Cytochrome *P*-450 facilitates phosphatidylcholine flip-flop in proteoliposomes

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1. INTRODUCTION

Transmembrane movement or flip-flop of phospholipids is an intriguing problem in structural dynamics studies of biological membranes. The following questions still remain unanswered: how can phospholipid molecules cross the lipid bilayer, what factors control the rate and specificity of transbilayer lipid migration and what is the role of membrane proteins in this process.

In order to test the hypothesis that membrane proteins could be responsible for the extremely rapid phospholipid flip-flop in rat liver microsomes we have studied reconstituted proteoliposomes containing two intrinsic microsomal proteins, cytochrome *P*-450 and cytochrome b_5. Phosphatidylcholine exchange protein from beef liver and bee venom phospholipase A_2 were used as membrane probes to measure the rate of flip-flop of [^{14}C]phosphatidylcholine in proteoliposomes.

2. MATERIALS AND METHODS

Phosphatidylcholine exchange protein was isolated from beef liver according to Kamp et al. [1]. Bee venom phospholipase A_2 was purified as described [2]. Cytochromes *P*-450 and b_5 were isolated from rabbit liver [3]. Proteoliposomes were prepared by cholate dialysis technique [4] from total lipid extract of rat liver microsomes containing phosphatidyl[*N-methyl*-^{14}C]choline. Protein-stimulated [^{14}C]phosphatidylcholine exchange was carried out at 37°C in 100 mM Tris–HCl buffer (pH 7.6) containing 1 mM EDTA; non-sonicated phosphatidylcholine liposomes (fraction sedimented at 9000 × *g*, 10 min) were used as acceptor membranes. Phospholipase A_2 hydrolysis was performed in the same buffer with addition of 6 mM $CaCl_2$.

3. RESULTS AND DISCUSSION

When proteoliposomes containing cytochrome *P*-450 were incubated with large excess of acceptor liposomes at high concentrations of the exchange protein, extensive exchange of [^{14}C]phosphatidylcholine was observed. Repetitive incubations of the cytochrome *P*-450–proteoliposomes with fresh portions of acceptor liposomes and the exchange protein resulted in practically complete removal of [^{14}C]phosphatidylcholine from the proteoliposomes (fig.1). On the contrary, [^{14}C]phosphatidylcholine was only partially exchangeable in proteoliposomes that contained cytochrome b_5. The percentage of the label in these proteoliposomes that could not be exchanged approached 28% after three successive incubations and that value did not change during two additional incubations.

Since the cytochrome *P*-450 proteoliposomes were found to be impermeable to proteins as big as trypsin [5,6], the phosphatidylcholine exchange protein of similar M_r also could be expected not to be able to enter the proteoliposomes. Therefore the complete exchangeability of phosphatidylcholine could be explained either by its predomi-

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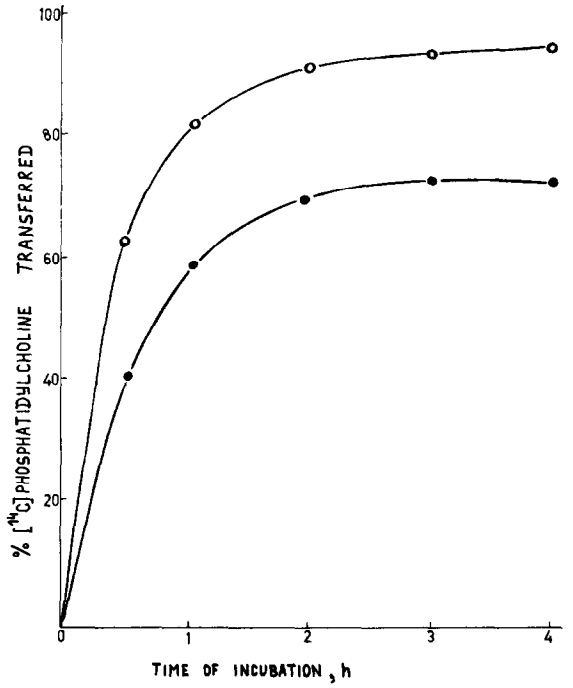

Fig.1. Percentage of [^{14}C]phosphatidylcholine exchange between unsonicated liposomes and proteoliposomes containing cytochrome *P*-450 (o-o-o) or cytochrome b_5 (•–•–•) in the presence of beef liver phosphatidylcholine exchange protein. Proteoliposomes (5 μg lipid P), unsonicated phosphatidylcholine liposomes (500 μg lipid P) and phosphatidylcholine exchange protein (40–60 μg) were incubated for 0.5 h at 37°C. Unsonicated liposomes were removed by centrifugation (22 000 × *g*, 15 min). The supernatant was subsequently reincubated (for 1 h) with the same amount of fresh unsonicated liposomes and an aliquot of exchange protein, and this procedure was repeated 4 times.

