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The low-affinity lipid binding site of the non-specific lipid transfer protein. Implications for its mode of action

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The non-specific lipid transfer protein (nsL-TP) from bovine liver was studied by using the following fluorescent lipid analogs: phosphatidylcholine species with a *sn*-2-pyrenylacyl-chain of different length [Pyr(x)PC], *sn*-2-pyrenyldecanoyl-labelled phosphatidylinositol [Pyr(10)PI], -phosphatidylinositol 4-phosphate [Pyr(10)PIP], -phosphatidylinositol 4,5-bisphosphate [Pyr(10)PIP_2] and dehydroergosterol. These analogs provided information on the effect of hydrophobicity and charge on lipid binding and transfer by nsL-TP. Binding of the Pyr(x)PC species decreased with increasing *sn*-2 acyl-chain length. Under equilibrium conditions, the fraction of nsL-TP that carried a PC molecule did not exceed 8%, which is consistent with a low affinity binding site. Also nsL-TP-mediated transfer of the Pyr(x)PC species decreased with increasing *sn*-2 acyl-chain length and was highly correlated with spontaneous transfer. Binding of the phosphoinositides increased in the order Pyr(10)PI < Pyr(10)PIP < Pyr(10)PIP_2, indicating that an increase in lipid negative charge stimulates binding. The transfer of the phosphoinositides, however, decreased in the same order, which suggests that a high negative charge impairs the dissociation of the phospholipid from nsL-TP. Cholesterol, at concentrations up to 50 mol% in the donor membrane, hardly affected binding and transfer of Pyr(6)PC, strongly suggesting that nsL-TP has no high binding affinity for cholesterol. In agreement with this, binding of dehydroergosterol to nsL-TP was not detectable. Despite this apparently negligible affinity, nsL-TP-mediated transfer of dehydroergosterol was in the same order as that of Pyr(6)PC. The results are interpreted to indicate that transfer of lipids by nsL-TP involves the formation of a putative low-affinity lipid-protein complex. This formation is enhanced when lipid hydrophobicity decreases or lipid negative charge increases. Based on the binding and transfer data, the mode of action of nsL-TP is discussed in terms of change in free energy.

Introduction

The non-specific lipid transfer protein (nsL-TP) also called sterol carrier protein-2, has been purified from rat, bovine, goat, and human liver cytosol [1–7]. NsL-TP has the unique property to stimulate the intermembrane-transfer of a wide range of lipid molecules such as cholesterol [2,8–11], phospholipids [2,9,12], sphingomyelin [2], gangliosides [13] and neutral glycosphingolipids [13]. NsL-TP is also able to bind lipids [10,12,14,15]. Recently, nsL-TP was shown to bind DPH-labelled PC and to form a water-soluble lipid-protein complex in which the DPH-moiety was completely immobilized following the rotation of nsL-TP

Abbreviations: nsL-TP, non specific lipid-transfer protein; DPH, 1,6-diphenyl-1,3,5-hexatriene (all-*trans*); NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; dehydroergosterol, $\Delta^{5,7,9(11),22}$-ergostatetraene-3β-ol; PC, phosphatidylcholine; PA, phosphatidic acid; Pyr(x)PC, *sn*-2-pyrenylacyl-phosphatidylcholine; Pyr(10)PI, *sn*-2-pyrenyldecanoyl-phosphatidylinositol; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; TNP-PE, *N*-[2,4,6-trinitrophenyl]phosphatidylethanolamine; DMSO, dimethylsulfoxide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol bis(β-aminomethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PC-TP, phosphatidylcholine transfer protein; PI-TP, phosphatidylinositol transfer protein.

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(Gadella et al., submitted). It is still unclear how the binding of lipids and the nsL-TP-mediated transfer of lipids are connected. It has even been suggested that nsL-TP may facilitate transfer without actually forming a water-soluble lipid carrier complex [16,17]. Consequently, one model was proposed in which nsL-TP enhances the rate of desorption of lipid monomers from the donor membrane by decreasing the energy barrier for the monomer-membrane equilibrium reaction [18], and a second model in which nsL-TP brings two membranes into contact with one another, thereby facilitating the equilibration of phospholipid monomers between the membranes [9,19]. By studying the binding and transfer of NBD-labelled PC, Nichols proposed a model in which nsL-TP acts both as a carrier and as a catalyst for spontaneous transfer [12]. In view of the possible role of nsL-TP in cholesterol, steroid hormone and bile acid metabolism [3,5,10,20–27], it is of great importance to establish if the transfer-mechanism of nsL-TP involves the binding of lipids to the protein. In this respect it is of note that in contrast to a catalyzed spontaneous transfer mechanism, only a carrier mechanism can result in a specific targeting of lipids to certain cellular organelles or enzymes. In this study we have further investigated the mode of action of nsL-TP by measuring binding and transfer of lipids by nsL-TP using pyrene-labelled PC and phosphoinositide analogues, and the fluorescent sterol analog dehydroergosterol. It will be shown that nsL-TP lowers the activation energy for the off-rate of lipid monomers from the membrane interface, which may be due to the presence of a low-affinity binding site.

