

Par j 1 and Par j 2, the two major allergens in *Parietaria judaica*, bind preferentially to monoacylated negative lipids

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Par j 1 and Par j 2 proteins are the two major allergens in *Parietaria judaica* pollen, one of the main causes of allergic diseases in the Mediterranean area. Each of them contains eight cysteine residues organized in a pattern identical to that found in plant nonspecific lipid transfer proteins. The 139- and 102-residue recombinant allergens, corresponding respectively to Par j 1 and Par j 2, refold properly to fully functional forms, whose immunological properties resemble those of the molecules purified from the natural source. Molecular modeling shows that, despite the lack of extensive primary structure homology with nonspecific lipid transfer proteins, both allergens contain a hydrophobic cavity suited to accommodate a lipid ligand. In the present study, we present novel evidence for the formation of complexes of these natural and recombinant proteins from *Parietaria* pollen with lipidic molecules. The dissociation constant of oleyl-lyso-phosphatidylcholine is $9.1 \pm 1.2 \mu\text{M}$ for recombinant Par j 1, whereas pyrene-dodecanoic acid shows a much higher affinity, with a dissociation constant of approximately $1 \mu\text{M}$ for both recombinant proteins, as well as for the natural mixture. Lipid binding does not alter the secondary structure content of the protein but is very efficient in protecting disulfide bonds from reduction by dithiothreitol. We show that Par j 1 and Par j 2 not only bind lipids from micellar dispersions, but also are able to extract and transfer negative phospholipids from bilayers.

Plant nonspecific lipid transfer proteins (ns-LTPs) have been found in a variety of tissues from mono- and dicotyledonous species [1,2]. Two main families have been characterized in plants: LTP1 with a molecular mass of approximately 9 kDa [3] and LTP2 with a molecular mass of approximately 7 kDa [4]. Their biological role remains unknown; their function was initially associated with their *in vitro* ability to transfer

phospholipids between membranes. On the basis of this ability, they were assumed to play a role in membrane biogenesis by mediating the transport of lipids from their site of biosynthesis to other membranes. The presence of a signal peptide in their sequence, on the other hand, suggests an extracellular location, and some studies have highlighted their *in vivo* role in pathogen defense reactions and/or responses to

Abbreviations

DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; LUV, large unilamellar vesicle; ns-LTP, nonspecific lipid transfer protein; OLPC, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; rPar j 1, recombinant Par j 1 expressed in *Pichia pastoris*; rPar j 2, recombinant Par j 2 expressed in *Pichia pastoris*; β -py-C₁₀-HPC, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine; β -py-C₁₀-HPG, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol.

environmental changes, cutin formation, embryogenesis and symbiosis [3,5–8]. Interestingly, *Parietaria judaica* LTPs have been shown to represent primarily intracellular proteins that are released from the pollen grains upon germination [9]. Moreover, it has been observed that, in some plant species, different isoforms are expressed differently, suggesting that different types of ns-LTPs with different tissue specificity (and presumably different function) may coexist in a given plant [10]. It appears that ns-LTPs could play a role in different biological functions through their ability to bind and/or carry lipophilic compounds. A comparison of their biochemical properties reveals several common characteristics [4]. They are all soluble, relatively small proteins, and their isoelectric point is, in general, basic. Furthermore, at the level of primary structure, they share a pattern of eight cysteines forming four disulfide bridges, and the tertiary structure is characterized by a single compact domain with four α -helices and a nonstructured C-terminal coil [11–13].

The identification, isolation and characterization of proteins responsible for IgE-mediated allergy is a necessary task for improving both the diagnosis and treatment of this important increasing clinical disorder. The knowledge of the biochemical role of novel allergens can improve the strategy for their purification and characterization and, more importantly, it can help to explain the relationships among biological function, protein structure and allergenic activity [14]. Unfortunately, a relatively small number of allergens have been biochemically characterized among the pollen allergens. Several members of the plant ns-LTP family have been identified as relevant allergens in foods [15]. This allergen family is particularly important in the Mediterranean area. In addition to foods, allergens of the LTP family have also been identified in other plant sources, such as latex of *Hevea brasiliensis* [16] and some pollens. In the latter, LTPs from *Ambrosia artemisiifolia* [17], *Olea europaea* [18], *Artemisia vulgaris* [19], *Arabidopsis thaliana* [20], *Platanus acerifolia* [21] and *P. judaica* pollens [22,23] have been described.

Parietaria is a genus of dicotyledonous weeds belonging to the *Urticaceae* family. The most common species are *P. judaica* and *Parietaria officinalis*, which are widely and abundantly distributed in the Mediterranean area, where *Parietaria* pollen is one of the most common causes of pollinosis [24]. The two major allergens of *P. judaica*, Par j 1 and Par j 2, have been cloned and sequenced, and their recombinant counterparts were able to induce histamine release from basophils of patients allergic to *P. judaica* pollen in a way comparable to that of the crude extract from natural *P. judaica* [23,24]. Although Par j 1 and Par j 2 display strong sequence divergence with respect to the ns-LTPs described to date, 3D modeling by homology suggests that both allergens belong to the ns-LTP protein family [25,26]. In support of this hypothesis, we have found significant molecular features of these modeled *Parietaria* proteins that are shared by other members of the family. More importantly, the ability of these allergens to bind and transfer lipids is demonstrated in the present study using both natural and fluorescently labeled ligands.

Results

Molecular model comparison

Previous molecular modeling analysis of Par j 1 and Par j 2 showed a common 3D structure similar to that of ns-LTPs [25,26], characterized by an α -helical fold stabilized by four disulfide bonds [3]. In addition, experimental assignment of the disulfide bridges in Par j 2 showed a pattern consistent with this fold [27]. Nevertheless, both *Parietaria* allergens display low sequence identity (24–29%) with respect to the ns-LTPs described to date, as well as larger molecular sizes (14.7 and 11.3 kDa, respectively). Only residues relevant from the structural point of view, such as cysteine, proline and glycine, are completely conserved in all sequences. Indeed, both Par j 1 and Par j 2 contain eight cysteines that could well be involved in a similar pattern of four disulfide links (Fig. 1).



Fig. 1. Amino acid sequence alignment of five plant ns-LTPs (barley, wheat, maize, rice and peach), together with Par j 1 and Par j 2. The C-terminal extensions of Par j proteins are not presented. The conserved residues in all seven proteins are boxed in yellow. Asterisks denotes residues that interact with lipid in ns-LTP_{maize}-palmitate complex (1mzm.pdb).

One dissimilar overall feature of Par j proteins with respect to ns-LTPs is the net charge. In general, plant ns-LTPs are basic proteins (pI 8–10). By contrast, Par j 1 and Par j 2, although containing many charged residues (17 positive and 16 negative side chains for Par j 2 versus eight positive and two negative side chains for maize ns-LTP), show almost neutral isoelectric points. The views of the electrostatic surface potential reveal an amphipathic overall Par j 1 structure compared to the basic surface of ns-LTP_{maize} (Fig. 2A,B). This seems to be a common feature of allergens in that they appear to contain more charged residues compared to their non-allergic counterparts.

The most relevant structural peculiarity of the ns-LTP family is the internal cavity that works as the binding site for different lipidic molecules. In the present study, VOIDOO software was used to calculate the van der Waals volumes of the hydrophobic cavities found in the modeled structures. The volume calculated for the cavity found in Par j 1 is 73 Å³ (Fig. 2E) and 200 Å³ in Par j 2 (Fig. 2F). Inspection of known structures shows that a palmitate molecule fills a 600 Å³ cavity in ns-LTP_{maize} (1mzm.pdb; Fig. 2D), and two molecules the same lipid span throughout the ns-LTP_{rice} molecule occupying an open tunnel of 1345 Å³ (1uvb.pdb; Fig. 2H). On the other hand, the empty cavity of ns-LTP_{rice} has 249 Å³ in the unligated form (1uva.pdb) [28]. Apparently, the volumes of the filled and empty hydrophobic cavities differ significantly with respect to several structures. Moreover, ns-LTPs are able to accommodate a wide range of lipidic ligands with little specificity due to the elasticity of the C-terminal loop (residue numbers 77–92), which points toward the hydrophobic cavity and blocks the lipid binding pocket in the free form [28] (Fig. 2G,H). According to this observation, it can be inferred that the volume of the empty cavity should not be critical in discriminating between potential ligands.