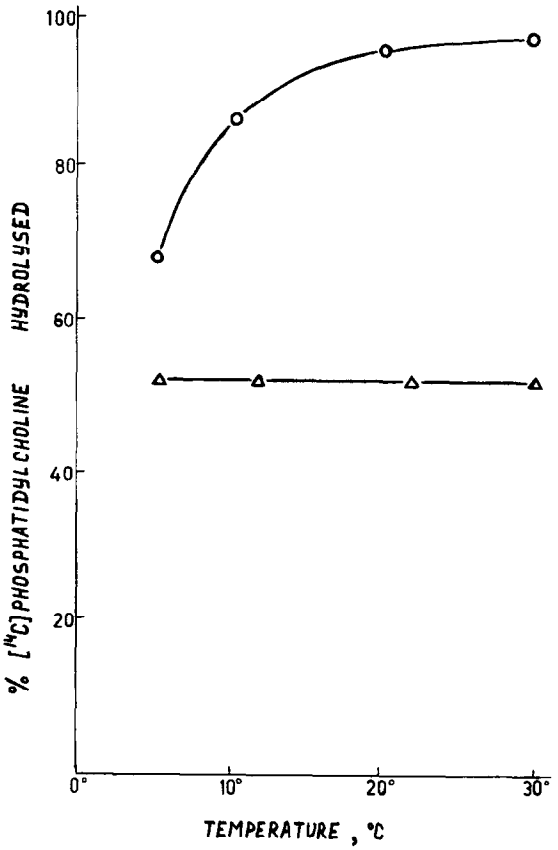

Fig.2. Phospholipase A_2 treatment of cytochrome *P*-450 containing liposomes (o-o-o) and sonicated liposomes prepared from total microsomal lipids (△–△–△). Proteoliposomes or sonicated liposomes (15 μg lipid P) were incubated with phospholipase A_2 (1.5 μg) at indicated temperatures for 10 min.

nant localization in the outer layer of the proteoliposomes or by rapid movement of phosphatidylcholine molecules from the inner to the outer layer.

Phospholipase A_2 digestion studies revealed that a substantial part of phosphatidylcholine was located in the inner layer of the cytochrome *P*-450–proteoliposomes (fig.2). While almost all the phosphatidylcholine was hydrolyzed at 20–30°C, the extent of hydrolysis was less than 70% at 5°C. In sonicated protein-free liposomes phosphatidylcholine hydrolysis was limited and temperature independent. Since the enzyme concentration was largely in excess of that required for complete hydrolysis of the accessible phosphatidylcholine even at low temperature, the limited low-temperature hydrolysis in the proteoliposomes indicates that at least 30% of the phosphatidylcholine molecules are not accessible to the enzyme. This non accessible pool can be supposed to be located in the inner layer of these proteoliposomes. Therefore, the complete availability of phosphatidylcholine for exchange and hydrolysis at elevated temperature suggests that cytochrome *P*-450 facilitates the

transbilayer movement of phosphatidylcholine. On the other hand, another intrinsic microsomal protein, cytochrome b_5, seems not to enhance the rate of flip-flop as can be judged from the limited accessibility of phosphatidylcholine for exchange in proteoliposomes containing cytochrome b_5.

As about 90% of phosphatidylcholine is exchanged in cytochrome *P*-450–proteoliposomes during the first hour of incubation at 37°C, the transbilayer movement of phosphatidylcholine in these proteoliposomes must have a halftime of 20 min or less. In protein-free phosphatidylcholine liposomes prepared by cholate dialysis, a halftime of 4 days or more was reported [7]*. Thus, the rate of transbilayer movement of phosphatidylcholine is enhanced by at least two orders of magnitude upon incorporation of cytochrome *P*-450 in proteoliposomes.

So far only a few intrinsic membrane proteins have been studied in respect to their possible action as mediators of phospholipid flip-flop. Enhanced transbilayer movements were observed upon incorporation of the intrinsic erythrocyte membrane proteins, glycophorin [9,10] and band 3 protein [11], into liposomes. On the other hand, another intrinsic membrane protein, mitochondrial cytochrome *c* oxidase, did not enhance phospholipid flip-flop in reconstituted proteoliposomes [7]. The biological significance of these findings is obscure because phospholipid flip-flop is a relatively rare event both in erythrocytes [12] and inner mitochondrial membranes [13]. On the contrary, extremely rapid transbilayer movement of phospholipids has been shown to occur in the microsomal membrane [14,15]. Several investigations have been undertaken to find out factors that could be responsible for this phenomenon. It has been demonstrated that lipid peroxidation [16] or presence of bile salts [17] and proteolipids [18] cannot account for the rapid phospholipid flip-flop in liver microsomes.

The present paper shows that one of the major intrinsic proteins of the microsomal membrane, cytochrome *P*-450, may be responsible for the rapid phospholipid flip-flop in microsomes. Although other explanations cannot be excluded, this suggestion is supported by our preliminary experiments on the fractionation of membrane proteins solubilized from microsomes. We found that only those protein fractions which contained cytochrome *P*-450 were able to enhance phosphatidylcholine flip-flop upon incorporation into liposomes.

* Control experiments demonstrated that residual traces of cholate did not facilitate transbilayer movement of phosphatidylcholine in liposomes prepared by cholate dialysis from total microsomal lipids. This is in agreement with results obtained by other authors under identical conditions [7,18]. The opposite conclusion was drawn by Kramer et al. [8] in a study of phosphatidylcholine exchange between cholate-containing liposomes and erythrocyte ghosts. The discrepancy might be related to the different nature of acceptor membranes used in the exchange experiments.

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