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Phospholipid binding and transfer by the nonspecific lipid-transfer protein (sterol carrier protein 2)

A kinetic model

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The nonspecific lipid-transfer protein (nsL-TP) from bovine liver was studied by measuring the binding and transfer of the fluorescent phospholipid 1-palmitoyl-2-[6-(1-pyrenyl)-hexanoyl]-*sn*-glycero-3-phosphocholine (PamPyrGroPCho). A kinetic model is presented involving three steps: (a) interaction of nsL-TP with a membrane surface; (b) equilibration of PamPyrGroPCho monomers between the membrane and nsL-TP; and (c) dissociation of the nsL-TP/PamPyrGroPCho complex from the membrane surface. Steady-state analysis of the model yielded theoretical equations describing both binding and transfer kinetics. Computer analysis, using these equations, showed good fits with the experimental results and several kinetic constants could be calculated. From these constants it was inferred that incorporation of acidic phospholipids into vesicles enhanced the interaction of nsL-TP with the membrane interface (step a), without affecting the equilibrium binding of phospholipid monomers to nsL-TP (step b). As a result, the rate of nsL-TP-mediated PamPyrGroPCho transfer from donor to acceptor vesicles was greatly affected. Under the conditions of incubation, incorporation of the acidic lipids in the donor membrane vesicles stimulated transfer, whereas incorporation of these lipids in the acceptor membranes could lead to a virtually complete inhibition of transfer. From the results it is concluded that the formation of a soluble lipid–nsL-TP complex is the key step in nsL-TP-mediated phospholipid transfer.

The nonspecific lipid-transfer protein (nsL-TP; also called sterol carrier protein-2) catalyses *in vitro* the transfer of a wide range of lipids between membranes [1–5]. nsL-TP forms a water-soluble complex with fluorescently labeled phospholipids present in vesicles [6–9]. A similar approach has failed to demonstrate complex formation with sterols [9]. Using time-resolved fluorescence spectroscopy, it was shown that the 1,6-diphenyl-1,3,5-hexatriene moiety of 1-palmitoyl-2-[3-(*p*-diphenylhexatrienyl)-propionyl]-*sn*-glycero-3-phosphocholine (PamDphGroPCho) bound to nsL-TP, is completely immobilized and follows the rotation of nsL-TP [8]. Attempts, however, to isolate a lipid/nsL-TP complex, have failed [6, 8, 10, 11]. As opposed to a carrier mechanism, several other modes of action were proposed. These included the formation of a ternary complex between nsL-TP and the

donor and acceptor membranes [11–15] as well as a mechanism in which nsL-TP binds to membranes and increases the off-rate of lipid monomers from the membrane interface [16, 17]. The high correlation between spontaneous and nsL-TP-mediated transfer [9, 17, 18] was taken into account by the latter mechanism. Recently, it was shown that acidic phospholipids stimulated the nsL-TP-mediated intermembrane transfer of lipids [9, 19]. This indicates that the interaction of nsL-TP with the membrane plays a significant role in the mode of action of the protein.

In this paper, a steady-state kinetic model for phospholipid binding and transfer by nsL-TP is presented. This model gives an accurate description of both equilibrium phospholipid binding and steady-state lipid transfer, and takes into account the influence of membrane surface charge on these two processes. The data are consistent with a model in which the formation of a phospholipid/nsL-TP complex is essential.

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Abbreviations. nsL-TP, nonspecific lipid-transfer protein; PtdCho-TP, phosphatidylcholine-transfer protein; PtdIns-TP, phosphatidylinositol-transfer protein; PtdCho, phosphatidylcholine; PtdOH, phosphatidic acid; PtdIns, phosphatidylinositol; PtdInsP, phosphatidylinositol 4-phosphate; PtdInsP₂, phosphatidylinositol 4,5-bisphosphate; Tnp-PtdEtn, *N*-[2,4,6-trinitrophenyl]-phosphatidylethanolamine; PamPyrGroPCho, 1-palmitoyl-2-[6-(1-pyrenyl)-hexanoyl]-*sn*-glycero-3-phosphocholine; PamDphGroPCho, 1-palmitoyl-2-[3-(*p*-diphenylhexatrienyl)propionyl]-*sn*-glycero-3-phosphocholine; Pam(NbdLau)GroPCho, 1-palmitoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine; Nbd, 7-nitro-2,1,3-benzoxadiazol-4-yl.

EXPERIMENTAL PROCEDURES

Materials

Egg-yolk phosphatidylcholine (PtdCho) and phosphatidic acid (PtdOH) were obtained from Sigma. 1-Palmitoyl-2-[6-(1-pyrenyl)-hexanoyl]-*sn*-glycero-3-phosphocholine (PamPyrGroPCho) was synthesized and purified as described [20]. *N*-[2,4,6-Trinitrophenyl]-phosphatidylethanolamine (Tnp-PtdEtn) was synthesized from egg-yolk phosphatidylethanolamine and trinitrobenzenesulfonic acid [21]. nsL-TP was purified from bovine liver according to Van Amerongen et

al. [18] and stored at -20°C in 60% (by vol.) glycerol. All other chemicals used were of analytical grade.

Phospholipid concentration

The concentration of PamPyrGroPCho was determined by measuring the absorbance in ethanol ($\epsilon_{342} = 42000 \text{ M}^{-1} \text{ cm}^{-1}$ [20]). Concentrations of the other phospholipids were estimated by phosphorous determination [22].

Fluorescence measurements

Fluorimetric spectroscopy was performed on a SLM-Aminco SPF-500C spectrofluorimeter equipped with a stirring device and a thermostatted cuvette holder. The temperature was kept at 25°C . Excitation and emission wavelength were 346 nm and 377 nm with corresponding bandpasses of 2.5 nm and 10 nm, respectively. The buffer used consisted of 20 mM Tris/HCl pH 7.4, 100 mM NaCl, 1 mM EGTA, 1 mM EDTA (Tris buffer) and was routinely filtered through a millipore filter (0.45 μm). The emission fluorescence was corrected for inner filtering if necessary.

Donor vesicle preparation

Solutions of PamPyrGroPCho, egg PtdCho, Tnp-PtdEtn and PtdOH in chloroform, were mixed and dried down by a stream of N_2 . The dried lipids were resuspended in the Tris buffer (0.1–0.5 mM phospholipid) and subjected to ultrasonication for 1 min with a Branson probe sonifier under a N_2 atmosphere at 0°C . The Tris buffer was saturated with argon prior to resuspension. This procedure did not alter the pyrene moiety as the fluorescence spectrum of the donor vesicle preparation dissolved in ethanol was identical to that of a standard PamPyrGroPCho solution.

