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The phosphatidylcholine transfer protein from bovine liver discriminates between phosphatidylcholine isomers. A monolayer study

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The activity of the phosphatidylcholine transfer protein from bovine liver toward phosphatidylcholine isomers carrying a long and a short fatty acyl chain on either the *sn*-1- or *sn*-2-position was determined by way of the monolayer-vesicle assay. In this assay equimolar mixtures of the isomers were spread at the air/water interface and their transfer measured to the vesicles in the subphase initiated by addition of the transfer protein. The following isomers were tested: 1-decanoyl-2-[^{3}H]oleoyl-*sn*-glycero-3-phosphocholine (C10:0/[^{3}H]C18:1-PC) and 1-oleoyl-2-decanoyl-*sn*-glycero-3-phospho[^{14}C]choline (C18:1/C10:0-[^{14}C]PC); 1-lauroyl-2-[^{3}H]oleoyl-*sn*-glycero-3-phosphocholine (C12:0/[^{3}H]C18:1-PC) and 1-oleoyl-2-[^{14}C]lauroyl-*sn*-glycero-3-phosphocholine (C18:1/[^{14}C]C12:0-PC); 1-myristoyl-2-[^{3}H]oleoyl-*sn*-glycero-3-phosphocholine (C14:0/[^{3}H]C18:1-PC) and 1-oleoyl,2-myristoyl-*sn*-glycero-3-phospho[^{14}C]choline (C18:1/C14:0-[^{14}C]PC). It was found that the protein transferred C10:0/[^{3}H]C18:1-PC twice as fast as C18:1/C10:0-[^{14}C]PC. Similar differences in rate were observed for C12:0/[^{3}H]C18:1-Pc and C18:1/[^{14}C]C12:0-PC but not for the isomers carrying myristic acid. We propose that the transfer protein can discriminate between PC isomers due to the presence of distinct binding sites for the *sn*-1- and *sn*-2-acyl chain (Berkhout et al. (1984) Biochemistry, 23, 1505–1513).

Introduction

The PC transfer protein from bovine liver acts as a specific carrier of PC between membrane interfaces [1–5]. This protein has a recognition site for the phosphorylcholine headgroup [6], and two independent binding sites for the *sn*-1- and *sn*-2-fatty acyl chain [7]. Phospholipase treatment failed to cleave the ester bonds of PC bound to the protein. This strongly suggests that this PC-molecule is shielded from the medium [8]. Binding of PC with nitroxide groups at various positions along the *sn*-2-fatty acyl chain, yielded electron spin resonance spectra which showed a strong interaction of this acyl chain over its whole length with the protein [9,10]. Strong binding of both acyl chains was demonstrated by time resolved fluo-

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Abbreviations: PC transfer protein, phosphatidylcholine transfer protein; di-C10:0-PC, 1,2-didecanoyl-*sn*-glycero-3-phosphocholine; di-C12:0-PC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine; di-C14:0-PC, 1,2-dimyristoyl-*sn*-3-phosphocholine; di-C18:1-PC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; C10:0/[^{3}H]C18:1-PC, 1-decanoyl-2-[^{3}H]oleoyl-*sn*-glycero-3-phosphocholine; C12:0/[^{3}H]C18:1-PC, 1-lauroyl-2-[^{3}H]oleoyl-*sn*-glycero-3-phosphocholine; C14:0/[^{3}H]C18:1-PC, 1-myristoyl-2-[^{3}H]oleoyl-*sn*-glycero-3-phosphocholine; C18:1/C10:0-[^{14}C]PC, 1-oleoyl-2-decanoyl-*sn*-glycero-3-phospho[^{14}C]choline; C18:1/[^{14}C]C12:0-PC, 1-oleoyl-2-[^{14}C]-lauroyl-*sn*-glycero-3-phosphocholine; C18:1/C14:0-[^{14}C]PC, 1-oleoyl-2-myristoyl-*sn*-glycero-3-phospho[^{14}C]choline.

rescence spectroscopy using PC which carried a parinaroyl chain at the *sn*-1- or at the *sn*-2-position [7]. Covalent coupling of photoactivatable PC analogues indicated that a hydrophobic peptide segment is part of the binding site for the *sn*-2-fatty acyl chain [11,12].

