

Steady-state tyrosine fluorescence to study the lipid-binding properties of a wheat non-specific lipid-transfer protein (nsLTP1)

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Received 21 December 1999; received in revised form 8 March 2000; accepted 17 March 2000

Abstract

The binding properties of a wheat non-specific lipid-transfer protein (nsLTP1) for different mono- and diacylated lipids was investigated. Lipids varied by their chain length, unsaturation and/or polar head group. In the case of fatty acid or lysophospholipid with a C10 chain length, no interaction can be measured, while poor affinity is reported for a C12 chain length. The dissociation constant (K_d) is about 0.5 μ M independent of chain length from C14 to C18. The same affinity is obtained for C18 fatty acids with one or two unsaturations, whatever the *cis-trans* double bond isomery. In all cases, the number of binding sites, n , by protein ranges between 1.6 and 1.9, suggesting that two lipids can fit within the protein. ω -Hydroxy-palmitic acid, a natural monomer of cutin polymer, is found to interact with nsLTP1 with a K_d of 1 μ M and $n = 2$. In contrast with previous data that reported the binding of the anionic diacylated phospholipid, DMPG (Sodano et al., FEBS Lett. 416 (1997) 130–134), nsLTP1 is not able to bind dimyristoylphosphatidylcholine, dimyristoylphosphatidic acid, palmitoyl-oleoylphosphatidylcholine or palmitoyl-oleoylphosphatidylglycerol added as liposomes or solubilized in ethanol. However, when both nsLTP1 and lipids are first solubilized in methanol, and then in the buffer, it was evidenced that the protein can bind these lipids. These results suggest that lipid–lipid interactions play an essential role in the binding process of plant nsLTP1 as previously mentioned for other lipid-transfer proteins. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Lipid-transfer protein; Tyrosine fluorescence; Interaction; Lipid; Binding

1. Introduction

Plant non-specific lipid-transfer proteins (nsLTP) are well known for their ability to enhance, in vitro, the inter-membrane exchange and/or transfer of various polar lipids including phospholipids and glyco-

lipids. These basic and cysteine-rich proteins are ubiquitous in the plant kingdom where they form a multigenic family. Two main families have been characterized, nsLTP1 with a molecular mass of about 9 kDa and nsLTP2 with a molecular mass of about 7 kDa [2]. NsLTPs share a common structural fold stabilized by four disulfide bonds and composed of four helices packed against a C-terminal arm formed by series of turns [3–6]. In contrast with many globular protein folds, the hydrophobic side chains do not enter in close contact in the hydrophobic core so that a cavity is revealed within the protein. The cavity of wheat nsLTP1 displays an unique plasticity with a volume increase from 250

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DMPA, dimyristoylphosphatidic acid; DMPG, dimyristoylphosphatidylglycerol; POPC, palmitoyl-oleoylphosphatidylcholine; POPG, palmitoyl-oleoylphosphatidylglycerol; nsLTP1, non-specific lipid-transfer protein; LPCx, lysophosphatidylcholine with x carbon chain length; MLV, multilamellar vesicles

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to 750 Å³ on lipid binding, without major changes in the overall three-dimensional structure of the protein [1]. Recently, resolution of the structure of lipid–wheat nsLTP1 complex by X-ray crystallography has unambiguously shown that two lysophosphatidylcholines can be loaded within the protein [7]. This allowed to characterize a hydrophobic tunnel rather than a cavity, crossing the long axis of the protein. The binding of two monoacylated lipids was also suggested by Kader et al. [8,9] from the fluorescence quenching of pyrene and anthroxyloxy fatty acid derivatives in the presence of maize and wheat nsLTP1. From these latter studies, it was interesting to note that plant nsLTP1 could bind several lipid derivatives including labelling with pyrene and anthroxyloxy polycycles. This is rather surprising since these aromatic molecules have rather voluminous and rigid structures. However, it has never been shown whether these lipids are completely loaded within the protein or if a part of their acyl chains protrude out of it.