Materials and methods

Materials

Egg yolk PC, phosphatidic acid (PA), PIP and PIP_2 were obtained from Sigma. PI was purified from yeast as described [28]. The pyrene-labelled PC species (Pyr(x)PC; x indicates an acyl chain of 6, 8, 10, 12 and 14 C-atoms in the *sn*-2 position, and a palmitoyl-chain in the *sn*-1 position) were a kind gift of Dr. P.J. Somerharju (University of Helsinki, Finland). Pyr(10)PI was synthesized and purified as described [29,30]. Pyr(10)PIP and Pyr(10)PIP_2 were synthesized by phosphorylation of Pyr(10)PI using PI- and PIP-kinase preparations [28]. Trinitrophenylphosphatidylethanolamine (TNP-PE) was prepared from egg-yolk-PE and trinitrobenzenesulfonic acid [31]. Dehydroergosterol (full name see abbreviations) was prepared as described [32] and was a generous gift of Dr. G. van Ginkel (University of Utrecht). All lipids used yielded one spot on thin-layer chromatography. NsL-TP was purified from bovine liver as described [9] and stored in 60% glycerol (v/v) at −20°C. Organic solvents used for absorbance or fluorescence measurements were of spectroscopic grade. All other chemicals used were of analytical grade.

Methods

The concentration of pyrene-labelled PC-species [Pyr(x)PC] was determined by measuring the absorbance in ethanol ($\varepsilon_{342} = 42\,000\ M^{-1}\ cm^{-1}$ [29]). The concentration of Pyr(10)PI, Pyr(10)PIP and Pyr(10)PIP_2 was estimated by determining the absorbance in ethanol/DMSO (75:25, v/v) ($\varepsilon_{342} = 39\,700\ M^{-1}\ cm^{-1}$ [28]), and by measuring the fluorescence emission spectrum in chloroform/methanol/0.6 M HCl (1:2:0.8, v/v) using pyrene-labelled phospholipid standards (excitation 346 nm, excitation slit 2.5 nm, emission slit 2 nm). Dehydroergosterol was quantitated by measuring the absorbance in 1,4-dioxane ($\varepsilon_{326} = 10\,600\ M^{-1}\ cm^{-1}$ [11]). The concentration of the other lipids used was assayed by phosphorous determination [33].

Fluorimetric spectroscopy. Fluorescence measurements were performed on a SLM-Aminco SPF-500C spectrofluorimeter equipped with a thermostated cuvette holder and a magnetic stirring device. For experiments with pyrene lipids, the excitation and emission were set at 346 and 378 nm (slits 2.5 and 10 nm, respectively). For experiments with dehydroergosterol, excitation and emission were set at 326 and 390 nm (slits 4 and 10 nm, respectively). The buffer used for the fluorescence measurements was filtered through a Millipore filter (0.45 μm). Measurements were performed at 25°C under continuous stirring.

Preparation of phospholipid vesicles. Donor vesicles containing Pyr(x)PC species were prepared by injecting 20 μl of a phospholipid-mixture (2 nmol) dissolved in DMSO/ethanol (25:75, v/v) into 2 ml of 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA (buffer A), yielding small unilamellar vesicles [34,39]. The lipid-mixture consisted of Pyr(x)PC and unlabeled PC in various ratios, together with 0.2 nmol TNP-PE to efficiently quench the fluorescence of the donor vesicle. Prior to use, the donor vesicles were equilibrated for 1 min. Donor vesicles containing phosphoinositides were prepared by brief sonication (3 min, 5 s on, 10 s off, 0°C, N_2 atmosphere) of a lipid mixture suspended in 1 ml of buffer A. The suspension consisted of 4 nmol of either Pyr(10)PC, Pyr(10)PI, Pyr(10)PIP or Pyr(10)PIP_2, 12 nmol PC and 1.6 nmol TNP-PE [28,35]. The integrity of the pyrene moiety was checked by recording an emission fluorescence spectrum of a small sample of the vesicle preparation in chloroform/methanol/0.6 M HCl (1:2:0.8, v/v). No significant degradation was observed as compared to control samples. Donor vesicles containing cholesterol or dehydroergosterol were prepared by injecting 20 μl of a lipid mixture (4 nmol) dissolved in DMSO/ethanol (25:75, v/v) into buffer A (2 ml) followed by a 3 min equilibration. In order to obtain acceptor vesicles, a

lipid mixture containing 7.6 μmol PC and 0.4 μmol PA, was suspended in 2 ml of buffer A and sonified for 3 min (without interruption) at 0°C under a stream of nitrogen, yielding a clear solution. Prior to sonication, buffer A was saturated with argon to prevent lipid oxidation.