Conversely, residues delineating the cavity in ns-LTPs could be considered to be the functionally relevant moieties. Therefore, the character of the side chains lining the cavities of Par j 1 and Par j 2 could provide more revealing insights into the proteins function than the cavity size. An asterisk in Fig. 1 indicates residues contacting the lipid in the ns-LTP_{maize} (1mzm.pdb). Most of these residues have a hydrophobic nature in all ns-LTPs and also in Par j 1 and Par j 2 sequences, which is consistent with their potential function as lipid binding proteins. Although apolar interactions provide the majority of contacts, there are two important exceptions in Arg46 and

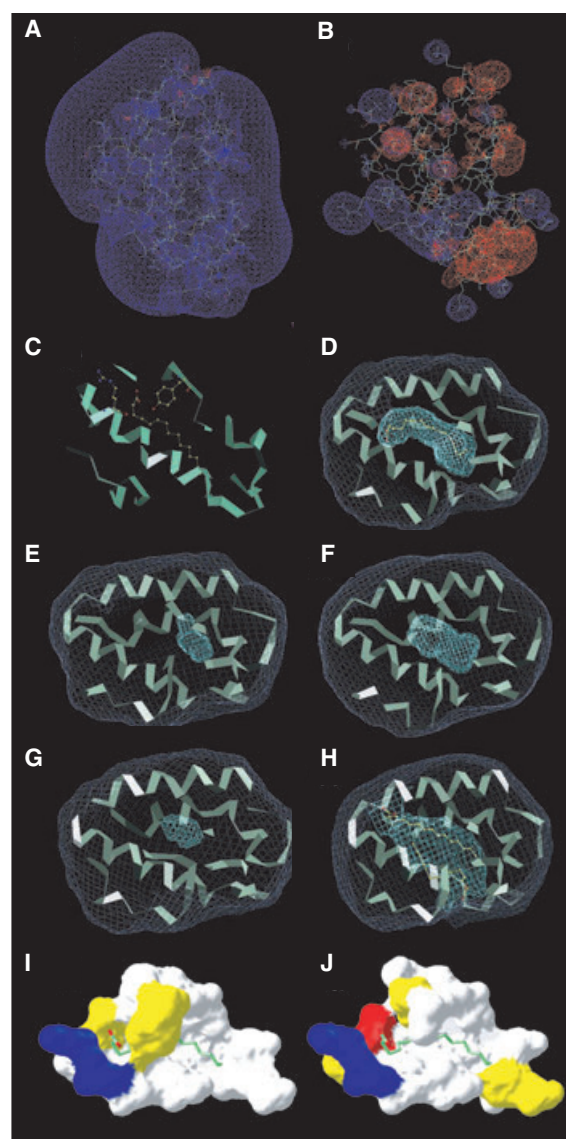


Fig. 2. (A) Electrostatic surface charge potential calculated for ns-LTP_{maize} (1mzl.pdb) and (B) Par j 1. (C) Ribbon diagram of ns-LTP_{maize} complexed with palmitate (1mzm.pdb). Tyr81 and Arg46 are shown as a ball and stick model. Surface of the cavities from ns-LTP_{maize}-palmitate complex (1mzm.pdb) (D), Par j 1 (E) and Par j 2 (F) models, unligated ns-LTP_{rice} (1rzl.pdb) (G) and ns-LTP_{rice}-(palmitate)₂ complex (1uvc.pdb) (H), and van der Waals surface representations of residues facing the cavity of ns-LTP_{maize} (I) and Par j 1 (J). Hydrophobic residues on the surface are shown in white, polar residues are shown in yellow, negative residues are shown in red and positive residues are shown in blue.

Tyr81 (number according to maize sequence) that are present in all the plant ns-LTPs. Both residues form hydrogen bonds with the carboxylate groups of fatty acids [29–31] and also act by filling the empty cavity, shifting significantly after lipid binding. Arg46 is

found in Par j 1 and substituted by a lysine in Par j 2, whereas Tyr81 is absent in both *Parietaria* sequences. The cavity of maize ns-LTP is highly polarized and mainly hydrophobic on one side, and polar and positively charged on the opposite side, where Arg46 and Tyr81 are located close to each other (Fig. 2I). This polarization appears to be ideally suited for an amphipathic negative molecule within the cavity. Tyr60, the single tyrosine residue found in Par j 1 sequence does not lie at the polar end as expected, but at the nonpolar side of the cavity (Fig. 2J). Moreover, the net charge of the cavity is neutral due to the presence of Asp37 that compensates the charge of Arg46.

CD

The overall structure of the ns-LTPs known to date is a four helix bundle with a long C-terminal loop. To control the correct folding of both proteins after purification, CD spectroscopy was performed. CD spectra obtained for the natural mixture were compared with those of individual recombinant Par j 1 and Par j 2 expressed in *Pichia pastoris* (rPar j 1 and rPar j 2, respectively). Very similar spectra are obtained for rPar j 2 and natural Par j 1–Par j 2, showing a minimum at 208 nm, a well defined shoulder at 222 nm, and a maximum at 190 nm. The ratio of intensities obtained at 222 and 208 nm, however, are significantly lower than those typical for all- α proteins, suggesting that β or/and unordered conformations are also present in significant amounts. The content of α -helix, β -sheet and unordered structure in Par j 2, as determined by the Fasman protocol [32], was 47%, 11% and 42%, respectively, in good agreement with secondary structure content in the Par j 2 model; 49 out of 102 residues adopt a helical conformation. The far-UV CD spectrum of rPar j 1 reveals a higher content in unordered conformations. Difference spectra of protein molar ellipticities indicate that the 37 extra residues of rPar j 1 are in an unordered conformation and could account for this deviation.

Lipid binding assayed through tyrosine intrinsic fluorescence

Tryptophan fluorescence is frequently used as a means to test protein conformational changes induced by unfolding, ligand binding and other protein transitions. Similarly to plant ns-LTPs, neither rPar j 1, nor rPar j 2 contain tryptophan residues. Although the tyrosine fluorescence quantum yield is lower and less

sensitive to environmental changes, in the absence of tryptophan residues, tyrosine provides an alternative intrinsic fluorophore. Indeed, Tyr81 (according to the maize numbering) fluorescence had been previously used to monitor lipid binding to ns-LTP_{maize} [11], ns-LTP_{barley} [33] and ns-LTP_{wheat} [30,34,35].

As indicated above, neither Par j 1, nor Par j 2 contain a Tyr residue at the corresponding position. However, in the model described for Par j 1, Tyr60 is facing the cavity and, in principle, it can be expected to be sensitive to lipid binding (Fig. 3). Par j 2 contains two Tyr residues, Tyr101 and Tyr102, that occupy the last two positions of the sequence. If the proposed models are correct, and these *Parietaria* proteins bind lipids, a saturable transition should be observed for Par j 1 with the addition of lipid, whereas Par j 2 fluorescence should remain unchanged. Figure 4 shows the results obtained for this experiment. The titration was performed with 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (OLPC) because this lipid can be suspended in water and does not cause major changes in sample turbidity when added sequentially to the protein preparation, unlike other lipids (e.g. oleic acid, also tested in the present study). Tyrosine fluorescence increased significantly in Par j 1 with the addition of OLPC (scattered light contribution of the lipid had been subtracted), whereas only



Fig. 3. Ribbon representation of maize ns-LTP structure (1mzl.pdb). Tyr81 is shown in red stick, whereas Tyr60 of Par j 1 is superimposed in green.