Interaction of the PC transfer protein with PC-containing interfaces results in the exchange of the bound PC molecule for one from the interface [2,8]. As for the mode of action we presume that the protein perturbs the membrane interface to the extent that PC can flip from the interface onto the protein whilst the bound PC is released into that interface [13]. The rate of exchange would then depend on the hydrophobic forces that govern the interaction between the PC-molecules in the interface and their interaction with the binding sites of the transfer protein exposed at the interface. In support of this concept PC vesicles in the gel state are much poorer substrates for the PC transfer protein than vesicles in the liquid-crystalline state [14,15]. In addition, the protein mediated transfer of PC increased with increasing unsaturation of the PC species [6,16,17]. In studies in which PC was in the fluid state, the transfer protein preferred long chain unsaturated species (i.e., di-C18 : 1-PC) over short chain saturated species (i.e., di-C14 : 0-PC) [14,18].

Here we present some experiments to demonstrate that the binding sites for the *sn*-1- and *sn*-2-fatty acyl chain are involved in the interfacial exchange process. For this purpose, PC isomers were synthesized which carry a long and a short acyl chain at either the *sn*-1- or *sn*-2-position. Transfer of these PC isomers was measured in the monolayer-vesicle assay [2,19]. It will be demonstrated that the PC transfer protein discriminates between PC isomers preferring the PC species with the long acyl chain at the *sn*-2-position.

Materials and Methods

Materials. [9,10-^{3}H]Oleic acid (2 Ci/mmol), [1-^{14}C]lauric acid (15 mCi/mmol), and [^{14}C]methyl iodide (58.5 mCi/mmol) were purchased from Amersham International, Amersham. Myristic acid was obtained from Fluka, oleic acid from Merck, and decanoic acid was a gift from Dr. Verhagen of the Department of Organic Chemistry in Utrecht. Dimethylaminopyridine, 1,4-diazobicyclo(2.2.2)octane, dimethylformamide, tetrahydrofuran, cyclohexylamine, and silica gel 60 thin-layer plates were purchased from Merck, Dicyclohexylcarbodiimide was obtained from Aldrich, and phospholipase A_2 (*Crotalus adamanteus*) from Koch Light Lab. Di-C10 : 0-PC, di-C12 : 0-PC. di-C14 : 0-PC, and di-C18 : 1-PC were purified by high performance liquid chromatography and were kindly provided by W.S.M. Geurts van Kessel of this Department. The PC transfer protein was purified from bovine liver and stored at −20°C in 55% (v/v) glycerol [20].

Synthesis of phosphatidylcholine isomers. The lyso-derivatives of di-C10 : 0-PC, di-C12 : 0-PC, di-C14 : 0-PC, and di-C18 : 1-PC were prepared by phospholipase A_2 treatment and were subsequently purified by precipitation in cold diethyl ether [21]. The preparations were finally extracted by the method of Bligh and Dyer [22] to remove traces of the phospholipase A_2. The anhydride of [^{3}H]oleic acid was prepared [23], added to the lyso-PC derivatives and the coupling reaction carried out for 2 h using dimethylaminopyridine as catalyst [24]. The ^{3}H-labeled PC analogues (i.e. C10 : 0/[^{3}H]C18 : 1-PC, C12 : 0/[^{3}H]C18 : 1-PC, C14 : 0/[^{3}H]C18 : 1-PC, C18 : 1/[^{3}H]C18 : 1-PC were isolated by TLC with $CHCl_3$/MeOH/NH_3/H_2O (90 : 54 : 5.5 : 5.5, v/v) as solvent. The products were analyzed for acyl chain migration by phospholipase A_2 treatment followed by TLC of the total digest. Release of [^{3}H]oleic acid amounted to 95–99%, showing that under the conditions of synthesis the migration of the *sn*-1-acyl chain varied between 1 and 5%.

The ^{14}C-labeled isomers of the PC species were synthesized as described above, starting with the lyso-derivative of di-C18 : 1-PC and the anhydrides of decanoic acid, [^{14}C]lauric acid and myristic acid. The isomers with decanoic acid and myristic acid were labeled in the polar headgroup by demethylation and remethylation with [^{14}C]methyliodide [25]. The migration was less than 5% as checked by phospholipase A_2 treatment followed by TLC (in the instance of C18 : 1/[^{14}C]C12 : 0-PC), or by gas-liquid chromatography of the fatty acid methyl esters (in the instance of C18 : 1/C10 : 0-[^{14}C]PC and C18 : 1/C14 : 0-[^{14}C]PC). The

methyl esters were prepared by dissolving the liberated fatty acids in 0.5 ml methylation reagent (14% BF_3 in methanol) followed by an incubation at 100°C for 10 min under nitrogen [26]. The methyl esters were dissolved in CS_2 and analyzed by gas-liquid chromatography. The corresponding unlabeled PC isomers were prepared by the same method.