Fluorescence binding assay. Binding of Pyr(x)PC to nsL-TP was determined by titration of donor vesicles with the protein similar to the method described [36–38]. Briefly, aliquots of nsL-TP (2.5 μg) were added to the donor vesicles (2 nmol phospholipid). The increase in pyrene-monomer fluorescence resulting from the uptake of Pyr(x)PC from the quenched donor vesicles by nsL-TP, was taken as a measure for lipid binding. An equilibrium situation was usually reached after 1–2 min of incubation. Binding of pyrenylacyl-labelled phosphoinositides and of dehydroergosterol were measured by a similar assay (for details, see legends).

Fluorescence transfer assay. The transfer assay was carried out as described before [37,39–41]. For the experiments with Pyr(10)PC, the incubation mixture contained 2 nmol donor vesicles, and an excess of acceptor vesicles (100 nmol). The spontaneous transfer of Pyr(x)PC from donor to acceptor vesicles was recorded by measuring the pyrene monomer fluorescence increase. Alter 1 min, nsL-TP (4.8 μg) was added to initiate protein-mediated transfer (final volume 2 ml). Transfer rates were corrected for spontaneous transfer which was always found to be less than 5% of the protein-mediated transfer. For the experiments with pyrenylacyl-labelled phosphoinositides, the reaction mixture contained 0.88 nmol donor vesicles and 25 nmol acceptor vesicles and the transfer was initiated by addition of 4.8 μg nsL-TP. The pyrene monomer fluorescence was quantitated by use of a standard vesicle preparation containing Pyr(10)PC, PC and PA (1:1900:100, mol/mol). Therefore, the transfer-rates could be expressed as pmoles transferred per minute. Transfer experiments with donor vesicles containing cholesterol or dehydroergosterol, were performed similarly as described for the Pyr(x)PC experiments, except that 4 nmol (total lipid) donor vesicles were used and that 14 μg of nsL-TP was added to initiate transfer. Dehydroergosterol fluorescence was calibrated with a vesicle preparation consisting of dehydroergosterol, PC and PA (1:95:5, mol/mol).

Results

Calibration of lipid binding.

Lipid binding was calibrated as shown in Fig. 1. After addition of an aliquot of nsL-TP (arrow B) to quenched donor vesicles containing Pyr(8)PC, a rapid increase of pyrene-monomer fluorescence was observed as a result of binding of Pyr(8)PC to nsL-TP.

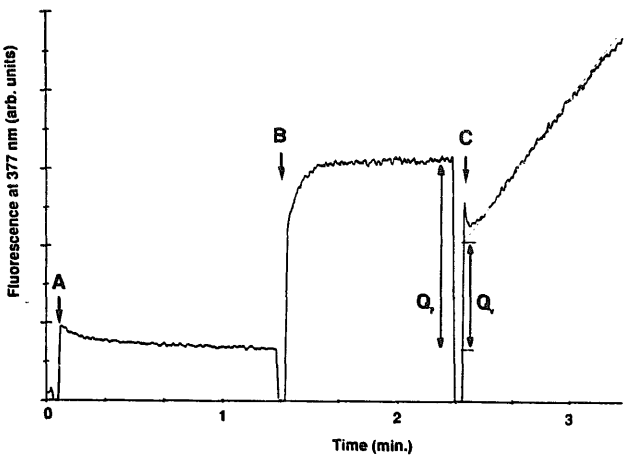

Fig. 1. Calibration of pyrene-lipid binding to nsL-TP. Pyrene-monomer fluorescence emission was measured at 378 nm (excitation 346 nm). Pyr(8)PC (1.8 nmol) and TNP-PE (0.2 nmol) dissolved in 20 μl ethanol were injected into the cuvette containing 1.88 ml of buffer A (arrow A). Arrow B indicates the addition of 4.8 μg nsL-TP (10 μl), and arrow C indicates the addition of 100 nmol sonicated acceptor vesicles (PC/PA, 95:5 mol/mol). The fluorescence quantum yield of Pyr(8)PC in nsL-TP relative to that in vesicles is calculated by dividing the distance Q_p by Q_v. Q_v was calibrated by measuring the pyrene monomer fluorescence of vesicles consisting of Pyr(10)PC, PC and PA (1:1900:100, mol/mol). The quantum yield Q_v was independent of the acyl chain length [39].

After equilibrium was reached (1 min), an excess of acceptor vesicles was added (arrow C, Fig. 1). At this point the pyrene-monomer fluorescence decreased, going through a minimum, after which it steadily increased with time due to protein-mediated transfer of Pyr(8)PC from donor to acceptor membranes. The decrease in fluorescence is explained by the rapid transfer of virtually all Pyr(8)PC bound to nsL-TP, to the acceptor vesicles. By extrapolating the protein-mediated transfer to the acceptor vesicles to zero time (as indicated in Fig. 1), one may obtain the quantum yield of Pyr(8)PC bound to the protein (Q_p) relative to the quantum yield in the vesicle (Q_v) The signal of the latter was compared to the signal of a standard vesicle preparation containing 0.05 mol% PyrPC (see Methods), enabling the estimation of what percentage of nsL-TP carried a lipid molecule. We observed that the fluorescence quantum yield of all the pyrenyl-labelled lipids used, was 1.7–1.85-times higher when the lipid was bound to nsL-TP than when the lipid was present in the acceptor vesicles (average of 1.75). This is comparable with the results obtained for PI-TP which yielded a factor of 1.6 [7].