Acceptor vesicle preparation

Acceptor vesicles consisting of PtdCho and varying amounts of PtdOH were prepared by drying down the lipid mixture from chloroform, followed by suspension of the lipids in the Tris buffer (1–5 mM phospholipid) and ultrasonication under a N_2 atmosphere at 0°C for 5 min with a Branson sonifier. Prior to suspension the Tris buffer was saturated with argon. Vesicles were kept at 0°C and were always used within one day. Vesicle (micellar) suspensions consisting of PtdOH, phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdInsP) or phosphatidylinositol 4,5-bisphosphate (PtdInsP₂) were prepared by ultrasonication of a lipid emulsion (50 μM) for 3 min (5 s on and 10 s off) as described above. Interruption of sonication was necessary to prevent degradation of PtdInsP and PtdInsP₂ [23].

Phospholipid binding assay

Binding of PamPyrGroPCho to nsL-TP was assayed by the method described for the phosphatidylcholine-transfer protein (PtdCho-TP) [24] or for the phosphatidylinositol-transfer protein (PtdIns-TP) [25]. Briefly, aliquots of nsL-TP (0.09–0.71 μM final concentration) were added to donor vesicles consisting of 50% (by mol) PamPyrGroPCho, 10% (by mol) Tnp-PtdEtn, egg PtdCho and PtdOH (0.25–10 μM total phospholipid, final volume of 2 ml). The increase in pyrene-monomer fluorescence was recorded as a function of time. The slope of the fluorescence increase (initial rate of

binding) and the increase at equilibrium (which was usually reached after 10–15 min) were determined. The fluorescence signal was calibrated as described [9], so that the binding could be expressed as the molar amount of PamPyrGroPCho bound to nsL-TP.

Phospholipid transfer assay

This assay is similar to the one described for PtdIns-TP [26] and is based on the pyrene-monomer fluorescence increase as a result of translocation of a PamPyrGroPCho molecule from a quenched donor to an unquenched acceptor vesicle. Briefly, acceptor vesicles (2.5–200 μM) were mixed with donor vesicles (0.25–10 μM) present in the cuvette containing the Tris buffer. The spontaneous transfer of PamPyrGroPCho from donor to acceptor vesicles was followed by recording the increase in pyrene-monomer fluorescence with time. Subsequently, nsL-TP was added (0.09–1.36 μM) to initiate the protein-mediated phospholipid transfer reaction (final volume of 2 ml). Transfer rates were corrected for the spontaneous transfer. The pyrene-monomer fluorescence was calibrated with a standard vesicle preparation which, apart from the 0.05% (by mol) PamPyrGroPCho, was identical to the acceptor vesicles used. Rates of transfer were expressed as pmol PamPyrGroPCho transferred/min.

Kinetic treatment

Kinetic analysis of transfer between donor and acceptor vesicles

The transfer process is described by the following set of reactions:

where P, D and A represent nsL-TP, donor membrane and acceptor membrane, respectively. PL designates nsL-TP with a bound PamPyrGroPCho molecule. $\text{P} \cdot \text{D}$ and $\text{P} \cdot \text{A}$ are complexes between nsL-TP void of bound lipid, with the donor and the acceptor membrane, respectively. Similarly, $\text{PL} \cdot \text{D}$ and $\text{PL} \cdot \text{A}$ represent complexes between nsL-TP that contains PamPyrGroPCho, with the donor membrane and the acceptor membrane. The binding reaction (reaction A) was divided into three steps: two steps concern the association/dissociation reactions between nsL-TP and the donor membrane (k_1 , k_{-1}), and one step concerns the equilibration of lipid monomers between nsL-TP and the donor vesicle (reactions k_d and k_{-d}). It is assumed that the association/dissociation reactions (k_1 , k_{-1}) are described by similar rate constants for both P or PL.

In this model the initial rate of transfer is given by the velocity at which PamPyrGroPCho is released in the acceptor membrane. This can be expressed as:

$$v_0 = k_{-a}[\text{PL} \cdot \text{A}] - k_a[\text{P} \cdot \text{A}]. \quad (1)$$

If steady-state conditions are assumed the following equations can be derived:

$$\begin{aligned} \frac{d[\text{P}]}{dt} &= -k_1[\text{P}][\text{D}] + k_{-1}[\text{P} \cdot \text{D}] \\ &\quad - k_2[\text{P}][\text{A}] + k_{-2}[\text{P} \cdot \text{A}] = 0 \end{aligned} \quad (2)$$

$$\begin{aligned} \frac{d[PL]}{dt} &= -k_1[PL][D] + k_{-1}[PL \cdot D] \\ &\quad - k_2[PL][A] + k_{-2}[PL \cdot A] = 0 \end{aligned} \quad (3)$$

$$\begin{aligned} \frac{d[P \cdot D]}{dt} &= k_1[P][D] - k_{-1}[P \cdot D] \\ &\quad - k_d[P \cdot D] + k_{-d}[PL \cdot D] = 0 \end{aligned} \quad (4)$$

$$\begin{aligned} \frac{d[PL \cdot D]}{dt} &= k_1[PL][D] - k_{-1}[PL \cdot D] \\ &\quad + k_d[P \cdot D] - k_{-d}[PL \cdot D] = 0 \end{aligned} \quad (5)$$

$$\begin{aligned} \frac{d[P \cdot A]}{dt} &= k_2[P][A] - k_{-2}[P \cdot A] \\ &\quad - k_a[P \cdot A] + k_{-a}[PL \cdot A] = 0 \end{aligned} \quad (6)$$

$$\begin{aligned} \frac{d[PL \cdot A]}{dt} &= k_2[PL][A] - k_{-2}[PL \cdot A] \\ &\quad + k_a[P \cdot A] - k_{-a}[PL \cdot A] = 0. \end{aligned} \quad (7)$$

The total protein concentration $[P_{tot}]$ is given by:

$$[P_{tot}] = [P] + [PL] + [P \cdot D] + [PL \cdot D] + [P \cdot A] + [PL \cdot A]. \quad (8)$$

From rewriting and substitution of Eqns (4) and (5) and from Eqns (6) and (7), Eqns (9) and (10), respectively, are obtained:

$$\begin{aligned} [P \cdot D] &= \frac{\alpha_d}{k_{-1}} [D][PL] + \left(\frac{\alpha_d}{k_{-1}} + \frac{\alpha_d}{k_{-d}} \right) [D][P]; \\ \alpha_d &= \frac{k_{-d}k_1}{k_{-1} + k_d + k_{-d}} \end{aligned} \quad (9)$$

$$\begin{aligned} [P \cdot A] &= \frac{\alpha_a}{k_{-2}} [A][PL] + \left(\frac{\alpha_a}{k_{-2}} + \frac{\alpha_a}{k_{-a}} \right) [A][P]; \\ \alpha_a &= \frac{k_{-a}k_2}{k_{-2} + k_a + k_{-a}}. \end{aligned} \quad (10)$$