Wheat nsLTP1 possesses two tyrosine residues and no tryptophan, but fluorescence of this aromatic side chain can be sensitive to lipid binding [1]. The observed increase of tyrosine fluorescence intensity provide titration curves which can be easily fitted with binding models, in contrast with the previous competitive binding experiments [8,9]. Finally, while structure determination gives valuable information on how the lipid can fit within the protein, intrinsic tyrosine fluorescence offers the possibility to quantify the stability of the complex. Moreover, this technique can reveal in a very fast way whether and how the lipid can interact with the protein.

2. Materials and methods

2.1. Lipids and wheat nsLTP

All lipids used in this study were from Sigma and were used as well. The concentration of the stock solutions varied between 4 and 6 mg/ml. Fatty acids were solubilized in methanol and lysophospholipids in phosphate buffer (50 mM sodium phosphate buffer pH 7).

Purification of wheat nsLTP1 was performed according to ref. [7]. Ten milligrams of protein was

weighted exactly and solubilized in phosphate buffer (10 ml) affording the stock solution (100 µM). A two-fold dilution of this stock solution was used for fluorescence experiments.

2.2. Fluorescence spectroscopy

Fluorescence intensity was measured at 25°C with a Fluoromax-Spex (Jobin et Yvon, France). Excitation was set at 275 nm while emission spectra were recorded from 280 to 340 nm. One milliliter of 50 µM nsLTP1 was poured in the cuvette and titration was performed by adding in a stepwise manner the lipid solution. Titration curves reported maximum fluorescence intensity at 305 nm vs. the molar ratio lipid/protein, R_i .

The fluorescence intensity of a lipid/protein solution is proportional to the concentration of free (P) and bound proteins (P_L) as $F = F_o(P) + (F_{max} - F_o)(P_L)$ where F_o and F_{max} are the fluorescence intensities of nsLTP1, free and at lipid saturation, respectively. The bound protein fraction, $\alpha = (P_L)/P_t$, is deduced from fluorescence data as $\alpha = (F - F_o)/(F_{max} - F_o)$, with P_t , the total protein concentration. Assuming n independent and identical sites, α versus R_i provides a second-order equation with one possible solution that is easily fitted with a non-linear Newton–Gauss function [10,11]. This fitting procedure returns both the dissociation constant, $K_d = (P)(L)/(P_L)$ where (L) is the concentration of free lipids, and n , the number of binding sites. As presented in Section 3, the number of sites, n , returned by this fitting method showed that two lipids could be bound by the protein. Therefore, a fitting procedure with two non-equivalent binding sites was also considered $K_{d1} = (P)(L)/(P_L)$ and $K_{d2} = (P_L)(L)/(P_{L2})$, P_{L2} being the concentration of protein with two bound lipids. This yielded a three-order equation that could be easily solved using mathematical procedure [12,13].

3. Results

3.1. Binding of saturated fatty acids

For these experiments, we have used fatty acid sodium salt and in the case of C16, both sodium

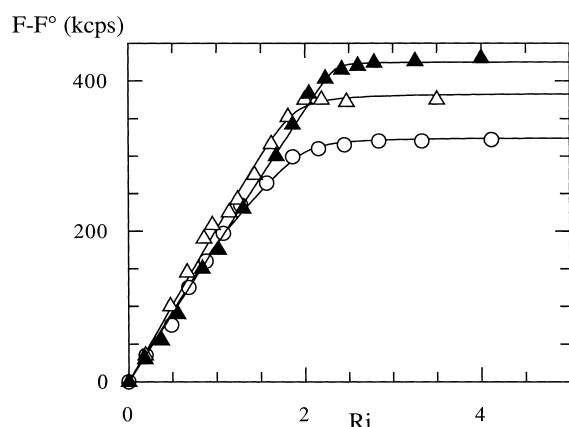


Fig. 1. Variation of the fluorescence, $F - F^\circ$, as a function of the molar ratio lipid/LTP, R_i , for fatty acids with various chain lengths, C14 (○), C16 (△) and C18 (▲). The curves represent the best fit obtained with the non-cooperative model described in Section 2. Accuracy is within symbol size.