Binding of the Pyr(x)PC species

To investigate the affinity of nsL-TP for PC species that carry a pyrenylacyl chain of different length in the *sn*-2 position, lipid binding studies were performed. In these studies, use was made of donor vesicles that contain varying amounts of Pyr(x)PC relative to unla-

beled PC. As shown in Fig. 2A, the binding of each Pyr(x)PC species increased linearly with the Pyr(x)PC mole fraction in the donor membranes. Apparently, there is no competition between pyrene-labelled PC and non-labelled PC for the lipid binding site of nsL-TP. In addition, binding decreases drastically with increasing acyl chain length. For example, with vesicles that contain 90 mol% Pyr(x)PC, about 8% of nsL-TP contained a Pyr(6)PC molecule, whereas 0.3 mol% of nsL-TP contained a Pyr(14)PC molecule. The binding process can be described by an equilibrium reaction (Eqn. 1)

$$P + D \rightleftharpoons PL^* + D \qquad (1)$$

in which D, P and PL* denote donor membrane, empty nsL-TP and nsL-TP occupied with Pyr(x)PC, respectively. When the donor lipids are present in excess over the transfer protein or when only a small fraction of the donor membrane lipids is bound to the transfer protein, the change in donor membrane composition after the binding reaction can be neglected, allowing the expression of the equilibrium constant K as the ratio of occupied/empty nsL-TP molecules (Eqn. 2),

$$K = [PL^*]/[P] \qquad (2)$$

$$\Delta G^\circ = -RT \ln(K) \qquad (3)$$

from which the free energy of the binding reaction (ΔG°) directly follows (Eqn. 3). R and T represent the gas constant and the absolute temperature. The ΔG° values have been calculated for the binding of the various Pyr(x)PC species under conditions where the donor membrane contained 90 mol% Pyr(x)PC. In the inset of Fig. 2A, these energy differences are plotted as a function of the *sn*-2 acyl-chain length. From this plot it follows that the free energy needed for Pyr(x)PC binding to nsL-TP increases linearly with the acyl chain length (about 1 kJ/mol per methylene unit).

Transfer of the Pyr(x)PC species

The nsL-TP mediated transfer of Pyr(x)PC species from donor to acceptor vesicles is shown in Fig. 2B. Rates of transfer increase linearly with the mol fraction of Pyr(x)PC present in the donor vesicle, and decrease sharply with an increase in acyl chain length as was also observed by Van Amerongen et al. [9]. It is evident that the transfer of the Pyr(x)PC species is highly correlated with their binding behaviour. From comparing Figs. 2A and 2B, one can see that the decrease of transfer with increasing chain length is more pronounced than the decrease of binding. It is of note that a logarithmic plot of the rate of transfer versus acyl-chain length (inset of Fig. 2B), yields a straight line (see Discussion).

All the donor vesicles used, contained 10 mol% TNP-PE as internal fluorescence quencher. In principle, dequenching of donor fluorescence could occur upon binding or transfer of TNP-PE by nsL-TP. However, the lack of fluorescence dequenching in the experiments with Pyr(14)PC, indicated that TNP-PE remained with the donor membrane. This reflects the negligible binding affinity of TNP-PE for nsL-TP, most likely due to the long-acyl chain composition of this lipid. To further show that TNP-PE has no effect, donor vesicles that contain 1.8 nmol Pyr(x)PC (x = 6, 8, 10, 12, 14) and 0.2 nmol PA were prepared. These vesicles do not display monomer fluorescence due to excimer formation. Using these vesicles in binding and