Substitution of Eqns (9) and (10) in Eqn (2) yields:

$$\begin{aligned} \frac{[P]}{[PL]} &= \frac{\alpha_d[D] + \alpha_a[A]}{K_d\alpha_d[D] + K_a\alpha_a[A]}; \\ K_d &= k_d/k_{-d}; \quad K_a = k_a/k_{-a}. \end{aligned} \quad (11)$$

Addition of Eqns (4) and (5) and of Eqns (6) and (7) yield Eqn (12) and Eqn (13) respectively:

$$[P \cdot D] + [PL \cdot D] = \{[P] + [PL]\}K_1[D]; \quad K_1 = k_1/k_{-1} \quad (12)$$

$$[P \cdot A] + [PL \cdot A] = \{[P] + [PL]\}K_2[A]; \quad K_2 = k_2/k_{-2}. \quad (13)$$

Substitution of Eqns (12) and (13) into Eqn (8) results in Eqn (14):

$$[P_{tot}] = \{[P] + [PL]\} \{1 + K_1[D] + K_2[A]\}. \quad (14)$$

Substitution of Eqn (11) into Eqn (14) yields:

$$[PL] = \frac{[P_{tot}]}{\{1 + K_1[D] + K_2[A]\} \left\{ \frac{\alpha_d[D] + \alpha_a[A]}{K_d\alpha_d[D] + K_a\alpha_a[A]} + 1 \right\}}. \quad (15)$$

By subsequently using Eqns (6) and (14), Eqn (1) can be rewritten as:

$$v_o = k_{-2}[P \cdot A] - k_2[P][A] = \alpha_a[A]\{[PL] - K_a[P]\}. \quad (16)$$

Finally substitution of Eqns (11) and (15) into Eqn (16) yields:

$$v_o = \frac{\alpha_d \alpha_a (K_d - K_a) [D] [A] [P_{tot}]}{\{\alpha_d(1 + K_d)[D] + \alpha_a(1 + K_a)[A]\} \{1 + K_1[D] + K_2[A]\}}. \quad (17)$$

The reaction constants k_d and k_a are dependent on the mole fraction of PamPyrGroPCho in the membrane [9]. As on time zero the mole fraction of PamPyrGroPCho in the acceptor vesicles is zero, k_a and hence also K_a become zero. Under these experimental conditions, Eqn (17) can be simplified to:

$$\begin{aligned} v_o &= \frac{\alpha_d \alpha'_a K_d [D] [A] [P_{tot}]}{\{\alpha_d(1 + K_d)[D] + \alpha'_a[A]\} \{1 + K_1[D] + K_2[A]\}}; \\ \alpha'_a &= \frac{k_{-a}k_2}{k_{-2} + k_{-a}}. \end{aligned} \quad (18)$$

Kinetic treatment of equilibrium binding

The binding process is described by reactions A. The equilibrium binding, therefore, can be directly derived from Eqn (15) by, substituting $[A] = 0$:

$$[PL] = \frac{K_d}{1 + K_d} \frac{[P_{tot}]}{\{1 + K_1[D]\}}. \quad (19)$$

This equation predicts that equilibrium binding is independent of the donor concentration at conditions where $K_1[D] \ll 1$.

RESULTS

Exchangeability of donor lipids

In view of the net-transfer capability of nsL-TP, it is of interest to check whether the protein has access to all lipids in the donor membrane, or only to the lipids in the outer leaflet. At low pyrene-phospholipid concentration (0–5%) in a PtdCho bilayer, the pyrene excimer fluorescence is linear with the pyrene surface concentration [27]. The exchangeability of PamPyrGroPCho from the donor vesicle can therefore be estimated by monitoring the pyrene-excimer fluorescence decrease in the presence of a large excess of unlabeled acceptor vesicles and nsL-TP. Since PamPyrGroPCho in the acceptor vesicles is greatly diluted, the excimer fluorescence in the acceptor vesicles is negligible. As shown in Fig. 1, 60% of PamPyrGroPCho in the donor membrane is transferred by nsL-TP, independent on the amount of PtdOH present in the acceptor membrane. In the absence of nsL-TP an identical amount of PamPyrGroPCho is transferred, though at a much slower rate. This experiment is taken to indicate that nsL-TP, like PtdCho-TP [28] and PtdIns-TP [29] has only access to phospholipids in the outer leaflet of the donor vesicle in agreement with previous studies [30, 31]. The donor vesicles used in the other experiments (see below) contained 10% (by mol) Tnp-PtdEtn, and hence the asymmetry of donor lipid accessibility to nsL-TP could not be determined for these vesicles. Although it is not critical for the proposed kinetic model, we assume that, for the donor vesicles described below, this accessibility is unchanged.

Fig. 1. Exchangeability of PamPyrGroPCho in the donor vesicle. Lipid transfer was measured between donor vesicles (10 μ M) consisting of PamPyrGroPCho, PtdOH and PtdCho, molar ratio 5:10:85, and acceptor vesicles (250 μ M) consisting of PtdOH and PtdCho, molar ratio 5:95 (○, ●) or of 10:90 (□, ■) which were incubated in the presence (●, ■) or absence (○, □) of 1.7 μ M nsL-TP (final volume 2 ml). The pyrene excimer fluorescence was obtained by exciting at 346 nm (bandpass 2 nm) and measuring the fluorescence at 470 nm (bandpass 10 nm).

Fig. 2. Fluorescence measurement of PamPyrGroPCho binding to nsL-TP. Arrow A indicates the addition of donor vesicles (1 μ M) consisting of PamPyrGroPCho, Tnp-PtdEtn and PtdCho (molar ratio 50:10:40). At arrow B the binding reaction was initiated by the addition of nsL-TP (0.34 μ M, final volume 2 ml). The dashed line indicates the extrapolated initial rate of binding. The fluorescence was recorded at excitation 346 nm (bandpass 2 nm) and emission 377 nm (bandpass 10 nm).

Binding kinetics

The result of a typical binding experiment is shown in Fig. 2. From each binding experiment two parameters were obtained: the equilibrium binding, and the initial rate of binding. These slow binding kinetics have not been observed for PtdCho-TP and PtdIns-TP [25] and indicate that the rate-limiting step is not diffusion-controlled.