salt and acid forms. For a long chain, such as C18, the lipid was not soluble in the buffer, forming crystals rather than micelles [14]. Then, all fatty acids from C10 to C18 chain length were prepared in methanol and injected directly in the fluorescence cuvette. The methanol concentration at the end of titration was below 10% and it was checked that such a concentration did not interfere with tyrosine fluorescence. The intrinsic fluorescence of pure wheat nsLTP1 originated from the signal of the two tyrosine residues within the protein. It is noteworthy that the fluorescence of nsLTP1 tyrosine residues was quenched since their fluorescence intensity was lower than the intensity of an equivalent free tyrosine concentration (100 μM) in aqueous buffer. Addition of fatty acids yielded a marked increase of fluorescence depending on the lipid chain length as illustrated in Fig. 1. For C10, no increase of fluorescence was observed suggesting that this lipid was not bound by nsLTP1. In the case of C12, the interaction of the lipid with the protein induced only a 10% increase of

fluorescence, and even at $R_i = 6$, the saturation was not reached (not shown). As a consequence, no fit could be produced and the affinity is supposed to be higher than 1 mM. For longer chain length, a typical non-cooperative behavior was observed for α versus R_i (Fig. 1). At the end-point titration, a 150% increase of fluorescence intensity was obtained in regard to that of free nsLTP1. Interestingly, the intensity of the diffusion signal at 280 nm remained constant at low R_i for C14 and C16 and markedly started to increase at $R_i = 2.7$ and 1.8, respectively. In the case of C18, the diffusion signal appeared as soon as one aliquot of the lipid was poured in the cuvette. These titration curves were fitted with the first model described above, and returned values for K_d and n (Table 1). Interestingly, both dissociation constant and stoichiometry are constant from C14 to C18 chain length, around 0.5 μM and 1.7, respectively. Since stoichiometry was higher than one and below two sites/protein, a non-cooperative binding model with two non-identical sites was used [12]. In the case of C14, this fitting returned the following set of data: $K_{d1} = 8 \pm 25$ nM; $K_{d2} = 1.2 \pm 0.4$ μM . The high standard deviation obtained for K_{d1} and the high difference between the values of K_{d1} and K_{d2} suggested that this fitting model was not adapted to the binding process. Therefore, it was no longer used for other lipids.

ω -Hydroxy palmitic acid is well represented in the family of cutin monomers [15]. The hydroxy fatty acid was solubilized as well in methanol and poured in the cuvette. As for the above fatty acids, it generated a 200% increase of fluorescence and the fitting procedure returned a K_d and n , of 1.3 μM and 2, respectively.

3.2. Binding of unsaturated fatty acids

For these titrations, we have used protonated oleic acid, *cis*-C18:1; elaidic, *trans*-C18:1; linoleic acid,

Table 1

Dissociation constant, K_d (μM), and number of sites, n , for the various fatty acids with and without insaturations

	C10	C12	C14	C16	C18	C18:1 c	C18:1 t	C18:2 c	C18:2 t
K_d	–	> 1 mM	0.7	0.3	0.7	0.5	0.9	0.8	0.5
n	–	nd	1.7	1.8	1.7	1.6	1.6	1.7	1.6

Data are obtained by fitting the experimental fluorescence curves as described in Section 2. 't' and 'c' stand for a *trans* and *cis* double bond, respectively. The standard deviation for K_d is ± 0.05 and ± 0.1 for n . nd, not determined.

cis-C18:2 and linoelaidic acid, *trans*-C18:2. The double bond is located at positions 9–10 for C18:1 and 9–10/12–13 for C18:2. As for the saturated fatty acids, titration yielded a marked increase of fluorescence with a saturation at 160% compared to that of pure nsLTP1. These data were fitted by the non-cooperative binding model with n identical and independent sites and the results were summarized in Table 1. The affinities of nsLTP1 for these four unsaturated fatty acids were quite similar to those of the C18 saturated fatty acid. As for saturated fatty acids, the number of binding sites was higher than one so that two of these could fit within the protein.