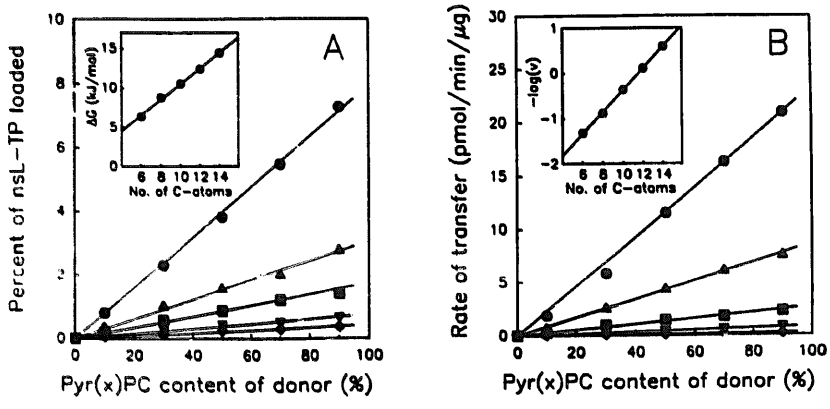

Fig. 2. Binding and transfer of Pyr(x)PC by nsL-TP. Vesicles containing Pyr(x)PC and egg-PC in varying amounts, together with 10 mol% TNP-PE (total of 2 nmol) were prepared as described in Methods. Binding (panel A) was determined by measuring the increase in pyrene-monomer fluorescence after titration with nsL-TP (four aliquots of 2.5 μg were added). The signal was calibrated as shown in Fig. 1. The inset represents the free energy of binding (ΔG°) as a function of the *sn*-2 acyl chain length. This was calculated from the experiments with donor vesicles containing 90 mol% Pyr(x)PC (see Results, Eqn. 3). Transfer (panel B) was measured after addition of unlabeled acceptor vesicles (100 nmol; PC/PA, 95:5, mol/mol) and of 4.8 μg nsL-TP to the donor vesicles described above. The inset represents a logarithmic plot of the rate of transfer for the experiments with the donor vesicles containing 90 mol% Pyr(x)PC. Binding and transfer are shown for Pyr(6)PC (●), Pyr(8)PC (▲), Pyr(10)PC (■), Pyr(12)PC (▼) and Pyr(14)PC (◆).

TABLE I

Binding and transfer of Pyr(10)PC, -PI, -PIP, and -PIP_2 by nsL-TP

Phospholipid substrate [a]	Binding [b] (% of nsL-TP loaded)	Rate of transfer [c] (pmol/min/μg)$\times 10^3$
Pyr(10)PC	0.62 ± 0.2	18
Pyr(10)PI	2.4 ± 0.3	131
Pyr(10)PIP	6.5 ± 0.1	36
Pyr(10)PIP_2	8.1 ± 0.5	6

[a] Donor vesicles consisted of 0.2 nmol of the listed lipids, 0.6 nmol egg-PC, and 0.08 nmol TNP-PE.

[b] Binding was measured after addition of nsL-TP (aliquots of 0.5 μg) to the donor vesicles.

[c] Transfer was measured after subsequent addition of 25 nmol of unlabeled sonicated acceptor vesicles (PC/PA, 95:5, mol%) and of nsL-TP (4.8 μg) to the donor vesicles.

transfer experiments, gave results very similar to the ones obtained with the donor vesicles quenched by TNP-PE (90 mol% Pyr(x)PC see Figs. 2A and B) (not shown). This confirms that TNP-PE in the donor vesicles is non-exchangeable both in the absence and presence of nsL-TP.

Binding and transfer of Pyr(10)-phosphoinositides

To further explore the requirements for phospholipid binding to nsL-TP we have investigated the binding of pyrene-labelled phosphoinositides, that vary in negative charge from -1 in case of PI to about -4 in case of PIP_2 [42]. As listed in Table I, binding of phosphoinositides by nsL-TP increased in the order of Pyr(10)PI < Pyr(10)PIP < Pyr(10)PIP_2 in parallel with the increased negative charge. Compared to Pyr(10)PC, binding was 4-fold higher for Pyr(10)PI to as much as 13-fold higher for Pyr(10)PIP_2. Under conditions where the donor vesicle contained 23 mol% of Pyr(10)PIP_2, 8% of nsL-TP was loaded with Pyr(10)PIP_2. From this it may be inferred that the affinity for Pyr(10)PIP_2 is 4-fold higher than for Pyr(6)PC (see Fig. 2A).

In contrast to what was observed with the Pyr(x)PC species, the rate of transfer of the respective phosphoinositides did not correlate with the binding (Table I). Pyr(10)PI was the preferred substrate and transferred at 7-fold higher rate than Pyr(10)PC. Despite its increased binding to nsL-TP, transfer of Pyr(10)PIP was decreased about 4-fold relative to Pyr(10)PI. Pyr(10)PIP_2 was very poorly transferred by nsL-TP suggesting that its high bind efficacy interferes with its release from the protein.

To check the influence of phosphoinositides on the activity of nsL-TP, donor vesicles were prepared consisting of 23 mol% unlabeled phosphoinositides, and 23 mol% Pyr(6)PC. As shown in Table II, PI, PIP and PIP_2 have barely an effect on the binding of Pyr(6)PC, despite their relatively high affinity for nsL-TP. Apparently, there is no competition between phosphoinositides and Pyr(6)PC for the lipid binding site. On the other hand, the transfer of Pyr(6)PC was increased by a factor of 2.7 for the vesicles that contained PI. Substitution of PI for PIP or PIP_2 slightly reduced the rate of transfer, but transfer was still a factor of two higher as compared to the control. The stimulatory effect of phosphoinositides on the transfer of Pyr(6)PC may be due to the high negative charge of the vesicles which may induce nsL-TP to accumulate at the vesicle surface.