Gas liquid chromatography. The fatty acid methyl esters were separated on a Packard series 805 gas chromatograph equipped with glass tubular columns (2 m × 2 mm internal diameter) packed with 3% Silar 5CP on Gas Chrom QII (80–100 mesh, Supelco Bellefonte). The samples were injected directly onto the column packing and eluted with nitrogen employing a temperature gradient of 80–180°C (5 Cdeg/min).

Preparation of vesicles. A mixture of egg yolk PC and phosphatidic acid (98 : 2, mol%) was suspended in a 10 mM Tris-HCl buffer (pH 7.4) at a concentration of 10 μmol/ml. Vesicles were prepared by sonication for 10 min at 4°C under nitrogen with a Branson sonifier (energy output 70 W).

The monolayer-vesicle assay. The transfer of PC as catalyzed by the PC-transfer protein was measured in the monolayer-vesicle assay as previously described [2,19]. Briefly an equimolar mixture of ^{3}H- and ^{14}C-labeled PC-isomers was spread at an air/water interface from a chloroform-methanol solution (9 : 1, v/v). The aqueous phase consisted of a 10 mM Tris-HCl buffer (pH 7.4) in a Teflon trough (5.4 × 5.8 cm) equipped with a 1.5 × 1.5 cm extended corner with a hole of 0.8 cm through which transfer protein and egg yolk PC vesicles were added to the subphase. The surface pressure was determined with a Beckman LM 500 electrobalance using a sand-blasted platinum plate.

All experiments were performed in a thermostated box at 25°C. Transfer of the [^{14}C]PC-isomer was determined directly by measuring the ^{14}C radioactivity with a gas-flow detector (Nuclear Chicago 8731) with a micromil window, 150 μg/cm^2, 4.2 × 1.3 cm. The gas used was helium/1.3% butane. The transfer of the ^{3}H-isomer was determined by taking samples from the subphase. The radioactivity was measured in a Packard Liquid Scintillation Counter using a double lable program. Instagel was used as a counting liquid.

As a control the ratio of the ^{3}H- and ^{14}C-label at the end of the experiment was determined.

Determination of force-area curves. Force-area measurements were performed at the air/water interface in a Teflon trough, 32.2 cm long and 17.2 cm wide, filled with 10 mM Tris-HCl (pH 7.4). The surface pressure was determined with a recording Beckman LM 500 electrobalance. The compression rate was 0.258 $nm^2 \cdot mol^{-1} \cdot min^{-1}$. The trough was placed in a thermostated box at 22°C. 50 nmol of lipid dissolved in chloroform was carefully released onto the air/water interface from an Agla micrometer syringe.

Results and Discussion

Pressure-area curves

The PC isomers containing decanoic, lauric, and myristic acid at either the *sn*-1- or *sn*-2-position formed stable monolayers at the air/water interface. This allowed the determination of their pressure-area curves (Fig. 1). At pressures upto 30 $mN \cdot m^{-1}$ each set of PC isomers had similar surface areas per molecule, although a consistent small shift to higher areas (i.e. 2 to 5 Å) was observed for the isomers with oleic acid at the *sn*-2-position. Di-C18 : 1-PC as a control, yielded a compression diagram which resembled that of C14 : 0/C18 : 1-PC and C18 : 1/C14 : 0-PC (Fig. 1). In previous experiments with di-C14 : 0-PC, diC12 : 0-PC and di-C10 : 0-PC, there was a tendency for the species with the shorter acyl chains to occupy a larger surface area [27]. A similar trend was observed for the PC species in this study in that at each pressure the surface area per molecule showed a small increase in the order: C18 : 1/C10 : 0-PC > C18 : 1/C12 : 0-PC > C18 : 1/C14 : 0-PC. Apparently, shortening of the acyl chains adversely affects the intramolecular interactions in the PC molecule, giving rise to larger molecular surface areas at the air/water interface.