3.3. Binding of lysophospholipids

The titration was performed with lysophospholipids of various chain lengths, from C10 to C18. These lipids were solubilized in a phosphate buffer. The aqueous LPC18 stock solution was turbid and non-homogeneous at room temperature. Therefore, the dispersion of this lipid was maintained at 50°C affording its perfect homogenization. Addition of lysophospholipids produced a 200% increase of fluorescence at the end-point titration. The highest enhancement of fluorescence intensity was obtained in the case of LPC14 (Fig. 2). In contrast with fatty acids, no diffusion signal was observed for lysophospholipids, probably because LPC formed small-sized

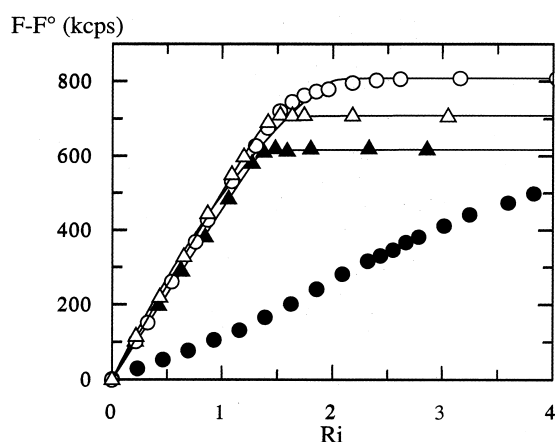


Fig. 2. Variation of the fluorescence, $F-F^0$, as a function of the molar ratio lipid/LTP, R_i , for lysolipids with various chain lengths, C12 (●), C14 (○), C16 (△) and C18 (▲). The curves represent the best fit obtain with the non-cooperative model described in Section 2. Accuracy is within symbol size.

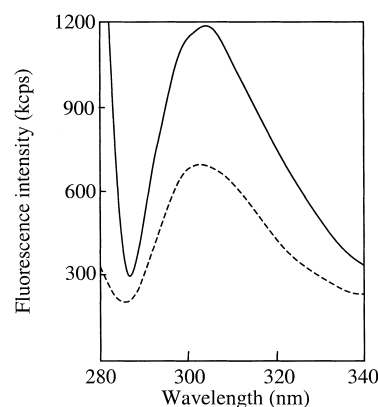


Fig. 3. Intrinsic tyrosine fluorescence spectra of nsLTP1, free (—) and complexed with DMPC (---) by the procedure described in the text.

micelles. As for the fatty acid with a C10 chain length, LPC10 did not produce any enhancement of fluorescence. In the case of LPC12, the fluorescence exhibited a typical cooperative behavior as illustrated in Fig. 2 and even at $R_i = 10$ (not shown), the saturation was not reached. As a consequence, these data could not be fitted with the model described above. For the other chain length, a non-cooperative behavior was obtained (Fig. 2) allowing to fit the data with the first model and the corresponding parameters were collected in Table 2. As for fatty acids, the affinity remained roughly constant for LPC14 to LPC18 with a stoichiometry around 1.6. It was interesting to recalculate the concentration of free lipids, (L) , in the cuvette as: $(L) = L_t - n\alpha P_t$. For LPC14, until $R_i = 1.5$, that is to say, at a total lipid concentration L_t of 75 μM , (L) remained below 10 μM , and then increased almost linearly. Interestingly, it reached a value of 50 μM at roughly $R_i = 3$ ($L_t = 150 \mu\text{M}$) what corresponded to the critical micellar concentration (CMC) of the free lipid [16,17]. In the case of LPC16, the CMC of the free lipid is 5 μM and during the titration by nsLTP1, this concentration was reached at $R_i = 2$, i.e. when the bind-

Table 2

Dissociation constant, K_d (μM), and number of sites, n , for the various lysophospholipids

	LPC10	LPC12	LPC14	LPC16	LPC18
K_d	—	> 1 mM	0.4	0.7	0.7
n	—	nd	1.6	1.7	1.7

Data are obtained by fitting the experimental fluorescence curves as described in Section 2. The standard deviation for K_d is ± 0.05 and ± 0.1 for n . nd, not determined.

ing sites were fully occupied. For LPC18, this occurred at $R_i = 1$ ($\text{CMC} = 0.5 \mu\text{M}$) so that at the beginning of the titration, nsLTP1 could interact both with monomers and micelles.