Effect of nsL-TP concentration

The effect of the concentration of nsL-TP on the binding kinetics is presented in Fig. 3. The equilibrium binding at a constant donor vesicle concentration (Fig. 3A) is clearly linearly dependent on the protein concentration. This supports Eqn (19). Also the initial rate of binding (Fig. 3B) exhibited a first-order dependency with the nsL-TP concentration which is in agreement with reaction A. Since steady-state conditions are not met at $t = 0$ (see Fig. 2), a kinetic analysis was not carried out on the initial binding process. Nichols

Fig. 3. Effect of nsL-TP concentration on PamPyrGroPCho binding to nsL-TP. The experiments were performed as indicated in Fig. 2. The donor vesicles (1 μ M) consisted of PamPyrGroPCho, Tnp-PtdEtn and PtdCho (molar ratio 50:10:40). In (A), the symbols represent the equilibrium concentration of the nsL-TP/PamPyrGroPCho complex. In (B), the symbols represent the initial rate of binding.

analyzed initial lipid-binding kinetics by nsL-TP (see Discussion [7]).

Effect of donor vesicle concentration

As seen in Fig. 4A, the amount of PamPyrGroPCho binding to nsL-TP increases with increasing donor vesicle phospholipid concentration up to 1 μ M, whereas above 1 μ M a zero-order dependency between donor vesicle concentration and the PamPyrGroPCho binding is apparent. The fact that, at $[D] > 1 \mu$ M, binding is independent of the donor vesicle concentration is in agreement with Eqn (19) and lends strong support for the proposed model. The decrease in lipid binding at low $[D]$ contradicts Eqn (19). It cannot be explained by exhaustion of the donor PamPyrGroPCho content, since, even at the lowest $[D]$, only 6% of the PamPyrGroPCho is bound to nsL-TP (8 nM equilibrium concentration cf. 125 nM total PamPyrGroPCho concentration). We propose an additional reaction where nsL-TP releases its bound lipid into the buffer (see Appendix). Under these conditions, the so-called equilibrium binding no longer reflects a true equilibrium, but a steady-state process. In this extended model, the release of bound lipid into the buffer is assumed to be a very slow process (minute timescale) only occurring to a significant extent at very low vesicle (lipid) concentration. Given these assumptions, we could optimize a K_d at 0.046, $k_3/\alpha'_d = 0.24 \mu$ M, and $K_1 = 7 \text{ mM}^{-1}$ (Eqn A9, see legend to Fig. 4). This gives rise to a β value of 0.24 μ M representing the donor vesicle concentration at which the release reaction of the bound lipid to the donor membrane (reaction A) and the release reaction to the buffer (reaction C) occur at identical rates. Therefore at $[D] > 1 \mu$ M, Eqn (A9) can be approximated by Eqn (19).

Fig. 4. Effect of donor vesicle concentration on PamPyrGroPCho binding by nsL-TP. The experiments were performed as described in Fig. 2. Donor vesicles consisted of PamPyrGroPCho, Tnp-PtdEtn and PtdCho (molar ratio 50:10:40). The nsL-TP concentration was 0.34 μM . In (A), the symbols represent the equilibrium concentrations of the nsL-TP/PamPyrGroPCho complex. Nonlinear least-squares analysis according to Eqn (A9) (see Appendix) yielded $K_d = 0.046 \pm 0.004$, $K_1 = 7 \pm 19 \text{ mM}^{-1}$ and $k_3/\alpha_d = 0.28 \pm 0.07 \mu\text{M}$. The curve as indicated was drawn based on these constants and showed a correlation coefficient $r = 0.979$. In (B), the symbols represent the initial rate of binding.

The initial binding velocity is strikingly linear with the donor vesicle concentration (see Fig. 4B). This indicates that the rate-limiting step in the binding process occurs in a first-order dependency with the donor vesicle concentration, and thereby lends further support for a model involving reaction A (see methods).

Effect of donor vesicle surface charge

In Fig. 5A it is shown that the equilibrium binding at three different nsL-TP concentrations is hardly dependent on the PtdOH surface concentration (or surface charge). Since the association of nsL-TP with these vesicles can be considered as minimal at these low $[D]$, (i.e. $K_1[D] \ll 1$, see also below), it is inferred that K_d (Eqn 19) is independent of the membrane surface charge. In other words the equilibration of lipid monomers on and off the protein at the lipid-protein interface (second step in reaction A) is not dependent on the charge of the membrane. This confirms the experiments described in another study [9]. The initial rate of binding as depicted in Fig. 5B, however, does increase with increasing membrane surface charge. Apparently the first step in reaction A (the association of nsL-TP with membranes) is accelerated by increasingly negatively charged membranes, resulting in a faster equilibration (see also discussion below).

Transfer kinetics

Then nsL-TP mediated transfer of PamPyrGroPCho from donor to acceptor vesicles was monitored as shown in Fig. 6.

Fig. 5. Effect of donor vesicle surface charge on binding of PamPyrGroPCho to nsL-TP. Measurements were carried out as described in Fig. 2. The donor vesicles (1 μM) consisted of PamPyrGroPCho, PtdOH, Tnp-PtdEtn and PtdCho (molar ratio 50:0:10:40 or 50:10:10:30 or 50:20:10:20). The nsL-TP concentration was 0.17 μM (■), 0.34 μM (▲) or 0.68 μM (●). In (A), the equilibrium binding and in (B) the initial rate of binding is plotted as a function of the PtdOH content of the donor vesicle.

Fig. 6. Fluorescence measurement of transfer of PamPyrGroPCho from donor to acceptor vesicles. Arrow A indicates the addition of 5 μM donor vesicles containing PamPyrGroPCho, Tnp-PtdEtn and PtdCho (molar ratio 20:10:70). Arrow B represents the addition of the acceptor vesicles (50 μM) consisting of PtdCho and PtdOH (molar ratio 95:5). At C, nsL-TP was added to give a final concentration of 0.34 μM (total volume 2 ml). The dashed line marked with v_b represents the extrapolated spontaneous transfer whereas the dashed line labeled v_{b+p} represents the extrapolation of the initial rate of transfer at time C. The nsL-TP-mediated PamPyrGroPCho transfer was obtained by subtraction of v_b from v_{b+p} . The pyrene fluorescence was recorded at excitation 346 nm (bandpass 2 nm) and emission 377 nm (bandpass 10 nm). For other experimental details see Experimental Procedures.

After addition of unlabeled acceptor vesicles (arrow B) to quenched donor vesicles containing PamPyrGroPCho, the spontaneous rate of transfer from donor to acceptor vesicles is measured (v_b). After addition of nsL-TP (arrow C) the rate of transfer is increased to a large extent. This nsL-TP-medi-

Fig. 7. Effect of nsL-TP concentration on nsL-TP-mediated transfer of PamPyrGroPCho. Transfer was measured as described in Fig. 6 at the indicated nsL-TP final concentrations. The donor and acceptor vesicle concentration and composition was identical to that described in Fig. 6.

ated transfer was always corrected for the spontaneous transfer. The kinetic model was tested by varying the concentration and/or composition of the three components.