TABLE II

Effect of phosphoinositides on binding and transfer of Pyr(6)PC

Phosphoinositide in donor vesicle [a]	Relative binding [b]	Relative rate of transfer [b]
PC (control)	1.00	1.0
PI (23 mol%)	0.85	2.7
PIP (23 mol%)	1.02	2.3
PIP_2 (23 mol%)	1.10	2.0

[a] Donor vesicles consisted of 0.2 nmol of the listed phospholipids, 0.2 nmol Pyr(6)PC, 0.4 nmol egg-PC, and 0.08 nmol TNP-PE.

[b] Binding and transfer were determined as described in the legend to Table I.

Effect of cholesterol on binding and transfer of Pyr(6)PC

In order to compare the affinity of Pyr(6)PC and of cholesterol for the lipid binding site of nsL-TP, a competition assay was used, employing donor vesicles containing Pyr(6)PC (40 mol%), TNP-PE (10 mol%) and varying amounts of cholesterol and unlabeled PC (total lipid 4 nmol). An increase in the cholesterol concentration from 0 to as much as 50 mol% in the donor vesicles had very little effect on Pyr(6)PC binding to nsL-TP (Fig. 3). Similarly, transfer of Pyr(6)PC was almost independent on the cholesterol mole frac-

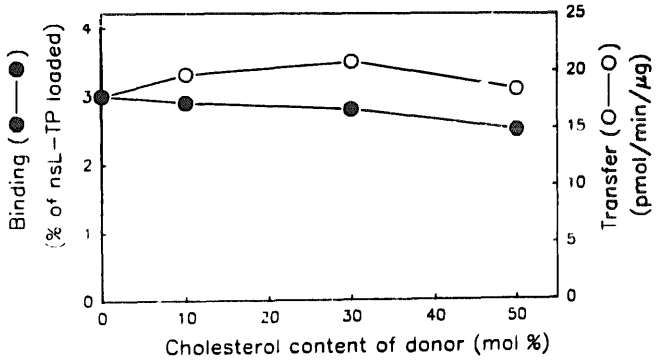

Fig. 3. Effect of cholesterol on Pyr(6)PC binding (●) and transfer (○) by nsL-TP. Donor vesicles (4 nmol total lipid) consisted of 40 mol% Pyr(6)PC, 10 mol% TNP-PE and 50 mol% cholesterol/egg-PC in various ratios. Binding experiments were carried out by titrating the donor vesicles with nsL-TP (aliquots of 2.8 μg) and subsequently monitoring the increase of pyrene-monomer fluorescence for 2 min. Transfer was measured by adding acceptor vesicles (100 nmol of phospholipid; PC/PA, 95:5, mol/mol) and (after 2 min) 6.4 μg nsL-TP to the donor vesicles.

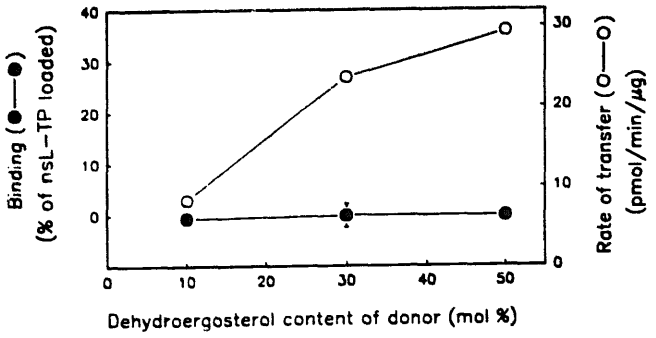

Fig. 4. Binding (●) and transfer (○) of dehydroergosterol to nsL-TP at various dehydroergosterol concentrations in mixed bilayers. Donor vesicles (4 nmol total lipid) consisted of 10 mol% TNP-PE and dehydroergosterol/egg-PC in various ratios. Binding was measured by titrating the vesicles with nsL-TP (aliquots of 7 μg) followed by a 2 min equilibration. The values were corrected for nsL-TP fluorescence and calibrated with a dehydroergosterol standard (see Methods). It was assumed that the quantum yield of dehydroergosterol inside nsL-TP was comparable with that of dehydroergosterol in vesicles. Transfer was measured by adding acceptor vesicles (100 nmol of phospholipid; PC/PA, 95:5, mol/mol) and (after 2 min) 14 μg nsL-TP to the donor vesicles. The rates of transfer were corrected for spontaneous transfer.

tion in the membranes. The same experiments carried out with rat-nsL-TP. gave comparable results (data not shown). From these experiments it is concluded that cholesterol does not compete with Pyr(6)PC both for binding and transfer by nsL-TP.

Binding and transfer of dehydroergosterol

Binding of dehydroergosterol was determined under similar conditions used in the competition assay. Donor vesicles consisted of varying amounts of dehydroergosterol and PC, together with 10 mol% TNP-PE (total lipid: 4 nmol). Due to the presence of TNP-PE (in the donor vesicles), the dehydroergosterol-fluorescence was efficiently quenched (> 90%). Therefore, both binding to nsL-TP and transfer to acceptor vesicles could be determined by continuously monitoring the increase in fluorescence intensity. Binding of dehydroergosterol by nsL-TP was determined at three different concentrations in the donor membrane (Fig. 4). At no concentration was there any evidence that dehydroergosterol was bound to nsL-TP. Here we assume that the quantum yields of dehydroergosterol in the vesicle and inside nsL-TP are of the same order of magnitude. Identical negative results have been obtained for rat-nsL-TP (data not shown). This lack of binding is remarkably different from the 1:1 stoichiometry reported by Schroeder et al. [14].