Monolayer-vesicle transfer

The transfer experiments were carried out at about 30 $mN \cdot m^{-1}$ at which pressure the surface area per molecule differed maximally from 60 Å for C18 : 1/C14 : 0-PC to 70 Å^2 for C10 : 0/C18 : 1-PC (Fig. 1). In the monolayer-vesicle assay, equimolar mixtures of the PC isomers were

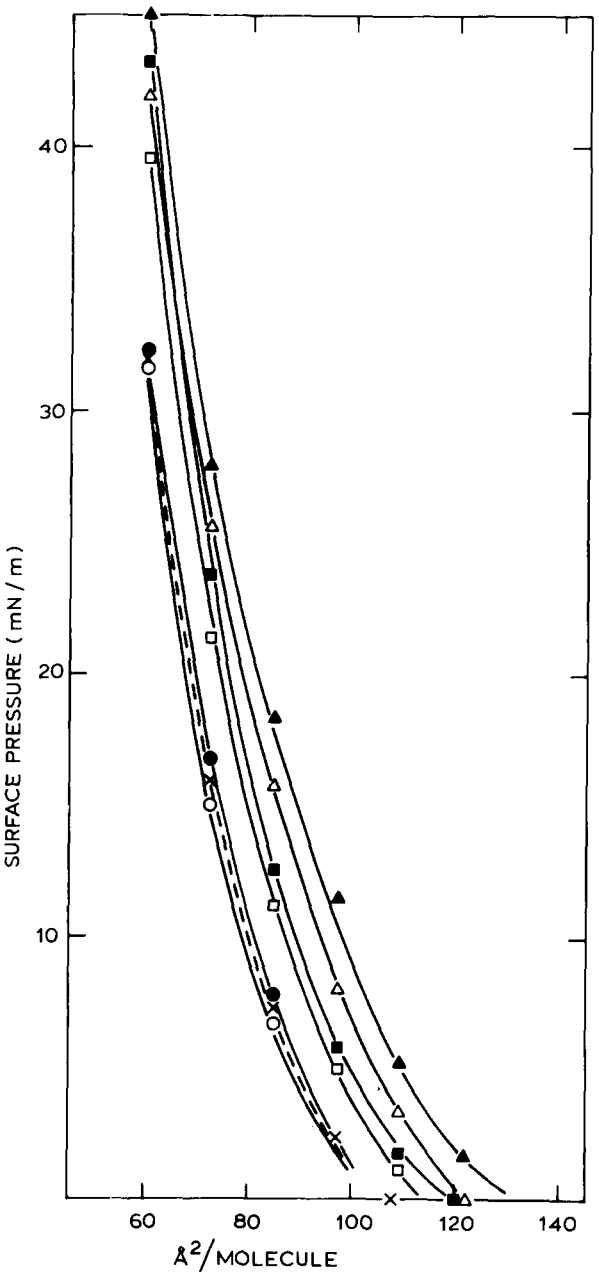

Fig. 1. Compression diagram of ×, C18:1/C18:1-PC; ▲, C10:0/C18:1-PC; △, C18:1/C10:0-PC; ■, C12:0/C18:1-PC; □, C18:1/C12:0-PC; ●, C14:0/C18:1-PC and ○, C18:1/C14:0-PC.

spread at the air-water interface and the transfer of [^{14}C]PC and [^{3}H]PC to the vesicles (egg PC/phosphatidic acid, 98:2, mol%) in the subphase measured. In the absence of PC-transfer protein,

TABLE I

PC-TRANSFER PROTEIN MEDIATED TRANSFER OF PC ISOMERS FROM A MONOLAYER TO VESICLES

For incubation conditions, see legend to Fig. 2. The error in the rates of transfer is in the order of 10%.

PC species	Initial rate of transfer (% monolayer PC/min)
C10:0/[^{3}H]C18:1-PC	0.9
C18:1/C10:0[^{14}C]-PC	0.4
C12:0/[^{3}H]C18:1-PC	2.6
C18:1/[^{14}C]C12:0-PC	1.1
C14:0/[^{3}H]C18:1-PC	8.8
C18:1/C14:0-[^{14}C]PC	8.8
C18:/C18:1-[^{14}C]PC	4.8

no spontaneous transfer of any of the PC isomers tested was observed in the course of a 60-min time period. The interfacial stability of C18:1/C10:0-PC and C10:0/C18:1-PC differs from bulk experiments in which a rapid, spontaneous transfer of these PC-species between vesicles and mitochondria was observed (unpublished data). As shown in Fig. 2, injection of PC transfer protein and vesicles under a mixed monolayer of C18:1/[^{14}C]C12:0-PC and C12:0/[^{3}H]C18:1-PC resulted in a decrease of the ^{3}H/^{14}C-surface radioactivity, i.e., in a transfer of both PC isomers to the subphase. Under the conditions of transfer the surface pressure remained virtually constant, indicating that the extraction of the radiolabeled PC isomers from the monolayer was compensated for by insertion of egg PC from the vesicles. From the initial decrease of surface radioactivity it was estimated that the protein transferred C12:0/[^{3}H]C18:1-PC approximately 2-times faster than C18:1/[^{14}C]C12:0-PC (Table I). A similar 2-fold difference in rate of transfer was observed for C10:0/[^{3}H]C18:1-PC as compared to C18:1/C10:0-[^{14}C]PC. This preference for isomers with oleic acid at the *sn*-2-position strongly suggests that the binding site for the 2-acyl chain has a greater effect on the efficiency with which the transfer protein extracts PC from the interface than the binding site for the 1-acyl chain. On the other hand, this preference was not longer ap-