Effect of nsL-TP concentration

The dependency of PamPyrGroPCho transfer on the nsL-TP concentration was studied by performing transfer experiments at variable nsL-TP concentrations. At a fixed donor and acceptor vesicle concentration, this dependency was found to be linear in a wide nsL-TP concentration range (0–1.5 μM, see Fig. 7), which lends support for the presented kinetic model (see Eqn 18).

Effect of acceptor vesicle concentration and surface charge

At a fixed donor vesicle (5 μM) and nsL-TP concentration (0.34 μM), a variation of the acceptor vesicle concentration and composition clearly influenced nsL-TP-mediated PamPyrGroPCho transfer (see Fig. 8). As seen from the curve corresponding to the acceptor vesicles containing 5 mol PtdOH/100 mol, the rate of transfer increased at acceptor vesicle concentrations up to 50 μM whereas above 50 μM the rate of transfer was almost constant. A curve analysis was performed with Eqn (18), using $K_d = 0.046$ and $K_1 = 7 \text{ mM}^{-1}$ determined from the binding experiments depicted in Fig. 4A. Three constants were optimized by non-linear least-squares methods yielding $\alpha_d = 378 \text{ pmol} \cdot \text{min}^{-1} \mu\text{M}^{-2}$, $\alpha'_a = 259 \text{ pmol} \cdot \text{min}^{-1} \mu\text{M}^{-2}$ and $K_2 = 1.4 \text{ mM}^{-1}$, from which the curve as drawn in Fig. 8 was calculated. (Note that here the transfer velocity v_0 is defined as pmol PamPyrGroPCho transferred/min. Consequently, the dimension of both α'_a and α_d equals $\text{pmol} \cdot \text{min}^{-1} \mu\text{M}^{-2}$. Since the volume of incubation is always 2 ml, the dimension of velocity can also be defined as $\text{nM} \cdot \text{min}^{-1}$. The values in Figs 3B, 4B, 5B, 7, 8 and 9, have then to be divided by 2 ml. Similarly, division by 2000 μl converts the dimensions of both α'_a and α_d to $\text{min}^{-1} \mu\text{M}^{-1}$.)

The experiments were repeated with acceptor vesicles with a higher PtdOH content. As seen from Fig. 8, this results in an inhibition of nsL-TP-mediated transfer at high acceptor vesicle concentration. As the other curves contain fewer data points, a constraint was put upon the fits to these curves by fixation of the constant α_d in addition to K_d and K_1 (which are all independent of the acceptor membrane). Conse-

Fig. 8. Effect of acceptor vesicle concentration and charge on the nsL-TP-mediated transfer of PamPyrGroPCho. Transfer was measured as described in Fig. 6. The incubation medium (2 ml) contained donor vesicles (5 μM) consisting of PamPyrGroPCho, Tnp-PtdEtn and PtdCho (molar ratio 20:10:70), nsL-TP (0.34 μM) and variable concentrations of acceptor vesicles consisting of PtdCho and PtdOH at molar ratios of 95:5 (●), 90:10 (▲), 85:15 (◆), or 80:20 (■). Nonlinear least-squares analysis was done using Eqn (18) (see Experimental Procedures) using $K_d = 0.046$ and $K_1 = 7 \text{ mM}^{-1}$. For the data corresponding to 5% PtdOH (●) three kinetic constants were determined: $\alpha_d = 378 \pm 8 \text{ pmol} \cdot \text{min}^{-1} \mu\text{M}^{-2}$, $\alpha'_a = 259 \pm 10 \text{ pmol} \cdot \text{min}^{-1} \mu\text{M}^{-2}$, and $K_2 = 1.4 \pm 0.2 \text{ mM}^{-1}$ ($r = 0.998$). For the other data two kinetic constants were obtained due to fixation of $\alpha_d = 378 \text{ pmol} \cdot \text{min}^{-1} \mu\text{M}^{-2}$ in addition to K_d and K_1 . For the data corresponding to 10% (▲), 15% (◆) and 20% (■) PtdOH, $\alpha'_a = 552 \pm 178 \text{ pmol} \cdot \text{min}^{-1} \mu\text{M}^{-2}$ and $K_2 = 8.5 \pm 0.9 \text{ mM}^{-1}$ ($r = 0.969$); $\alpha'_a = 974 \pm 161 \text{ pmol} \cdot \text{min}^{-1} \mu\text{M}^{-2}$ and $K_2 = 23.4 \pm 0.9 \text{ mM}^{-1}$ ($r = 0.997$); and $\alpha'_a = 1446 \pm 103 \text{ pmol} \cdot \text{min}^{-1} \mu\text{M}^{-2}$ and $K_2 = 59.9 \pm 0.7 \text{ mM}^{-1}$ ($r = 0.9998$) were determined respectively. The curves drawn in the figure are calculated from these constants.

quently, only two parameters were optimized: K_2 and α'_a (for actual values, see the legend to Fig. 8). The results as indicated by the curves drawn in Fig. 8 indicate good fits with the experimental data, despite the constraint, and thereby support the model (Eqn 18). From the kinetic constants it is clear that both K_2 and α'_a increase with increasing negative surface charge in the acceptor vesicles.

Effect of donor vesicle concentration and surface charge

The effect of the donor vesicles on nsL-TP-mediated transfer was tested at a fixed acceptor vesicle concentration (50 μM, 5%, by mol, PtdOH) and nsL-TP concentration (0.34 μM), see Fig. 9. When PtdOH was included in the donor vesicles, the rate of transfer increased significantly. This is in agreement with previous studies [9, 19]. Also the deviation from linearity became more pronounced when the PtdOH concentration was increased in the donor vesicle (see Fig. 9). Kinetic analysis was performed with Eqn (18) using $\alpha'_a = 259 \text{ pmol} \cdot \text{min}^{-1} \mu\text{M}^{-2}$, $K_2 = 1.4 \text{ mM}^{-1}$, and $K_d = 0.046$ (determined from previous experiments) so that α_d and K_1 could be optimized by least-squares analysis. The results of the analysis are represented by the curves drawn in Fig. 9 (see legend for the constants). In agreement with the experiments described in Fig. 8, α_d and K_1 also increase with increasing negative surface charge in the donor membrane.