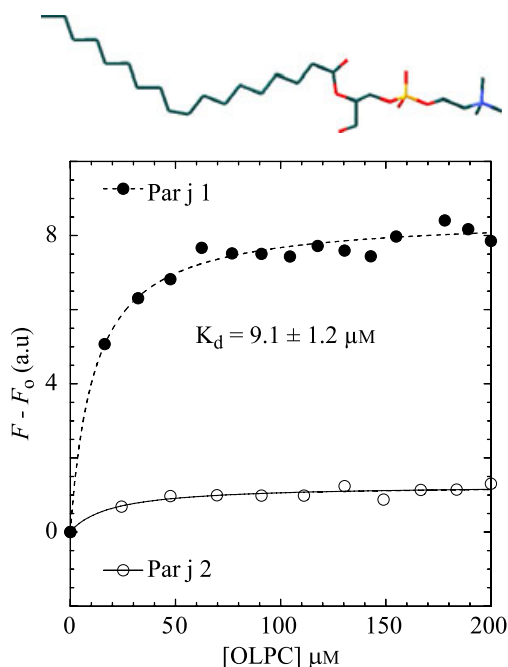


Fig. 4. Tyrosine intrinsic fluorescence data (excitation at 270 nm, emission at 310 nm) recorded after the addition of increasing amounts of an aqueous stock solution of OLPC 2 mM to a 1.5 μ M protein (filled circles, Par j 1; open circles, Par j 2) preparation in 20 mM NaCl/P_i. Contributions of identical additions of lipid in the absence of protein are subtracted. Lines correspond to data fitting to Eqn (1).

minor changes were observed for the fluorescence corresponding to the two remote tyrosine residues in the Par j 2 sequence. Data could be fitted to a single binding site using Eqn (1), and an estimated $K_d = 9.1 \pm 1.2 \mu\text{M}$ was found for the complex rPar j 1–OLPC. An identical result was obtained when Eqn (2) was used for fitting ($n = 1$).

Lipid binding assayed with a fluorescent lipid probe

Pyrene is an extrinsic fluorophore that exhibits fluorescence emission maxima at 375 and 395 nm (excitation at 345 nm), attributed to a monomeric pyrene moiety. In addition, it displays an additional fluorescence emission peak at longer wavelengths (~ 470 nm), which occurs only when two pyrene rings reside within 10 Å of each other and form an excited state dimer, usually called an excimer. In the present study, the fluorescence of 1-pyrenedodecanoic acid was monitored for increasing concentrations of the ligand in the presence of the Par j proteins. Fluorescence data, measured in the titration of the two recombinant proteins and the

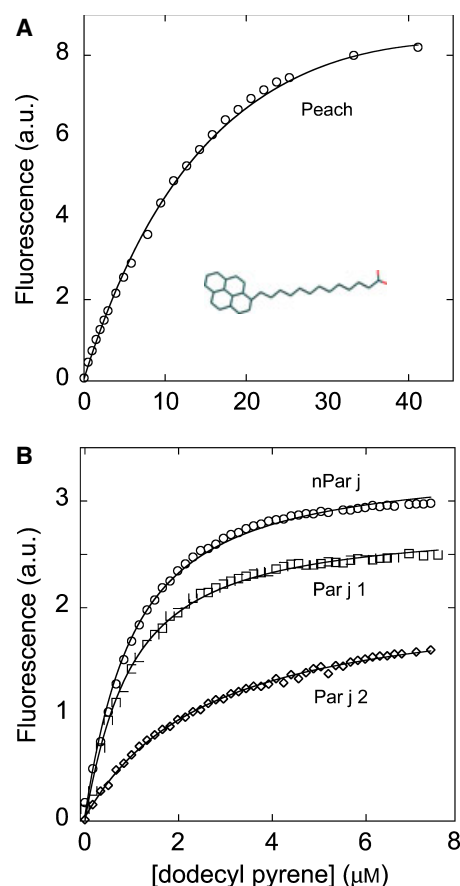


Fig. 5. 1-Pyrenedodecanoic acid fluorescence data (excitation at 345 nm emission at 375 nm) for increasing concentrations of the probe in the presence of ns-LTP_{pearl} in (A), and the natural mixture nPar j 1–nPar j 2 (open circles), rPar j 1 (squares) and rPar j 2 (diamonds) in (B), at 0.15 μ M protein concentration in 20 mM NaCl/P_i.

natural mixture of 0.15 μ M protein in 20 mM sodium phosphate (pH 7.0), are shown in Fig. 5 (lower panel). Equation (2) is used to fit $(F - F_0)$ for calculation of the K_d . Very similar values are obtained for the three proteins: $0.82 \pm 0.03 \mu\text{M}$ for nPar j 1–Par j 2, $0.76 \pm 0.03 \mu\text{M}$ for rPar j 1 and $1.6 \pm 0.06 \mu\text{M}$ for rPar j 2. These K_d values are comparable to those calculated for the binding of other ns-LTPs to monoacylated lipids [11] and much lower than the $K_d = 27.9 \pm 0.03 \mu\text{M}$ observed for the binding of 1-pyrenedodecanoic acid to ns-LTP_{pearl}, as also measured in the present study (Fig. 5A).

Lipid transfer activity

Large unilamellar liposomes (LUVs) preformed with pure 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol (β -py-C₁₀-HPG) at 9 μ M concentration

(donor vesicles) were preincubated with 360 μM LUVs of 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG, acceptor vesicles) for at least 100 s in the fluorescence cuvette before the addition of 0.15 μM protein. Fluorescence signal of pyrene moiety was registered ($\lambda_{\text{ex}} = 344$; $\lambda_{\text{em}} = 397$ nm) for some minutes (Fig. 6B). The same experiment was performed with the neutral phosphocholine derivatives with 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (β -py- C_{10} -HPC) LUVs as donors and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) LUVs as acceptors (Fig. 6, lower panel). The activity calculated for the canonical ns-LTP_{peach} was 35 $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein, similar to the reported ns-LTP_{maize} activity [36]. The values obtained for *Parietaria* proteins are somewhat lower: 10.3 and 7 $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein for rPar j 1 and rPar j 2, respectively. Activity values calculated with neutral phospholipids are two orders of magnitude lower. However, rPar j 2 transfers lipids more efficiently than ns-LTP_{peach} when the neutral β -py- C_{10} -HPC/DOPC pair is used.

Thermal behaviour of native proteins

As indicated above, a K_d constant could not be calculated for the complex rPar j 2-OLPC, due to the absence of tyrosine residues in the hydrophobic cavity of this protein. Alternatively, the binding of a substrate can be demonstrated by the stabilization induced on the protein. In the absence of reducing agents, temperature scans of nPar j 1-Par j 2 preparation up to 90 °C failed to show a complete melting transition (Fig. 7); instead, a typical baseline shift together with a steep slope at high temperature was observed. The same behaviour was observed for rPar j 1 and rPar j 2 (data not shown). The CD signal at 222 nm changed by less than 5% upon heating to 90 °C. After cooling down to the original temperature, an identical spectrum was obtained and a second temperature scan rendered a perfectly superimposable trace. This result suggests that the three protein preparations are in an oxidized thermoresistant, native form. Although significant conformational changes are observed in the C-terminal loop of some ns-LTPs upon lipid binding, these do not imply variation in the balance of regular versus non regular structure. In agreement with this, OLPC addition to 170 mM did not exert any changes in the far-UV CD spectra of Par j proteins (Fig. 7). Thus, the addition of a reducing agent appears to be essential for comparing the thermostability of these proteins and the effect of OLPC binding. Par j proteins contain eight cysteine residues potentially involved in four disulfide bridges.