3.4. Binding of diacylphospholipids

The binding studies were performed with both multilamellar and small unilamellar vesicles in the case of DMPG, DMPC and POPC. DMPG was used as reference in these experiments since it was previously shown that it is bound by wheat-LTP [1]. Similar data were obtained with higher affinities for DMPG dispersed as SUV than for the lipid dispersed as MLV. Unexpectedly, n was higher than 1 what could suggest, as in the case of fatty acids and lysophospholipids, that more than one lipid could fit within the protein. However, as done in the case of LPC, calculation of (L) revealed that even at low R_i , the concentration of free lipids in the cuvette, was higher than the critical vesicle concentration (CVC , $\approx 10^{-10}\text{M}$ [17]) and then, vesicles coexisted in solution with free and bound nsLTP1. This was in agreement with the presence of a diffusion signal at 280 nm as soon as one aliquot was poured in the cuvette. Since vesicles consist of bilayers and then possess an internal core, this means that the effective concentration of lipids that could indeed interact with the nsLTP1 was overestimated yielding a number of sites higher than 1. This was consistent with the fact that n decreased when passing from MLV to SUV since the lipids exposed to the nsLTP1 was greater in the latter case. In the same way, the affinity was improved what could be also associated with the fact that the lipid–lipid interactions are lower in the case of SUV.

DMPC, DMPA, POPC and POPG were solubilized in the buffer. It must be mentioned that these lipids formed spontaneously very large vesicles yielding turbid solutions. These four lipids did not produced any enhancement of fluorescence upon titration, indicating that they were not bound by nsLTP1. After sonication, only a slight increase of tyrosine fluorescence was observed and any saturation was obtained. As for fatty acids, aliquot solutions were prepared in methanol, ethanol or isopropanol affording its solubilization as monomers. In each case, titration experiments did not yield any increase of

fluorescence, while a huge diffusion signal could be observed at 280 nm. This suggested that, as soon as the lipid was poured in the cuvette, it instantaneously formed vesicles before having time to interact with nsLTP1. Then, we prepared in methanol, stock solutions of nsLTP1 and the following diacyl lipids, DMPC, DMPA, POPG or POPC. Fifty microliters of the protein solution was poured in glass tubes and 10 μl of the different lipids was added such as the molar ratio, R_i , was 3. Then, 1 ml of buffer was slowly added to the tubes affording a concentration of 50 μM nsLTP1. A reference tube was prepared as described, except that no lipids were added. These solutions were subjected to fluorescence analysis as in the above cases only for determining the signal intensity. In the case of the pure nsLTP1, the fluorescence intensity at 305 nm was identical to that of the nsLTP1 directly solubilized in buffer. For all other solutions including lipids, the intensity exhibited an increase ranging from 180 to 200% (Fig. 3), showing that there was binding by the nsLTP1. Concomitantly, a diffusion signal appeared at 280 nm since the lipid was in excess.

4. Discussion

Plant nsLTP1s are today among the rare examples of a tryptophan-free lipid-binding protein in which intrinsic tyrosine fluorescence can be used to monitor lipid binding. They can be regarded even as unique since the other known examples are peptides or proteins which interact only with lipid membranes [18,19]. In wheat nsLTP1, the intrinsic fluorescence originates from the two tyrosine residues within the protein, Tyr-16 and Tyr-79. However, directed mutagenesis studies have shown that only Tyr-79 is involved in the increase of fluorescence intensity when lipids are bound [20]. The hydroxyl group of Tyr-79, or of the corresponding residues in other plant nsLTP1s, forms hydrogen bonds with the glycerol, phosphate or carboxyl of polar lipids [4,7]. As a consequence, when a lipid is bound to the protein, Tyr-79 is probably less mobile and therefore less quenched by the possible internal quencher [21]. Intrinsic tyrosine fluorescence is not useful to probe the binding properties of all nsLTP1s since for example, only a weak increase of tyrosine fluorescence inten-