Inhibition experiments

To further explore the affinity of nsL-TP for negatively charged surfaces, inhibition experiments were performed em-

Fig. 9. Effect of donor vesicle concentration and charge on the nsL-TP-mediated transfer of PamPyrGroPCho. Transfer was measured as described in Fig. 6. The incubation medium (2 ml) contained acceptor vesicles (50 μ M) consisting of PtdCho and PtdOH (molar ratios 95:5), nsL-TP (0.34 μ M) and donor vesicles at variable concentrations containing PamPyrGroPCho, PtdOH, Tnp-PtdEtn and PtdCho at molar ratios of 20:0:10:70 (●), 20:5:10:65 (▲), 20:10:10:60 (◆), or 20:20:10:50 (■). The experimental data were fitted to Eqn (18) by using $\alpha'_a = 259 \text{ pmol} \cdot \text{min}^{-1} \mu\text{M}^{-1}$, $K_2 = 1.4 \text{ mM}^{-1}$ and $K_d = 0.046$. For the data corresponding to 0% (●), 5% (▲), 10% (◆), and 20% (■) PtdOH, $\alpha_d = 378 \pm 8 \text{ pmol} \cdot \text{min}^{-1} \mu\text{M}^{-2}$ and $K_1 = 23 \pm 6 \text{ mM}^{-1}$ ($r = 0.997$); $\alpha_d = 924 \pm 57 \text{ pmol} \cdot \text{min}^{-1} \mu\text{M}^{-2}$ and $K_1 = 35.9 \pm 1.8 \text{ mM}^{-1}$ ($r = 0.9997$); and $\alpha_d = 2450 \pm 55 \text{ pmol} \cdot \text{min}^{-1} \mu\text{M}^{-2}$ and $K_1 = 52 \pm 2 \text{ mM}^{-1}$ ($r = 0.997$) were determined respectively. The curves drawn in the figure were calculated from these constants.

Fig. 10. Inhibition of nsL-TP-mediated transfer by acidic phospholipid vesicles/micelles. The transfer of PamPyrGroPCho was measured as described in Fig. 6 with donor vesicles (2 μ M) containing PamPyrGroPCho, Tnp-PtdEtn, and PtdCho (molar ratio 10:10:80), acceptor vesicles (50 μ M) containing PtdCho and PtdOH (molar ratio 95:5) and nsL-TP (0.34 μ M). The sonicated acidic phospholipids PtdIns (●), PtdOH (○), PtdInsP (▲) and PtdInsP₂ (■) were added prior to the initiation of transfer by nsL-TP.

ploying donor and acceptor vesicles and a third vesicle/micelle population consisting of pure negatively charged phospholipids. As seen from Fig. 10, relatively low concentrations of these inhibitory vesicles/micelles may have a severe effect on nsL-TP-mediated transfer. An addition of as little as 5 μ M of PtdInsP₂ micelles completely inhibited nsL-TP-mediated PamPyrGroPCho transfer (see Fig. 10). The inhibition is explained by an association of nsL-TP with the PtdInsP₂ micelle after which the protein is incapable of transferring donor lipids to the acceptor membranes.

DISCUSSION

Validation of the kinetic model

The main reason for proposing a kinetic model is to obtain a better understanding of the mode of action of nsL-TP-mediated transfer of PtdCho. In this model both binding and transfer characteristics are combined. The validation of reactions A and B (see Experimental Procedures), is given by the observation that nsL-TP is able to extract a phospholipid monomer from a membrane and accommodate it into an endogenous lipid binding site [6–9] after which the phospholipid follows the rotation mobility of a free dissolved nsL-TP monomer [8]. In the model, the binding reaction is divided into three steps: an association of nsL-TP with a donor membrane; a step where one phospholipid monomer translocates from the membrane to nsL-TP; and another association (dissociation) reaction of the nsL-TP/phospholipid complex with the membrane. The translocation step is very likely dependent on the membrane lipid class and its surface concentration, whereas the association steps are more dependent on physical parameters like membrane charge and size. This division allowed us to determine kinetic constants describing these individual steps.

Two assumptions are made in the model. The first is that the association constants K_1 (and K_2) are independent of whether the protein contains an endogenous phospholipid molecule or not. This assumption is based on the facts that (a) upon binding of PtdCho the charge on nsL-TP will not change; (b) the mass of nsL-TP will increase only 5% after PtdCho binding; (c) without this assumption a very complex equation is obtained for v_0 (not shown) with which it is practically impossible to determine the various constants; and (d) the correctness of a model is likely to increase with the simplicity of the model [32]. The second assumption pertaining to the steady-state conditions is based on the near linearity of the fluorescence increase after addition of nsL-TP to a mixed donor/ acceptor vesicle solution, extending over a long time range (see Fig. 6). This last assumption has also been made for the analysis of the transfer kinetics of PtdCho-TP and PtdIns-TP [26, 33, 34].

An alternative model invoked by the correlation between spontaneous and nsL-TP-mediated transfer [9, 17, 18] which involves pathways where lipid monomers dissociate spontaneously from the membrane into the aqueous phase after which they eventually may bind to nsL-TP, is excluded since such a mechanism would lead to nonlinear relationships between binding and transfer with [nsL-TP] which we did not observe (see Figs 3 and 7). Furthermore such a model cannot explain a stimulation of transfer by nsL-TP because the rate-limiting step in spontaneous transfer is the dissociation of a lipid monomer from the membrane interface [16]. Models in which nsL-TP binds to the membrane and increases the off-rate of lipid monomers or in which nsL-TP forms a ternary complex between donor and acceptor vesicles [11–16] are also excluded. These models can only explain the fluorescence increase observed in the binding experiments in terms of a complex between nsL-TP, the donor membrane and the fluorescent lipid (like [PL · D] in reaction A). First it is expected that such a complex will be accessible to quenching by the trinitrophenyl moieties of the Tnp-PtdEtn present in the donor membrane (so we do not expect a substantial increase in fluorescence intensity upon formation of such a complex). Secondly, these models would predict an increase of equilibrium binding with increasing association of nsL-TP with the donor vesicle which is not observed. Consequently,

the zero order dependency with $[D]$ in Fig. 4A and the results in Fig. 5A can only be explained with these alternative models if we assume that 100% of nsL-TP is bound to the donor membrane (at $[D] > 1 \mu\text{M}$) which would give 100-fold higher association constants than the ones observed in Fig. 8.