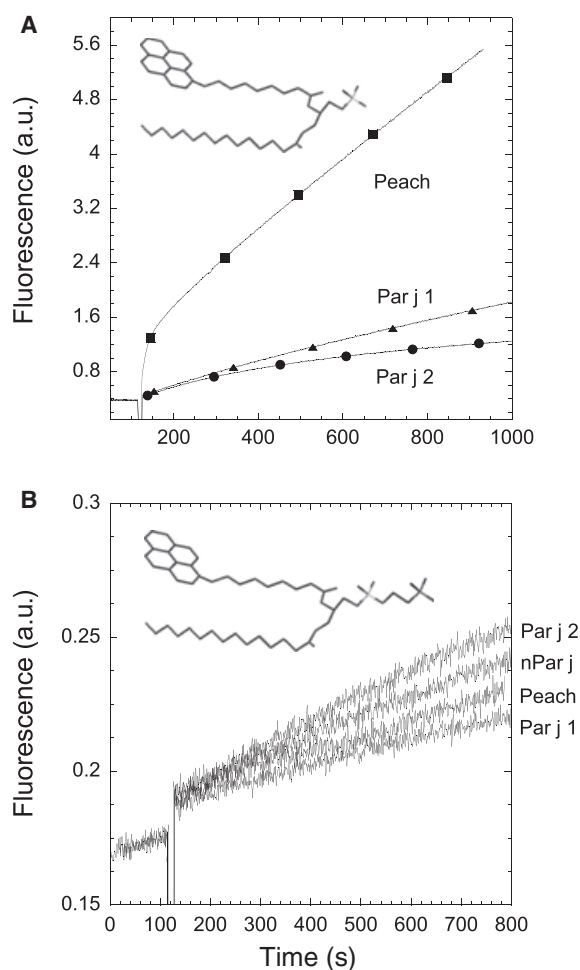


Fig. 6. (A) Time trace of fluorescence signal (excitation at 345 nm, emission at 375 nm) after the addition of 0.15 μM ns-LTP_{peach} (squares), Par j 1 (triangles) and Par j 2 (circles) to two populations of preformed liposomes with 9 μM β -py- C_{10} -HPG and 360 μM DOPG in 50 mM Hepes buffer. (B) Time trace of fluorescence signal as in (A) but with 9 μM β -py- C_{10} -HPC and 360 μM DOPC.

Figure 8 illustrates the effect of 15 h of incubation of the nPar j 1-Par j 2 with 1, 5 or 10 mM dithithreitol. Reduction induced a dramatic fall in ordered secondary structures in all cases. The decrease in the CD signal at 222 nm was higher for Par j 2 than for Par j 1. Incubation with 10 mM dithithreitol almost completely destroyed any ordered structure in Par j 2; a flat thermal trace was obtained for the reduced denatured protein under these conditions. Reduced Par j 1, on the other hand, still retained 50% CD signal at 222 nm. This reminiscent structure is lost after thermal melting and is not recovered after cooling down to 10 °C.

The natural mixture is assumed to contain both Par j 2 and Par j 1, although the presence of other iso-

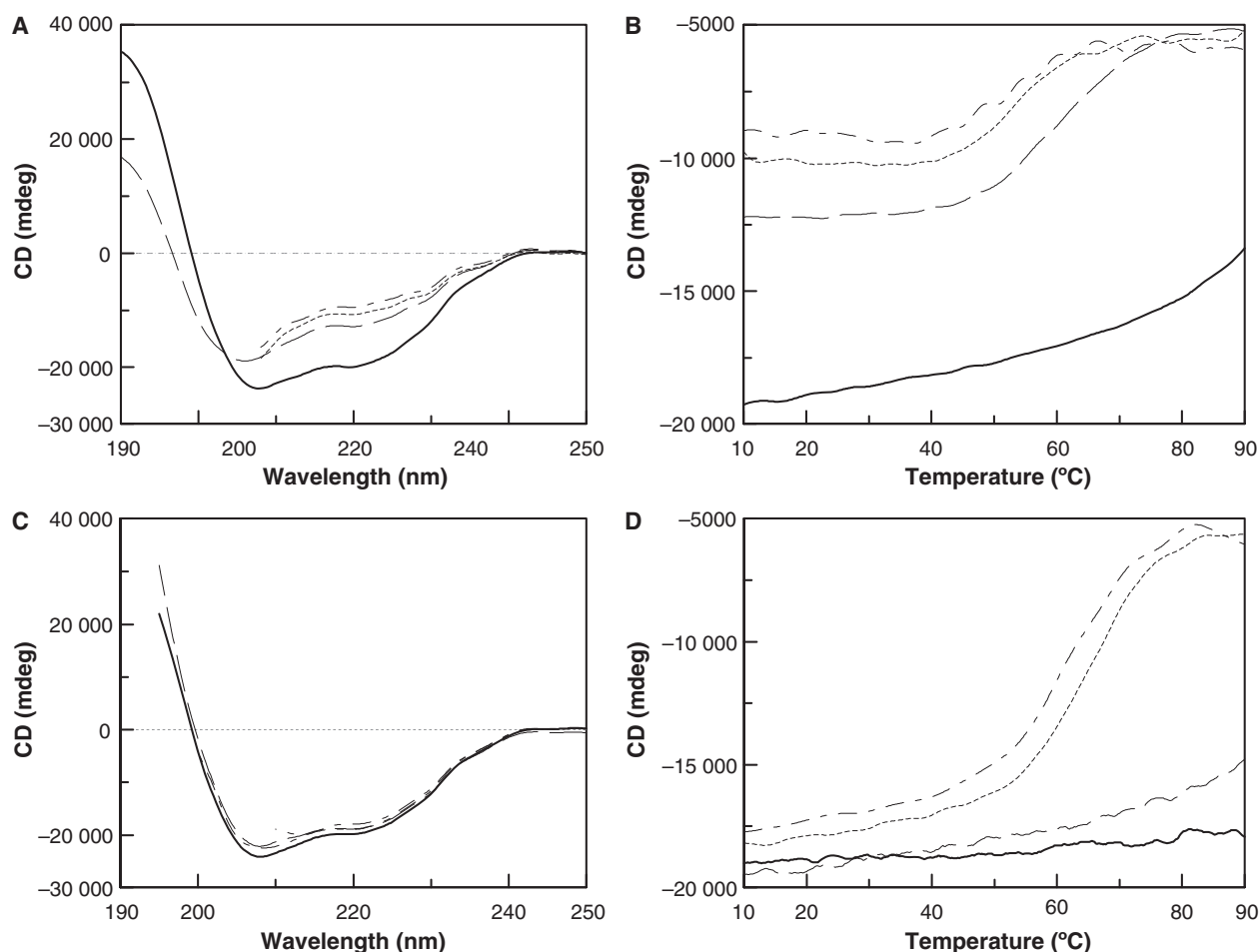


Fig. 7. CD spectra (A, C) and temperature scans recorded at 222 nm (B, D) obtained for nPar j 1–Par j 2 in 0 mM (continuous), 1 mM (dashed), 5 mM (dotted) and 10 mM dithiothreitol (dash-dotted) in the absence (A, B) and presence (C, D) of OLPC 170 μ M (20 mM NaCl/Pj).

forms of similar molecular weight cannot be discarded. The behaviour observed in the present study was intermediate between those of Par j 1 and Par j 2, compatible with the above assumption.

