly independent on protein concentration.
b) Relative experimental error about 3 %.
c) Mean translational diffusion coefficient at 20 °C in standard buffer.
d) Q is a measure for the polydispersity of the vesicle preparation and thus for the homogeneity (for definition see (24, 12)).
e) Approximate Rotational correlation times τ_R were calculated based on the Stokes equation $\tau_R = 4\pi \cdot \eta R^3/(3kT)$, with η = 1.87 mPa·s at 20 °C (corresponding to the viscosity of the standard buffer).
f) Molares detergent/lipid ratio.
g) Average data from ref. (22,2).

ratio of 2:1:0.02 was found smaller (50-70 %) than the incorporation into ML vesicles (75 %), whereas lipid and reductase incorporation were of comparable degree.

Examination of the vesicle fraction by freeze-fracturing electron microscopy shows the presence of predominantly large, smooth and round proteo-liposomes. The typical smooth fracture faces represented in Fig. 2 represent unilamellar vesicles because this was supported by rare cross fractures through their internal volume.

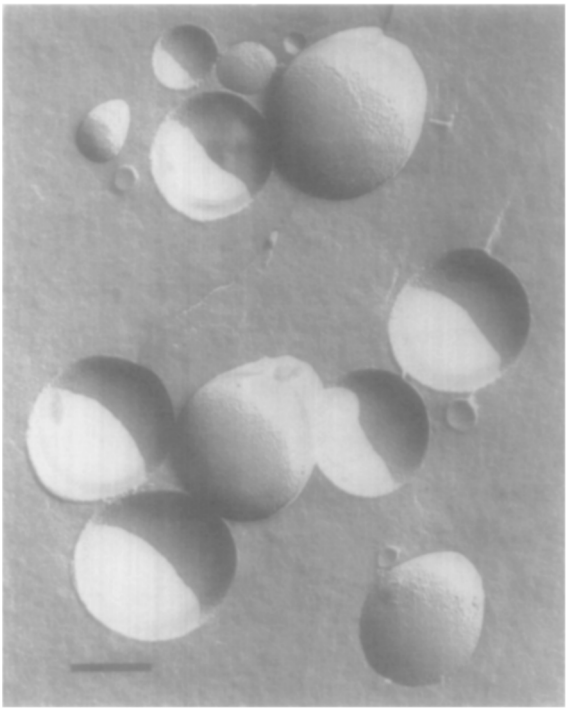

Fig. 2 Freeze-fracture electron micrograph of large unilamellar PC/PE/PA vesicles containing P-450LM2. Lipid/protein = 30:1 (w/w), other values as in Fig. 1. Bar = 200 nm.

Average diameters of the vesicles determined by dynamic light scattering of numerous preparations under different experimental conditions are summarized in Table 1. Diameters around 300 nm for pure and protein-containing PC/PE/PA vesicles are characteristic of preparations with 10 mg/ml of lipid. This large liposomal size results in a more than 130 times slower rotational diffusion rate of the vesicles compared with that of cholate vesicles, which are usually five times smaller in diameter (see τ_R values in Table 1).

Octylglucoside is removed during the reconstitution process as determined by use of [^{14}C]octylglucoside. The kinetics of detergent removal and the amount of residual octylglucoside was found to be similar for pure and protein-containing vesicles as well. Residual detergent after dialysis was about 0.05 % of what

was added initially. After gel filtration on Sepharose 4B the amount could be further diminished to 0.035 % in the vesicular preparation corresponding to about 1 detergent molecule/280 lipid molecules.

The described reconstitution procedure was applied to the incorporation of P-450LM2 and reductase into large unilamellar vesicles from (i) a microsomal lipid extract, (ii) a mixture of PC/PE/PA (2:1:0.02), and (iii) PC alone, up to lipid concentrations of at least 30 mg/ml whereby the size of the liposomes further increased. The proteo-liposomes obtained in this way are better suited for studies of rotational diffusion of proteins in membranes by optical and EPR methods because no additional alterations of the sample conditions are required to cancel vesicle rotation. It can be expected that reconstitution by means of octylglucoside dialysis allows the incorporation of other typical membrane proteins, too. Of course, octylglucoside reconstituted vesicles exhibit all other advantages reported already by other laboratories, especially that octylglucoside can be removed more quickly than other detergents (8-12).

According to the criteria discussed by Bösterling et al. (22) for the preparation of a reconstituted vesicle system with microsome-like properties one can state that the octylglucoside reconstituted vesicles have a lower amount of residual detergent and are very similar to liver microsomes. This holds true not only with regard to bilayer structure, lipid composition, negative surface charge, high protein content, and enzymatic activity, but additionally in size which is similar compared with microsomes (about 300 nm in diameter). The size of the vesicles results in vesicle tumbling which is at least one order of magnitude slower than usual rotational diffusion rates of membrane proteins and thus can be neglected. This system, therefore, seems to be at

present the most appropriate model for studying protein-protein and protein-lipid interaction in the endoplasmic reticulum.

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