Binding experiments

One additional pathway (k_3) was needed to explain the binding data (see Appendix). This reaction was found to be of minor importance at $[D] > 1 \mu\text{M}$. Therefore we excluded this pathway from the analysis of the transfer experiments. From combining $\alpha'_d = 372 \text{ pmol} \cdot \text{min}^{-1} \mu\text{M}^{-2}$ (or $0.19 \text{ min}^{-1} \mu\text{M}^{-1}$), (see Figs 8 and 9) with $k_3/\alpha'_d = 0.24 \mu\text{M}$ (Fig. 4A) it is inferred that k_3 equals 0.05 min^{-1} . This means that only 5% of the nsL-TP/phospholipid complex dissociates into nsL-TP and phospholipid/min (regardless of donor, or acceptor, concentration), which supports the notion that at high $[D]$ and $[A]$ this pathway is insignificant. It is of note that this pathway explains the failure to isolate the nsL-TP/phospholipid complex [6, 8, 10] since, upon separation of donor membranes from nsL-TP, $[D] \ll 1 \mu\text{M}$ after which nsL-TP releases its bound phospholipid into the medium.

In a previous study, Nichols also investigated lipid-binding kinetics by nsL-TP [7]. In that study the initial binding kinetics were studied as opposed to the equilibrium (or steady-state) binding described here. Based on the experiments with 1-palmitoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-dodecanoyl]-sn-glycero-3-phosphocholine [Pam-(NbdLau)PyrGroPCho], Nichols concluded that lipid binding and release was a complex mechanism involving not only a collisional pathway (reaction A) but also a monomer-diffusion one. In this study, we could adequately describe our experimental data without including a monomer-diffusion step. Also the linear relationships between lipid-vesicle concentration and nsL-TP concentration with initial binding rates (Figs 3B and 4B) are in agreement with a collisional step determining the initial binding process. In a previous study, in which we described the acyl-chain dependency of equilibrium binding and steady-state transfer, we proposed that nsL-TP lowers the energy barrier for lipids to leave the membrane, but the relative tendency for lipids to leave the membrane is not altered by nsL-TP: short acyl-chain lipids bind faster and to a higher extent to nsL-TP. Perhaps the difference between our results and those of Nichols is explained by the fact that, in contrast to the pyrene moiety of PamPyrGroPCho, the Nbd moiety of Pam(NbdLau)GroPCho is located near the membrane interface [35] and hence it is expected that Pam(NbdLau)GroPCho dissociates from the membrane with a different mechanism.

A $K_d = 0.046$ was optimized from the binding experiments. In a previous study, it was shown that this parameter is linearly dependent on the PamPyrGroPCho mole fraction in the donor membrane [9]. The linear relationship of K_d with the mole fraction of various pyrenyl-acyl-labeled PtdCho species as shown in [9] is indicative of the absence of competition of unlabeled PtdCho for the lipid-binding site on nsL-TP. Eqn (2) in [9] can be directly derived from our Eqn (11) by substitution of $[A] = 0$. This means that at 0% PamPyrGroPCho surface concentration, $K_d = 0$ (or $K_a = 0$), a condition which was used to convert Eqn (17) into Eqn (18). Interestingly, the equilibrium binding (or K_d) did not change upon increasing the negative surface charge in the donor membrane, whereas the initial binding velocity was markedly

Fig. 11. Relationship of kinetic constants with membrane negative surface charge. (A) K_1 (●) and K_2 (○); (B) α_d (●) and α'_d (○).

stimulated. This invariance of K_d with negative surface charge confirms a previous study [9] and was taken into account in the fit of the transfer data to Eqn (18). The inverse correlation of K_d with acyl chain length and the positive correlation with phospholipid headgroup polarity [9] strongly suggest that the equilibration of $P \cdot D$ into $PL \cdot D$ (reaction A) is passive in nature. In that study we proposed that by lowering the energy barrier for lipid monomers to dissociate from the membrane surface, nsL-TP only accelerates the natural tendency of lipids to leave the membrane.

Transfer kinetics

Eqn (17), which can be considered as a general equation describing phospholipid transfer, has some interesting features. First of all it resembles the equation derived by van den Besselaar et al. which applies to PtdCho-TP- and PtdIns-TP-mediated transfer of PtdCho [26, 33]. Secondly, v_0 reflects mass transfer instead of exchange (as for PtdCho-TP and PtdIns-TP). It can be seen from Eqn (17) that the sign of v_0 is positive (i.e. net transfer from donor to acceptor) when $K_d > K_a$ whereas v_0 is negative when $K_a > K_d$ (i.e. net transfer from 'acceptor' to 'donor'). Since a previous study revealed that K_d is linear with the surface concentration [9], it is inferred that nsL-TP will only catalyze transfer of phospholipids down a surface concentration gradient irrespective of the charge or association constant with the membranes.

From the transfer experiments in Figs 8 and 9, the association constants K_1 and K_2 and the kinetic quantities α'_d and α_d were determined as a function of membrane surface charge. We plotted these constants as a function of surface charge as depicted in Fig. 11 after considering the charge of -1 for Tnp-PtdEtn and of -1.2 for PtdOH [36]. Here it is clearly seen that all four constants increase markedly with increasing negative charge and that there is a high correlation between the constants derived for the acceptor and donor membrane. This is taken as a strong support for Eqn (18) (and 17) which are symmetric in nature with respect to $[A]$ and $[D]$. The

association constants K_1 and K_2 are much higher than those found for the interaction of PtdCho-TP or PtdIns-TP with negatively charged membranes [26, 33, 34]. This is probably due to the fact that nsL-TP is positively charged and PtdCho-TP and PtdIns-TP are negatively charged at physiological pH. The association constant K_2 is responsible for the decreased rate of transfer at higher acceptor vesicle concentrations. In fact, it may be concluded that in the experiment with acceptor vesicles containing 20% (by mol) PtdOH at a lipid concentration of 100 μM , about 85% of nsL-TP was associated with the acceptor vesicles and hence was no longer available for transfer. It is of interest that, in a time-resolved fluorescence study [8], it was shown that, in the presence of 100 μM phosphatidylcholine vesicles containing 20% (by mol) PtdOH, the rotational motion of nsL-TP was retarded (1.5-fold). From this it was inferred that the interaction of nsL-TP with membranes containing acidic phospholipids results in a partial rather than in a complete immobilization of the protein [8]. The inhibition experiments as depicted in Fig. 10 further emphasize the marked association of nsL-TP with negatively charged membrane surfaces. The inhibition increases in the order PtdIns < PtdOH < PtdInsP < PtdInsP₂ in analogy with the increasing negative charge of these lipids [36, 37]. It is of note that at a PtdInsP₂/nsL-TP molar ratio of 7:1, the nsL-TP-mediated transfer is already inhibited more than 50%. A similar study with PtdIns-TP yielded comparable results [26].