sity is observed on lipid binding to the barley nsLTP1s [22]. However, with the barley protein, it has been shown that the fatty acid or fatty acylCoA adopts a different orientation within the protein cavity and Tyr-79 is involved only in hydrophobic interaction with the aliphatic chain, while no hydrogen bond can be formed with the lipid polar head group [22,23].

The stoichiometry returned by the binding model is higher than 1 consistent with recent X-ray crystallography data showing that two C14 lysophosphatidylcholine molecules with a head-to-tail orientation are loaded within wheat nsLTP1 cavity [7]. Our results confirm that such a stoichiometry is also valid for monoacylated lipids with longer acyl chains. A two-site/monomer was also suspected to occur in the case of binding of pyrenyl fatty acid to maize nsLTP1, due to a fluorescence-quenching effect at lipid saturation [8,9]. For both fatty acids and lysophospholipids, the stoichiometry provided by the fitting procedure is about 1.7. This means that a non-cooperative binding model with two identical sites is not appropriate. The use of a non-cooperative model with two independent sites with different affinity was shown to be also not well-suited for fitting our data. Finally, a stoichiometry of 1.7 means that some cooperativity can occur in the binding process. Such a hypothesis is consistent with X-ray crystallography which show an interaction between the two acyl chains in the wheat nsLTP1-lysoPC complex [7]. However, since the free lipid concentration cannot be experimentally obtained from intrinsic tyrosine fluorescence spectroscopy, fitting with a cooperative binding model could not be performed. Nevertheless our fitting, although not totally appropriate, provides a rather good view of the affinity of wheat nsLTP1 with monoacylated lipids.

As expected, an increase of binding affinity from C10 to C14 was observed for both fatty acids and lysophospholipids. These results are well correlated with their aqueous solubility, suggesting an important contribution of the free energy of transfer between the aqueous phase and the hydrophobic interior of the nsLTP1 cavity. This explanation does not work for monoacylated lipids of C14 to C18 chain length, since K_d remains constant, even in the case of unsaturated C18 fatty acids. The crystal/monomer and micelle/monomer equilibria are apparently not

involved in this behavior since similar K_d and stoichiometry are obtained for protonated and deprotonated fatty acids as well as lysophospholipids. Similar K_d values are also obtained for protonated and deprotonated palmitic acid. Furthermore, the absence of variability between fatty acids and lysophospholipids show that the structure of the polar head group is apparently not a discriminating structural parameter in the affinity. However, structure studies have previously shown that the C-terminal tyrosine residue is involved in a hydrogen bond with either the carboxylate group of fatty acids [4] (or the hydroxyl group of the glycerol phospholipid backbone [7]). Therefore, as long as the hydrogen bond with Tyr-79 is formed, only hydrophobic interactions should be considered. This means also that from C14 to C18 no apparent changes occur in the strength of hydrophobic interactions in the stabilization of the lipid-protein complex. As mentioned above, from structural data, the supplementary CH₂ could be involved more in lipid-lipid than in lipid-protein interactions in the complex.

In the case of the C18 chain length, the presence of double bonds, either with *cis* or *trans* conformation does not influence the affinity for the wheat nsLTP1. In a first attempt this is not surprising since the cavity can adapt its volume for binding voluminous lipid molecules [1]. Moreover, even for a saturated fatty acid, the acyl chain does not adopt an all-*trans* conformation within the protein [4,7,23]. As a consequence, the kink that is imposed by the presence of double bonds is not unfavorable for the interaction with the nsLTP1. This is not in agreement with the data of Kader et al. [8,9], who observed that for more than one double bond, the affinity decreases. However, it must be reminded that these experiments were obtained with an indirect procedure, based on competitive binding experiments of natural and anthroxyloxy labelled fatty acids.