The same experiment, conducted in the presence of 170 μ M OLPC, revealed a significant protection versus protein reduction, suggesting effective complex formation. Additionally, thermal denaturation of the partially or totally reduced samples took place at higher temperatures. Figure 8 summarizes the reminiscent CD signal collected at 222 nm at 10 °C after incubation with various dithiothreitol concentrations for 15 h. Figure 8A shows that 1 mM dithiothreitol had the same reducing power for the free Par j 1 as a 10-fold concentration had for its complex with OLPC. A 5 °C shift of the melting transition was observed for this protein upon lipid binding (data not shown). The effect was more pronounced for Par j 2; although being more sensitive to reduction in its free

form that Par j 1, Par j 2 became more protected in the presence of OLPC, with a significant preservation of secondary structure. More remarkable is the effect observed in the natural mixture, in which 10 mM dithiothreitol caused only minor changes in secondary structure at 10 °C and the thermal transition shifted by almost 10 °C, whereas the protein was still thermostable in 1 mM dithiothreitol (Fig. 7). The degree of protection induced by 170 μ M OLPC, calculated as the ratio of [dithiothreitol]_{1/2} concentrations in the presence and absence of the ligand, is 8.5, 13 and 87 for rPar j 1, rPar j 2 and nPar j 1–Par j 2, respectively.

Although the K_d value could only be accurately determined for the complex Par j 1–OLPC by the titration monitored by tyrosine fluorescence, the CD results suggest that OLPC binds with a higher affinity to Par j 2 and results in a very stable complexes with the natural mixture nPar j 1–Par j 2.

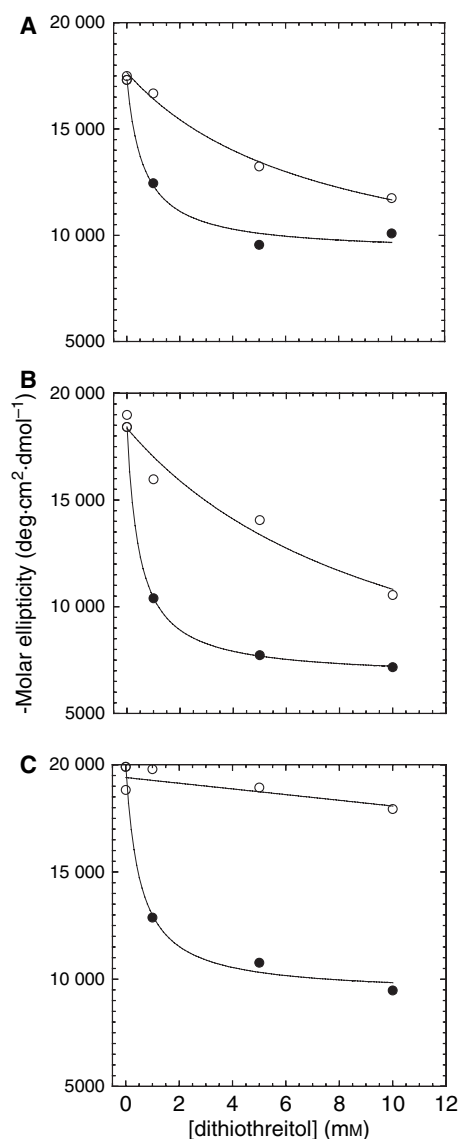


Fig. 8. CD signal recorded at 222 nm and 10 °C after 15 h of incubation with varying concentrations of the reducing agent dithiothreitol in the absence (filled circles) and presence (open circles) of 170 μ M OLPC: (A) rPar j 1, (B) rPar j 2 and (C) nPar j 1-Par j 2.

Discussion

Allergens are found in only 2% of all sequence-based and 5% of all structural protein families [37]. Sequences encoded in plant genomes, included in the prolamin superfamily (cereal storage proteins, nsLTPs, 2S storage albumins and inhibitors of trypsin and α -amilase), account for 65% of plant food allergens [38]. ns-LTPs bind a variety of lipidic molecules from fatty acids to phospholipids and are able to transport lipids *in vitro*. In the present study, the

ability of two pollen allergens to bind lipids was investigated. The relative sequence homology with ns-LTP together with the functional characterization would confirm Par j 1 and Par j 2 as being members of this protein family.

In the present study, we have shown that a monoacylated lipid such as OLPC is able to alter the fluorescence of the intrinsic probe Tyr60 in the Par j 1 sequence and also stabilizes the purified proteins against reduction. Intrinsic fluorescence was used to monitor lipid binding to ns-LTPs and the observed signal increase fitted known binding models. A single well-resolved transition is observed with a $K_d = 9.1 \pm 1.2 \mu$ M compatible with a 1 : 1 complex. This value compares well with the K_d calculated for lysophospholipids and other ns-LTPs. K_d values of 10.1 μ M and 1.9 μ M were obtained for lyso-C16 (1-palmitoyl-l- α -lysophosphatidylcholine) with ns-LTP_{wheat} and ns-LTP_{maize}, respectively [11], although values of 28.9 μ M have also been reported for the complex of lyso-C16 with ns-LTP_{wheat} [35]. Dissociation constants of approximately 0.5 μ M were measured for the complexes of ns-LTP_{wheat} and lysophospholipids and phospholipids with side chains from C14 to C18, independent of the presence of one or two insaturations [30]. Furthermore, a $K_d = 7.5 \mu$ M was reported for the interaction of dimyristoyl phosphatidylglycerol small liposomes with ns-LTP_{wheat} [34]. Although Par j 2 intrinsic fluorescence is insensitive to lipid binding and a K_d could not be measured, the CD experiments suggested that Par j 2 is binding with a higher affinity to OLPC than Par j 1.

The K_d for the interaction between 1-pyrenedodecanoic acid and Par j 1 and Par j 2 was also measured. A value in the micromolar range was calculated for the interaction with the three protein preparations assayed in the present study: Par j 1: $0.76 \pm 0.03 \mu$ M, Par j 2: $1.6 \pm 0.06 \mu$ M and nPar j 1-Par j 2: $0.82 \pm 0.03 \mu$ M. Zachowski *et al.* [39] showed that ns-LTP_{wheat} and ns-LTP_{maize} can bind two molecules of 1-pyrenedodecanoic acid by means of the fluorescence quenching of pyrene that followed the first signal increase. By contrast to OLPC, the colocalization of two molecules of analogues in the binding site would induce a fluorescence quenching. A K_d could not be calculated for ns-LTP_{wheat} and ns-LTP_{maize}, although there are data available [39,40] suggesting that the affinity is much higher than for OLPC, with a K_d in the submicromolar range. No apparent decrease in fluorescence signal was observed after the first saturation in the titration of Par j proteins, suggesting that Par j proteins offer a single binding site for 1-pyrenedodecanoic acid. ns-LTP_{peach} has also been

assayed in the present study for comparison (Fig. 5). A much higher $K_d = 27.8 \pm 4 \mu\text{M}$ was obtained in this case.

An analysis of the structural details of the molecular models of Par j 1 and Par j 2 revealed a notable resemblance with other ns-LTPs, together with some distinct structural details that could be relevant for protein function: (a) important residues are absent in the sequence of *Parietaria* proteins; (b) Par j 1 and Par j 2 are markedly less basic than other ns-LTPs; and (c) their internal hydrophobic cavities seem to be smaller and less polarized compared to other members of the family.