It is difficult to ascribe one process to the quantities α'_a and α_d since they are composed of many rate constants. It is however interesting to consider two extremes. First where $k_{-d} \gg k_{-1}$ and $k_{-a} \gg k_{-2}$, then $\alpha'_a = k_2$ and $\alpha_d \approx k_1$ (since $K_d \ll 1$). This would imply that the association rate of nsL-TP with membranes increases with increasing membrane negative charge (see Fig. 11) whereas the dissociation from these membranes would be hardly affected (since K_1 and K_2 increase proportionally to α'_a and α_d). This would be exactly opposed to the results obtained for PtdIns-TP where k_2 remained constant and k_{-2} decreased with increasing membrane negative charge [26]. The second situation to consider is $k_{-d} \ll k_{-1}$ and $k_{-a} \ll k_{-2}$, then $\alpha'_a = K_2 k_{-a}$ and $\alpha_d \approx K_1 k_{-d}$ (since $K_d \ll 1$). This would imply that k_{-d} and k_{-a} hardly change upon variation of the negative charge of the membrane, and would be consistent with the observation that K_d is invariant with membrane surface charge. We tentatively favor this last possibility.

In conclusion, we have shown that the kinetic model of PamPyrGroPCho binding and transfer by nsL-TP is consistent with a mode of action of nsL-TP involving the formation of a freely dissolved lipid/protein complex (reaction A). From the binding experiments it became evident that the equilibrium binding of PamPyrGroPCho to nsL-TP (second step in reaction A) is independent of membrane surface charge. The non-specific character of nsL-TP must find its origin in this second step. We propose a passive role of nsL-TP, lowering the energy barrier for lipids to leave the membrane interface [9]. This also explains why the access of nsL-TP to the lipids in the membrane is restricted to the outer leaflet (Fig. 1) [30, 31]. An increase in membrane surface charge significantly influences the binding kinetics as well as the nsL-TP-mediated transfer of PamPyrGroPCho between vesicles (Figs 5B, 7 and 8). These effects can be explained by a preferential interaction of nsL-TP with vesicles containing negative charge, which probably reflects rather aspecific electrostatic interactions between the positively charged protein [38] with the negatively charged vesicle surface. In view

of the high membrane concentration in cells (about 40 mM [7]) it may well be that in cells the majority of the nsL-TP molecules is associated with membranes. From the phospholipid binding experiments it was inferred that at very low membrane concentrations the protein may release its bound phospholipid in the aqueous phase. In view of the high lipid concentrations found in cells, this pathway is of minor importance. In other words, in the cell nsL-TP acts most likely as a phospholipid carrier, in agreement with the conclusions by Nichols [7]. This release pathway, however, may be more significant for sterols as the formation of a nsL-TP sterol complex could not be demonstrated in similar binding experiments [9]. In fact, the assumption that, for sterols, pathways C and D (see Appendix) are predominant (i.e. $k_3 \gg \alpha_d$) may explain why, despite the lack of sterol binding, sterols are transferred by nsL-TP. This statement requires further study but, if true, would have major implications for the targeting of sterols in cells.

APPENDIX

Binding kinetics with irreversible dissociation of lipid monomer from nsL-TP

A decrease of lipid binding with decreasing donor concentration can be explained by inclusion of an irreversible dissociation reaction of the complex between nsL-TP and PamPyrGroPCho (PL) into P and L (a lipid monomer in the aqueous phase) and a subsequent reassociation of this lipid monomer with the donor membrane. The binding process is then described by reactions A, C and D:

By inclusion of this irreversible step, binding will no longer be an equilibrium process but a steady-state reaction. It is of note that any binding model that assumes equilibrium conditions and includes reaction A will always yield the expression for [PL] as given in Eqn (19). By assuming steady-state conditions, this new model involving reactions A, C and D can give an expression of the steady-state concentration [PL]. The steady-state assumption yields Eqns (A1–A5):

$$\frac{d[\text{P}]}{dt} = -k_1[\text{P}][\text{D}] + k_{-1}[\text{P} \cdot \text{D}] + k_3[\text{PL}] = 0 \quad (\text{A1})$$

$$\frac{d[\text{PL}]}{dt} = -k_1[\text{PL}][\text{D}] + k_{-1}[\text{PL} \cdot \text{D}] - k_3[\text{PL}] = 0 \quad (\text{A2})$$

$$\frac{d[\text{P} \cdot \text{D}]}{dt} = k_1[\text{P}][\text{D}] - k_{-1}[\text{P} \cdot \text{D}] - k_d[\text{P} \cdot \text{D}] + k_{-d}[\text{PL} \cdot \text{D}] = 0 \quad (\text{A3})$$

$$\begin{aligned} \frac{d[\text{PL} \cdot \text{D}]}{dt} &= k_1[\text{PL}][\text{D}] - k_{-1}[\text{PL} \cdot \text{D}] \\ &+ k_d[\text{P} \cdot \text{D}] - k_{-d}[\text{PL} \cdot \text{D}] = 0 \end{aligned} \quad (\text{A4})$$

$$\frac{d[\text{L}]}{dt} = k_3[\text{PL}] - k_4[\text{L}][\text{D}] + k_{-4}[\text{D} \cdot \text{L}] = 0. \quad (\text{A5})$$

It is of note that Eqns (A3) and (A4) are identical to Eqns (4) and (5). The total protein concentration is given by:

$$[P_{\text{tot}}] = [P] + [PL] + [P \cdot D] + [PL \cdot D]. \quad (\text{A5})$$

Addition of Eqns (A3) and (A4) and substitution into Eqn (A5) yields:

$$[P_{\text{tot}}] = \{[P] + [PL]\} \{1 + K_1[D]\}. \quad (\text{A6})$$

From Eqns (A3) and (A4), Eqn (A7) (which is identical to Eqn 9) can be generated by elimination of $[PL \cdot D]$:

$$[P \cdot D] = \frac{\alpha_d}{k_{-1}} [D][PL] + \left(\frac{\alpha_d}{k_{-1}} + \frac{\alpha_d}{k_{-d}} \right) [D][P];$$

$$\alpha_d = \frac{k_{-d}k_1}{k_{-1} + k_d + k_{-d}}. \quad (\text{A7})$$

Substitution of Eqn (A7) into Eqn (A1) yields:

$$\frac{[P]}{[PL]} = \frac{\alpha_d[D] + k_3}{K_d\alpha_d[D]}. \quad (\text{A8})$$

Finally substitution of Eqn (A8) into Eqn (A6) yields:

$$[PL] = \frac{K_d}{1 + K_d} \frac{[P_{\text{tot}}]}{\{1 + K_1[D]\}} \frac{[D]}{[D] + \beta};$$

$$\beta = \frac{k_3}{(1 + K_d)\alpha_d}. \quad (\text{A9})$$

At $[D] \gg \beta$, Eqn (A9) becomes equal to Eqn (19).

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