Despite the failure to observe binding, nsL-TP very effectively mediated the transfer of dehydroergosterol from donor to acceptor vesicles with a halftime of approx. 1.5 min under the employed conditions (Fig. 4). The nsL-TP-controlled rate of transfer was clearly higher than the rate of spontaneous transfer and increased with increasing dehydroergosterol content of the donor membrane. However, in contrast to the Pyr(x)PC experiments, no linear dependency was observed, which may be attributed to a tighter packing of the vesicles at increasing sterol concentration. Similar results were obtained with rat nsL-TP (not shown). After 20 min of incubation, an equilibrium situation was reached at which, in agreement with other studies [43,44], about 80–90% of the dehydroergosterol present in the donor vesicles was transferred to the acceptor vesicles. This indicates that dehydroergosterol is subject to fast 'flip-flop' in membranes.

Discussion

Binding and transfer of pyrenylacyl-labelled phospholipids by nsL-TP were investigated to get more insight in the mode of action of this protein. From Fig. 1 one can see that the binding of Pyr(8)PC from the donor vesicles (arrow B) as well as the release of bound lipid to the acceptor vesicles (arrow C) is in the range of seconds. A calculation based on the constants presented by Nichols [12] for NBD-PC binding (using the combined model), yielded halftimes of about 9 and 3.5 s for the binding and release reactions, respectively. These figures compare favourably with the time-scale of binding and release observed in our experiments.

Binding of the Pyr(x)PC species is clearly dependent on acyl chain length (Fig. 2A). Binding of lipids to nsL-TP is governed by an equilibrium reaction (Results, Eqn. 1) in which the amount of lipid that moves from the vesicle onto the protein, is determined by the free energy difference ΔG. The low percentage of Pyr(x)PC-binding indicate that this binding reaction is clearly endergonic. From the linear correlation between the binding energy (ΔG) and the *sn*-2 acyl-chain length of Pyr(x)PC (insert of Fig. 2A), it can be concluded that for each additional methylene unit in the *sn*-2 acyl-chain about 1 kJ/mol of extra energy is required for binding of Pyr(x)PC to nsL-TP. This is in support of the notion that nsL-TP has a very low affinity for natural, long acyl-chain phospholipids and that, therefore, in the presence of membranes, the bulk of nsL-TP is empty. The failure of isolating the nsL-TP/lipid complex [2,15,45] is in agreement with the endothermic binding characteristics.

From the linear dependence of Pyr(x)PC binding and the Pyr(x)PC mole fraction in the donor membrane (Fig. 2A) it can be inferred that there is no competition between unlabeled PC and Pyr(x)PC for the lipid-binding site on nsL-TP. This lack of competition is a direct consequence of the fact that under equilibrium conditions the bulk of the protein (more than 90%) is empty. The energetics of Pyr(x)PC-binding to nsL-TP are completely different from binding of Pyr(x)PC to PC-TP or PI-TP. Since in binding experi-

ments with similar donor vesicles PC-TP or PI-TP are always loaded with a PC or Pyr(x)PC molecule [36,39], the binding reactions with these lipids are exergonic. As a consequence, PC and Pyr(x)PC strongly compete for the lipid binding site of PC-TP or PI-TP. In contrast to what was observed for nsL-TP (Fig. 2A), this competition results in non-linear binding characteristics [37,38], with Pyr(10)PC as the preferred substrate [36,39]. Another major difference is the stability of the PC-TP/lipid or PI-TP/lipid complexes in the absence of vesicles [46,47].

The transfer profiles (Fig. 2B) show a remarkable resemblance with the binding profiles (Fig. 2A), suggesting that binding of Pyr(x)PC by nsL-TP is the controlling step in the transfer reaction. On the other hand, the linear dependency of the transfer of Pyr(x)PC by nsL-TP on the Pyr(x)PC mole fraction in the donor membrane also correlates very well with spontaneous transfer [12,48]. In this respect it is of interest that the logarithmic plot of the rates of transfer versus the *sn*-2 acyl-chain length is linear (inset of Fig. 2B). This linear dependency is described by:

$$-\log(v_p) = 0.24n + \alpha \qquad (4)$$

in which v_p is the rate of transfer, n is the number of carbon atoms in the *sn*-2 acyl chain, and α is a constant depending on the donor vesicle and protein concentration. For spontaneous transfer the following correlation was derived:

$$\log(T_{1/2}) = 0.23n + \beta \qquad (5)$$

in which $T_{1/2}$ is the halftime of spontaneous transfer, and β represents a constant depending on the number of double bonds of the acyl chain [49]. Since the halftime and initial velocity (v_s) of spontaneous transfer are inversely correlated [50], Eqn. 5 can be expressed as:

$$\log(v_s) = 0.23n + \beta' \qquad (6)$$

The remarkable resemblance between Eqns. 4 and 6 strongly suggests that nsL-TP only enhances the natural tendency of a lipid molecule to leave the membrane interface. This tendency may be envisaged as a (partial) outward movement of the lipid from the plane of the membrane. With a nsL-TP-molecule in the vicinity of the interface, this lipid molecule may then bind to the protein. In this way, nsL-TP lowers the energy barrier for the lipid molecule to leave the membrane. If it is assumed that lipid binding is the most energy consuming step in the lipid transfer process, then the activation energy for the nsL-TP-mediated lipid transfer would be about 6.3 kJ/mol for 1-palmitoyl-2-Pyr(6)PC as compared to about 93 kJ/mol required for the spontaneous transfer of 1-lauroyl-2-Pyr(9)PC [51], a species with comparable hydrophobicity. It is very likely, however, that also the binding reaction has a certain activation energy. Therefore the 6.3 kJ/mol should be regarded as a minimum value for the activation energy.

The binding and transfer of phosphoinositides is clearly dependent on the degree of phosphorylation (see Table I). In view of the net-positive charge of nsL-TP [52], it is very plausible that the ΔG of lipid binding decreases with increasing negative charge on the phospholipid. This stimulates phosphoinositide binding to nsL-TP to the extent that, for instance, Pyr(10)PIP$_2$-binding is an order of magnitude higher than Pyr(10)PC-binding under similar conditions (Table I). The transfer of the phosphoinositides was inversely correlated with the binding (Table I). This apparent discrepancy is not explained by an increase in negative charge on the donor vesicle due to the presence of the phosphoinositides, which could possibly slow down the dissociation velocity of nsL-TP from the donor vesicle. In control experiments it was shown that increased negative surface charge increased rather than decreased the rate of transfer of, for example, Pyr(6)PC (see Table II). Butko et al. [53] have also reported the stimulatory effect of negative donor vesicle charge on nsL-TP-mediated lipid transfer. Therefore, the 7-fold higher rate of Pyr(10)PI as compared to Pyr(10)PC transfer is a reflection of both this increased negative surface charge and of the increased binding affinity for Pyr(10)PI (factor 4). In our view, the decreased transfer of Pyr(10)PIP and even more for Pyr(10)PIP$_2$ is best explained by a decreased tendency of these lipids to leave nsL-TP once bound to it. Calibration studies similar to the one in Fig. 1, yielded further evidence for this hypothesis since the rate of release of the phosphoinositides from nsL-TP into the acceptor vesicle decreased with increasing phosphorylation degree (not shown). Apparently, the activation energy for this release reaction is increased. This may be caused by a decreased interaction of the nsL-TP/phosphoinositide complex with the negatively charged acceptor membrane, as a result of a (partial) charge reduction of nsL-TP. An alternative explanation is that the stronger electrostatic interaction within the complex hampers the release reaction.

From the very small effect of cholesterol on Pyr(6)PC binding (Fig. 3), we conclude that cholesterol does not compete with Pyr(6)PC for the lipid binding site and does not form a 1:1 complex with nsL-TP as reported by Schroeder et al. [14]. In support of this conclusion, the binding of the fluorescent sterol dehydroergosterol by nsL-TP was negligible (at most 2%), i.e. very endothermic (Fig. 4). In agreement, with this observation, Butko et al. [53] did not find a change in dehydroergosterol-polarisation upon addition of nsL-TP to vesicles containing dehydroergosterol. These findings confirm

the observations by Van Amerongen et al. [9] that injection of nsL-TP under a monolayer containing radiolabeled cholesterol did not result in a decrease in surface radioactivity. In contrast to these observations, Schroeder et al. [14] reported a 1:1 stoichiometry between nsL-TP and dehydroergosterol. In this study pure dehydroergosterol instead of mixed bilayers were mixed with an excess of nsL-TP. We believe that these extreme conditions most likely have given rise to the discrepancy with our results.

Despite the very low binding, dehydroergosterol was transferred quite readily by nsL-TP (Fig. 4). By comparing dehydroergosterol with Pyr(6)PC (Fig. 2B) one can see that the rates of transfer are similar if one takes into consideration that the donor vesicle concentration in the dehydroergosterol-experiments is twice as high. This indicates that nsL-TP very efficiently lowers the energy barrier for sterol transfer without forming a stable (low ΔG) sterol-nsL-TP complex. In our view, sterol transfer by nsL-TP comes very close to the model proposed by Nichols and Pagano [18] in which nsL-TP increases the off-rate of lipid-monomers from the bilayer. Initially dehydroergosterol couid have been bound to nsL-TP. However, upon nsL-TP leaving the membrane, dehydroergosterol may dissociate from nsL-TP almost immediately. In view of the lack of sterol binding observed in this study, we feel that the designation sterol carrier protein-2 should not be used for this protein.

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