Comparison of the binding modes of different ns-LTPs suggests that, although the sequences and the 3D structures are very similar among plant ns-LTPs, the binding modes of these proteins differ substantially. The binding site of ns-LTPs is a hydrophobic groove in the globular helical structure, which is covered by the C-terminal peptide. The majority of the residues lining the cavity are hydrophobic, with few exceptions. A major role has been conferred to these polar side chains. For some plant ns-LTP complexes, the highly conserved Arg46 and Tyr81 form hydrogen bonds with the carboxylate groups of fatty acids [29–31]. They also act by filling the empty cavity and they both shift significantly after lipid binding. This highly conserved tyrosine among ns-LTPs is absent in Par j 1 and Par j 2. The ns-LTP tunnel has a wide opening in one end and a narrow opening in the other end [41]. The wide opening is considered to be the entrance. Most polar residues reside in this side and it is also where the carboxylate binds. The narrow opening is considered to be a closed exit. This is where the methyl group binds, surrounded by hydrophobic side chains from the protein. Thus, the binding site results in a polarized cleft in the interior of a basic container with two main openings to bulk solvent, which appears to be ideally suited for fitting an negative amphipathic small molecule. Lipid–protein 1 : 1 complexes for ns-LTP_{maize} [31,41], ns-LTP_{wheat} [29,42] and ns-LTP_{rice} [28] indicate that this appears to be the preferred mode of binding and ligand orientation. However, the opposite orientation has been observed in complexes with ns-LTP_{barley} [43,44]. With ns-LTP_{barley}, it has been shown that the fatty acid or fatty acylCoA adopts a different orientation within the protein cavity and Tyr81 is involved only in hydrophobic interaction with the aliphatic chain, whereas no hydrogen bond can be formed with the lipid polar head group. The inversion of the coordination of the ligand in ns-LTP_{barley} has been related to the charged Lys9 replacing the corresponding

conserved hydrophobic position of the other ns-LTPs [44]. Accordingly, it appears that minor sequence differences are able to switch from one binding mode to the other. Both orientations can even coexist in the same complex in ns-LTP_{rice} [28], ns-LTP_{wheat} [29] and ns-LTP_{peach} [45]. In both conformations, the apolar part of the lipid would be in the interior of the cavity, whereas the polar head can be to either extremes of the cavity, providing two modes of binding exactly opposite to each other. Likewise, two binding sites have also been proposed from spectroscopic studies for ns-LTP_{barley} [33], ns-LTP_{wheat} [30,40] and ns-LTP_{maize} [39,40]. Moreover, in some known complexes, the lipidic chain stretches out of the binding pocket with the polar head group protruding out, facing the solvent [45], with no interactions with the protein. This may explain why the calculated K_d values for lipid complexes of Par j proteins rank in the same order as their homologues, despite the absence of the highly conserved Tyr81.

Another molecular distinct feature that is shown to have little effect is the net protein charge as also illustrated by the surface electrostatic potential. Of the two monoacylated lipidic derivatives used in the present study, the negative 1-pyrenedodecanoic acid binds with a higher affinity than the zwitterionic OLPC to Par j 1. Also, negative phospholipids are transferred more efficiently than neutral homologues from LUVs. These results suggest that the loss of the net positive charge of the protein is not related to a marked preference for neutral lipids. This overall feature is more likely to be related to the location where these proteins exert their *in vivo* function rather than any specificity for particular lipids.

Nonetheless, the experimental data obtained in the present study explain certain differences that were visible in the models. The small cavity detected by VOIDOO software is shown experimentally to provide a single binding site for monoacylated lipids under conditions where other ns-LTPs are able to bind two lipid molecules. The tunnel volume of Par j 1 and Par j 2 models are rather small compared to other ns-LTPs, mainly due to some bulky side chains together with a one and two residue insertion, respectively, in the C-terminal loop. It is demonstrated that ns-LTPs have a considerable capability of expansion. The present results, however, suggest that the Par j 1 and Par j 2 cavities do not appear to be able to spread out to accommodate two lipidic ligands. It is possible that *Parietaria* proteins are more specialized in monoacylated lipids, whereas other plant ns-LTPs are designed to accept bigger ligands, such as diacylated phospholipids. This

is partially demonstrated when the affinities for monoacylated lipids and activities for diacylated lipids are compared for *Parietaria* proteins and the canonical ns-LTP_{peach}. 1-Pyrenedodecanoic acid binds with a higher affinity to *Parietaria* proteins. Conversely, ns-LTP_{peach} is able to transfer β -py-C₁₀-HPG with greater efficiency.

Plant LTPs are prefixed nonspecific (ns) because they show very broad specificity. In the present study, four very dissimilar lipid derivatives are shown to be able to bind to these *P. judaica* allergens, with affinities similar to other ns-LTPs. However, the K_d calculated for 1-pyrenedodecanoic acid is one order of magnitude lower than for OLPC, and rPar j 2 transfers β -py-C₁₀-HPG 10-fold more efficiently than β -py-C₁₀-HPC (i.e. this ratio is 40-fold for rPar j 1 and 100-fold for ns-LTP_{peach}). This suggests a certain degree of specificity for monoacylated negative phospholipids for *Parietaria* proteins. On the other hand, Par j 2 binds and transfers neutral phospholipid better than Par j 1, whereas Par j 1 works better with negative lipids. The bigger cavity of 200 Å³ found in the model of Par j 2 compared to the 73 Å³ cavity of Par j 1 may explain this preference because phosphocholine is bigger than the phosphoglycerol moiety. However this cannot be stated clearly in the absence of an experimentally determined 3D structure, because, in general, the lipid molecules interact with the ns-LTPs binding cavity mainly through hydrophobic interactions. Although some known complexes exhibit definite hydrogen bonds between protein side chains with the carboxylate group of fatty acids or the hydroxyl group of the glycerol phospholipid backbone, in other complexes, the polar head group is not in contact with the protein [44]. Regrettably, crystallization trials with rPar j 1 and rPar j 2 have so far proved unsuccessful, most probably due to flexibility of C-terminal extensions.

These versatile, malleable and nonspecific proteins are able to bind hydrophobic molecules in different cellular contexts. It is not expected that discriminating structural features essential for ligand binding will readily become apparent. Conversely, binding modes and clues appear to be redundant and unspecialized, which, together with the coexistence of isoforms [46–48], suggests that promiscuity is probably of major functional relevance. The present study provides some evidence that Par j 1 and Par j 2 are structurally and functionally related to this group of proteins and are able to transfer lipids *in vitro*. LTPs have been usually identified from *in vitro* activities. Only recently has strong evidence become available for lipid transfer in living cells [49–51].

Experimental procedures

Purification of natural and recombinant *P. judaica* major allergens

Natural allergens (natural Par j 1–Par j 2 mix) were immunopurified from defatted pollen from *P. judaica* (Iber-pollen, Málaga, Spain) using polyclonal rabbit anti-Par j 1–Par j 2 sera coupled to a CNBr-activated sepharose 4B column, as described previously [52]. Coding regions of Par j 1 and Par j 2 were amplified and cloned in pPIC9 and expressed in the methylotrophic yeast *P. pastoris* as described previously [53]. Purification of the recombinant proteins was carried out by immunoaffinity chromatography as described previously [53]. Protein concentration was determined using the method of Gill and Von Hippel [54]. ns-LTP_{peach} was obtained as previously described [55].

Molecular modeling of Par j 1 and Par j 2

The homology model of the N-terminal region of Par j 1 and Par j 2 (Fig. 1) was generated using SWISS-MODEL at the EXPASY molecular biology server (<http://www.expasy.ch/swissmod/SWISS-MODEL.html>) [56]. Ns-LTP_{maize} (Protein Databank code: 1mzl) was selected as modeling template (33% and 34% identity with Par j 1 and Par j 2, respectively. Par j 1 is 52% identical to Par j 2). Despite of the low sequence identity, the four cysteines impose powerful restraints, largely assisting homology modeling. The final total energy of the calculated model is 1946 KJ·mol⁻¹. The lowest energy structure was subject to 100 cycles of unrestrained Powell minimization using CNS [57].

Cavity volume calculations and display

Cavity volumes within Par j 1 and Par j 2 were computed with VOIDOO software [58] using a probe radius of 1.4 Å, and were visualized with the o software [59].

Fluorescence spectroscopy

Titration experiments with OLPC were conducted at 25 °C with a SLM Bowman Series 2 luminiscence spectrometer (Aminco, Lake Forest, CA, USA). Tyrosine fluorescence was monitored with excitation and emission wavelengths at 275 and 310 nm, with 2 and 4 nm bandwidth, respectively. Buffer contributions were corrected and inner filter effect was negligible, with a sample absorbance lower than 0.05 units. One microliter aliquotes of 200 μ M to 20 mM OLPC preparations in 20 mM sodium phosphate (pH 7.0) were added stepwise to a cuvette containing 100 μ L of a 1.5 μ M Par j 1 solution in the same buffer. Volume changes were also taken into account (i.e. a maximum increase of 15% at the end of the titration).