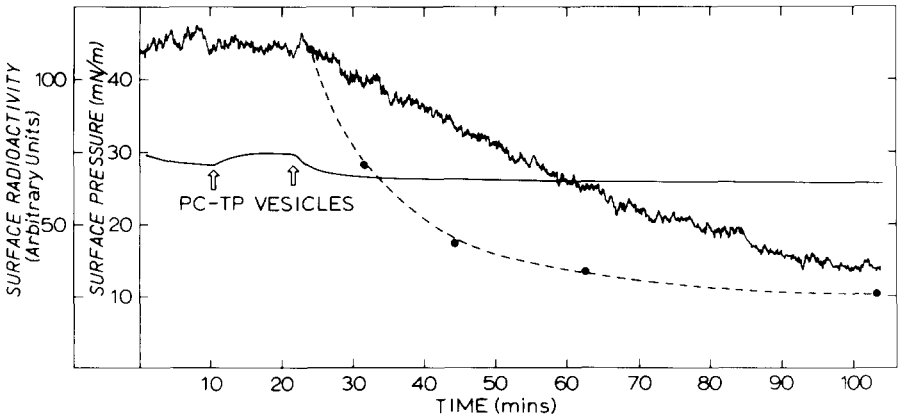

Fig. 2. Protein-mediated transfer of ^{3}H- and ^{14}C-labeled isomers of PC from monolayer to vesicles. The monolayer consisted of an equimolar mixture of C12:0/[^{3}H]C18:1-PC and C18:1/[^{14}C]C12:0-PC spread at the air-water interface at a surface pressure of 30 mN/m. The subphase contained 1 μmol of vesicles (PC/phosphatidic acid, 98:2, mol%) and 2 nmol of PC transfer protein in 22 ml of 10 mM Tris-HCl (pH 7.4). ^{14}C-label (recorded line); ^{3}H-label (●— — —●).

parent when the transfer of C18:1/C14:0-[^{14}C]PC and C14:0/[^{3}H]C18:1-PC were compared (Table I). These results may mean that for the transfer protein to discriminate between isomers, the acyl chains at the *sn*-1- and *sn*-2-position should differ sufficiently in length.

It is evident from Table I that the rates of transfer markedly diminished when the length of the acyl chain on either the *sn*-1- or *sn*-2-position was reduced from myristic acid to decanoic acid. In fact, those PC species which have the greatest tendency to spontaneously transfer in bulk experiments (i.e. C10:0/C18:1-PC and C18:1/C10:0-PC) were transferred the least by the protein. These data strongly suggest that a reduction of hydrophobicity on the part of the PC molecule adversely affects the binding to the transfer protein and, hence, results in diminished rates of transfer. A further increase of the hydrophobicity by replacing myristic acid for oleic acid at *sn*-2-position (i.e. C18:1/C18:1-[^{14}C]PC) resulted in a decrease of transfer as well. It is unlikely that this decrease reflects an inadequate accomodation of di-C18:1-PC in the two fatty acid binding sites of the transfer protein as both C14:0/C18:1-PC and C18:1/C14:0-PC gave optimal rates of transfer. Rather we propose that interactions between di-C18:1-PC molecules in the monolayer become stronger, leading to reduced rates of transfer. Hence, it appears that we are dealing with an optimum in the rate of transfer. This may reflect the subtle competition between the transfer protein and the monolayer proper for the individual PC molecules.

Concluding remarks

The above experiments provide clear evidence that the PC transfer protein can discriminate between PC isomers present in a one-to-one mixed monolayer. This discrimination most likely reflects the hydrophobic forces that, at the interface, govern the interaction of the 1- and 2-acyl chain with the corresponding binding sites on the protein. The fact that the isomers with oleic acid at the *sn*-2-position are transferred faster, may reflect on the mechanism by which the transfer protein extracts PC from the monolayer. In this respect it may be of consequence that the ester bond of the 2-acyl chain is closer to the bulk aqueous phase than the 1-acyl chain [28].

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