Interestingly, nsLTP1 binds ω -hydroxy-palmitic acid which is one of the main component of cutin [15]. The affinity is slightly lower than that obtained for the other lipids, but the number of site is equal to 2. This indicates that the non-cooperative and identical binding sites model fits well with such a hydroxy fatty acid. Therefore it can be suggested that both sites exhibit the same affinity for the corresponding fatty acid. In regard to the structure of nsLTP1 com-

plexed with 2 lysoC14 [7], it can be suggested that the two hydroxy fatty acids would adopt an antiparallel orientation in the hydrophobic cavity of the protein. In this way, the carbonyl region of one fatty acid would be at proximity of the hydroxyl part of the other lipid what could favor intermolecular hydrogen bonds between the two lipid molecules. This could lead to a symmetric rod-like structure with polar head at each side, and, in a sense, this lipid dimer can behave as a single molecule for binding to nsLTP1.

nsLTP1 can bind diacylated lipids within its cavity as already revealed by both NMR and intrinsic tyrosine fluorescence in the case of DMPG. It appeared that the binding process strongly depends on the physical state of the lipid itself since different K_d were returned when this phospholipid was dispersed as SUV or as MLV [1]. The importance of liposome size for the interaction with the nsLTP1 can be related in part to monolayer experiments [24] where it was shown that above a given surface pressure, nsLTP1 is unable to capture any lipid. The lipid packing is certainly higher when the curvature of liposomes decreases. Furthermore, when nsLTP1 interacts with vesicles, only the lipids that are at the external surface of the liposomes are concerned. Therefore, the higher number of accessible lipid molecules in SUV than in MLV can also lead to a lowering of the K_d . Therefore, the calculation of K_d is obviously hampered by the presence of vesicles at the beginning of the titration. Finally, the absence of complex formation with long chain diacylated lipids used herein (e.g. DMPC, POPC, DMPA, POPG) dispersed as SUV or MLV can be due to both high lipid packing in the corresponding liposomes.

In the case of fluorescence experiments, titration where diacylated phospholipids were first solubilized in ethanol does not lead to any complex formation. The most interesting feature is the co-solubilization in methanol of both the lipid and nsLTP1. This experimental procedure allows to clear the lipid–lipid interactions and seems to favor lipid binding to nsLTP1. It must be outlined that the complex formation can also arise because the mixture is prepared in methanol (see Section 3) where the initial concentration of LTP is 0.5 mg/50 μ l. This high concentration of both constituents can aid the interaction between them. This co-solubilization procedure in

methanol has no direct biological relevance because these lipids are rarely soluble as monomers. However, one can expect that a co-solubilization like-procedure, such as a mixture of DMPC in DMPG vesicles, could produce the same result, that is to say, the binding of DMPC by nsLTP1.

This study shows that wheat nsLTP1 is, above all, a lipid-binding protein since it is capable of capturing lipids within a cavity. Our experiments show that nsLTP1 is capable of binding both monoacylated and diacylated lipid species with a K_d in the micromolar range which is compatible with the nanomolar to micromolar ranges observed for other lipid-binding proteins [25,26]. For diacylated phospholipids, our study shows that the aggregation state (i.e. the lipid–lipid interaction) is a limiting factor for lipid binding and this is probably a major cause of the low transfer activity of wheat nsLTP1 in regard, for example, to maize nsLTP1 [27]. This is in agreement with previous data which have shown that the maize nsLTP1 penetrates phospholipid monolayers at higher surface pressures than the wheat protein [28]. Interestingly, our study shows that nsLTP1 is capable of binding all sorts of fatty acids, including the hydroxy-fatty acids, which are important components of cutin polymer and waxes of the plant cuticles [15]. This is consistent with the postulated role of nsLTP1 in the formation of plant cuticles [29] and/or in the mobilization of storage seed lipids [30].

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