The binding of a fluorescent lipid derivative, 1-pyrenedodecanoic acid, was also tested. The lipid was added from a concentrated stock solution in ethanol to 0.15 μM protein preparations in 20 mM sodium phosphate (pH 7.0). Emission signals at 375 nm with excitation at 345 nm through 2–4 nm slits were collected at 25 °C. A baseline experiment was carried out in the absence of protein under the same conditions.

Dissociation constant determination

It is possible to determine the K_d by monitoring tyrosine intrinsic fluorescence changes induced by lipid binding. When maximum bound lipid is considerably lower than K_d , a major fraction of the added ligand remains free in solution. The following equation can be used for those cases:

$$F = F_0 + \frac{(F_{\max} - F_0) \cdot [\text{lipid}]_{\text{free}}}{K_d + [\text{lipid}]_{\text{free}}} \quad (1)$$

where F is the fluorescence value recorded at 275 nm excitation and 310 nm emission, F_0 is the fluorescence of the free protein before lipid addition, $F_{\max}$ is the fluorescence value at saturating lipid concentrations and $[\text{lipid}]_{\text{free}}$ is the concentration of lipid that remains unbound. Particularly for low K_d values, $[\text{lipid}]_{\text{free}}$ should be calculated by subtracting bound lipid from the total added concentration:

$$[\text{lipid}]_{\text{free}} = [\text{lipid}]_{\text{T}} - n \cdot [\text{protein}] \cdot \frac{(F - F_0)}{(F_{\max} - F_0)} \quad (2)$$

where n is the number of binding sites per protein molecule and $[\text{lipid}]_{\text{T}}$ is the total concentration of added lipid. A 1 : 1 ratio has been contemplated here for Par j proteins with OLPC because a single binding transition is observed.

Alternatively, K_d can be calculated using the equation:

$$F = F_0 + (F_{\max} - F_0) \times \frac{(n \cdot [\text{protein}] + K_d + [\text{lipid}]_{\text{T}}) - \sqrt{(n \cdot [\text{protein}] + K_d + [\text{lipid}]_{\text{T}})^2 - (4 \cdot n \cdot [\text{protein}] \cdot [\text{lipid}]_{\text{T}})}}{2 \cdot n \cdot [\text{protein}]} \quad (3)$$

where all symbols are as indicated above. Curve fitting was carried out using KALEIDAGRAPH software (Sinergy Software, Reading, PA, USA).

Vesicle preparation

β -py-C₁₀-HPC and β -py-C₁₀-HPG were purchased from Molecular Probes (Eugene, OR, USA). DOPG and DOPC were obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA).

For liposome preparation, phospholipids were dissolved in chloroform : methanol (2 : 1, v/v), and the mixture was

evaporated to dryness under a stream of nitrogen. Traces of solvent were removed by evacuating the samples under high vacuum for at least 2 h. The samples were hydrated at 45 °C in 50 mM Hepes (pH 7.4), helping dispersion by stirring with a glass rod. The solution was frozen in liquid nitrogen and defrozen at 45 °C 10 times. LUVs were prepared by the extrusion method [60], using polycarbonate filters with a pore size of 0.1 μm (Nuclepore, Pleasanton, CA, USA). Vesicle sizes were determined by dynamic light scattering using a Malvern Zetasizer instrument (Malvern Instruments, Malvern, UK). The average vesicle diameter was 90–100 nm. Four independent liposomes populations were prepared with β -py-C₁₀-HPC, β -py-C₁₀-HPC, DOPG and DOPC in 50 mM Hepes.

For the lipid transfer experiments, 0.15 μM protein was added to a mixture of 9 μM β -py-C₁₀-HPC liposomes and 360 μM DOPC liposomes in 50 mM Hepes. The same was carried out with a mixture of 9 μM β -py-C₁₀-HPG liposomes and 360 μM DOPG liposomes. The increase in pyrene fluorescence signal was measured at 375 nm after excitation at 345 nm through 2 and 4 nm slits, respectively, in an SLM Bowman Series 2 luminiscence spectrometer (Aminco) at 25 °C.

CD

Far-UV (190–250 nm) CD spectra were recorded with a Jasco J-810 spectropolarimeter (Jasco Analitica Spain S.L., Madrid, Spain), which was previously calibrated with D-10-camphorsulphonic acid. The device was equipped with a Jasco PTC-423S temperature controller and cuvettes were thermostatted at 20 °C. The protein concentration was 0.035 mg·mL⁻¹ in 20 mM NaCl/P_i (pH 7.0) in a 0.2 cm cuvette. Proteins were diluted to the final concentrations required for CD analysis in the presence of the desired additives and incubated overnight to allow the reduction reaction to proceed completely. Just

before measurement, samples were centrifuged for 15 min at 14 000 *g* in an Eppendorf microcentrifuge at 4 °C. All the spectra were subtracted by the appropriate background and converted to mean residue ellipticity. Secondary structure content was determined in the spectral range 190–240 nm by means of several methods of analysis, compiled in DICROPROT software [61]. Thermally induced unfolding was monitored by CD at 222 nm in 0.2 cm pathlength cuvettes in the temperature range 277–363 °K. The temperature was increased stepwise by 0.2 °K at a rate of 60 °K·h⁻¹, and the ellipticity was recorded with a 1 nm bandwidth and a 2 s response. Melting temperatures

(T_m) were calculated as the maxima of the first derivatives of the temperature transition curves.

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References

- Yamada M (1992) Lipid transfer proteins in plants and microorganisms. *Plant Cell Physiol* **33**, 1–6.
- Kader JC (1997) Lipid transfer proteins: a puzzling family of plant proteins. *Trends Plant Sci* **2**, 66–70.
- Kader JC (1996) Lipid-transfer proteins in plants. *Annu Rev Plant Physiol Plant Mol Biol* **47**, 627–654.
- Douliez JP, Pato C, Rabesona H, Molle D & Marion D (2001a) Disulfide bond assignment, lipid transfer activity and secondary structure of a 7-kDa plant lipid transfer protein, LTP2. *Eur J Biochem* **268**, 1400–1403.
- García-Olmedo F, Molina A, Segura A & Moreno M (1995) the defensive role of non-specific lipid-transfer proteins. *Trends Microbiol* **3**, 72–74.
- Ge X, Chen J, Sun C & Cao K (2003) Preliminary study on the structural basis of the antifungal activity of a rice lipid transfer protein. *Protein Eng* **16**, 387–390.
- Buhot N, Gomes E, Milat ML, Ponchet M, Marion D, Lequeu J, Delrot S, Coutos-Thevenot P & Blein JP (2004) Modulation of the biological activity of a tobacco LTP1 by lipid complexation. *Mol Biol Cell* **15**, 5047–5052.
- Carvalho AO & Gomes VM (2007) Role of plant lipid transfer proteins in plant cell physiology. *Peptides* **28**, 1144–1153.
- Vega-Maray AM, Fernández-González D, Valencia-Barrera R, Polo F, Seoane-Camba JA & Suárez-Cervera M (2004) Lipid transfer proteins in *Parietaria judaica* L. pollen grains: immunocytochemical localization and function. *Eur J Cell Biol* **83**, 493–497.
- Blein JP, Coutos-Thevenot P, Marion D & Ponchet M (2002) From elicitors to lipid-transfer proteins: a new insight in cell signalling involved in plant defence mechanisms. *Trends Plant Sci* **7**, 293–296.
- Gomar J, Petit MC, Sodano F, Sy D, Marion D, Kader JC, Vovelle F & Ptak M (1996) Solution structure and lipid binding of a nonspecific lipid transfer protein extracted from maize seeds. *Protein Sci* **5**, 565–577.
- Lee JY, Min K, Cha H, Shin DH, Hwang KY & Suh SW (1998) Rice non-specific lipid transfer protein: the 1.6 Å crystal structure in the unliganded state reveals a small hydrophobic cavity. *J Mol Biol* **276**, 437–448.
- Poznanski J, Sodano P, Suh SW, Lee JY, Ptak M & Vovelle F (1999) Solution structure of a lipid transfer protein extracted from rice seeds. Comparison with homologous proteins. *Eur J Biochem* **259**, 692–708.
- Aalberse RC (2000) Structural biology of allergens. *J Allergy Clin Immunol* **106**, 228–238.
- Salcedo G, Sánchez-Monge R, Díaz-Perales A, García-Casado G & Barber D (2004) Plant non-specific lipid transfer proteins as food and pollen allergens. *Clin Exp Allergy* **34**, 1336–1341.
- Beezhold DH, Hickey VL & Kostyal DA (2003) Lipid transfer protein from *Hevea brasiliensis* (Hev b 12), a cross-reactive latex protein. *Ann Allergy Asthma Immunol* **90**, 439–445.
- Hiller KM, Lubahn BC & Klapper DG (1998) Cloning and expression of a ragweed allergen Amb a 6. *Scand J Immunol* **48**, 26–36.
- Tejera ML, Villalba M, Batanero E & Rodríguez R (1999) Identification, isolation, and characterization of Ole e 7, a new allergen of olive tree pollen. *J Allergy Clin Immunol* **104**, 797–802.
- Díaz-Perales A, Lombardero M, Sánchez-Monge R, García-Selles FJ, Pernas M, Fernández-Rivas M, Barber D & Salcedo G (2000) Lipid transfer proteins as potential plant panallergens, cross-reactivity among proteins of *Artemisia* pollen, *Castanea* nut and *Rosaceae* fruits, with different IgE-binding capacities. *Clin Exp Allergy* **30**, 1403–1410.
- Chardin H, Mayer C & Senechal H (2003) Lipid transfer protein 1 is a possible allergen in *Arabidopsis thaliana*. *Int Arch Allergy Immunol* **131**, 85–90.
- Lauer I, Miguel-Mocin MS, Abel T, Foetisch K, Hartz C, Fortunato D, Cistero-Bahima A, Vieths S & Scheurer S (1997) Identification of a plane pollen lipid transfer protein (Pla a 3) and its immunological relation to the peach lipid-transfer protein, Pru p 3. *Clin Exp Allergy* **27**, 261–269.
- Costa MA, Colombo P, Izzo V, Kennedy H, Venturella S, Cocchiara R, Mistrello G, Falagiani P & Geraci D (1994) cDNA cloning, expression and primary structure of Par j 1, a major allergen of *Parietaria judaica* pollen. *FEBS Lett* **341**, 182–186.
- Duro G, Colombo P, Costa MA, Izzo V, Porcasi R, Di Fiore R, Locotorondo G, Mirisola MG, Cocchiara R & Geraci D (1996) cDNA cloning, sequence analysis and allergological characterization of Par j 2.0101, a new major allergen of the *Parietaria judaica* pollen. *FEBS Lett* **399**, 295–298.
- Colombo P, Bonura A, Costa MA, Izzo V, Passantino R, Locorotondo G, Amoroso S & Geraci D (2003) The allergens of *Parietaria*. *Int Arch Allergy Immunol* **130**, 173–179.
- Colombo P, Kennedy D, Ramsdale T, Costa MA, Duro G, Izzo V, Salvadori S, Guerrini R, Cocchiara R,

- Mirisola MG *et al.* (1998) Identification of an immunodominant IgE epitope of the *Parietaria judaica* major allergen. *J Immunol* **160**, 2780–2785.
- 26 Asturias JA, Gómez-Bayón N, Eseverri JL & Martínez A (2003) Par j 1 and Par j 2, the major allergens from *Parietaria judaica* pollen, have similar immunoglobulin E epitopes. *Clin Exp Allergy* **33**, 518–524.
- 27 Amoresano A, Pucci P, Duro G, Colombo P, Costa MA, Izzo V, Lamba D & Geraci D (2003) Assignment of disulphide bridges in Par j 2.0101, a major allergen of *Parietaria judaica* pollen. *Biol Chem* **384**, 1165–1172.
- 28 Cheng HC, Cheng PT, Peng P, Lyu PC & Sun YJ (2004) Lipid binding in rice nonspecific lipid transfer protein-1 complexes from *Oryza sativa*. *Protein Sci* **13**, 2304–2315.
- 29 Charvolin D, Douliez JP, Marion D, Cohen-Addad C & Pebay-Peyroula E (1999) The crystal structure of a wheat nonspecific lipid transfer protein (ns-LTP1) complexed with two molecules of phospholipid at 2.1 Å resolution. *Eur J Biochem* **264**, 562–568.
- 30 Douliez JP, Michon T & Marion D (2000) Steady-state tyrosine fluorescence to study the lipid-binding properties of a wheat non-specific lipid-transfer protein (nsLTP1). *Biochim Biophys Acta* **1467**, 65–72.
- 31 Han GW, Lee JY, Song HK, Chang C, Min K, Moon J, Shin DH, Kopka ML, Sawaya MR, Yuan HS *et al.* (2001) Structure basis of non-specific lipid binding in maize lipid-transfer protein complexes revealed by high-resolution x-ray crystallography. *J Mol Biol* **308**, 263–278.
- 32 Vennyaminov SY, Yang JT & Fasman GD (eds) (1996). *Circular Dichroism and the Conformational Analysis of Biomolecules*, pp. 69–107. Plenum, New York, NY.
- 33 Douliez JP, Jégou S, Pato C, Molle D, Vinh Tran V & Marion D (2001) Binding of two mono-acylated lipid monomers by the barley lipid transfer protein, LTP1, as viewed by fluorescence, isothermal titration calorimetry and molecular modelling. *Eur J Biochem* **268**, 384–388.
- 34 Sodano P, Caille A, Sy D, de Person G, Marion D & Ptak M (1997) 1H NMR and fluorescence studies of the complexation of DMPG by wheat non-specific lipid transfer protein. Global fold of the complex. *FEBS Lett* **416**, 130–134.
- 35 Lullien-Pellerin V, Devaux C, Ihorai T, Marion D, Pahin V, Joudrier P & Gautier MF (1999) Production in *Escherichia coli* and site-directed mutagenesis of a 9-kDa nonspecific lipid transfer protein from wheat. *Eur J Biochem* **260**, 861–868.
- 36 Moreau F, Davy de Virville J, Hoffelt M, Guerbette F & Kader JC (1994) Use of a fluorimetric method to assay the binding and transfer of phospholipids by lipid transfer proteins from maize seedlings and *Arabidopsis* leaves. *Plant Cell Physiol* **35**, 267–274.
- 37 Radauer C, Merima B, Wagner S, Mari A & Breiteneder H (2008) Allergens are distributed into few protein families and possess a restricted number of biochemical functions. *J Allergy Clin Immunol* **121**, 847–852.
- 38 Jenkins JA, Griffiths-Jones S, Shewry PR, Breiteneder H & Mills ENC (2005) Structural relatedness of plant food allergens with specific reference to cross-reactive allergens: an in silico analysis. *J Allergy Clin Immunol* **115**, 163–170.
- 39 Zachowski A, Guerbette F, Grosbois M, Jolliot-Croquin A & Kader JC (1998) Characterisation of acyl binding by a plant lipid-transfer protein. *Eur J Biochem* **257**, 443–448.
- 40 Guerbette F, Grobois M, Jolliot-Croquin A, Kader JC & Zachowski A (1999) Comparison of lipid binding and transfer properties of two lipid transfer proteins from plants. *Biochemistry* **38**, 14131–14137.
- 41 Shin DH, Lee JY, Hwang KY, Kim KK & Suh SW (1995) High resolution crystal structure of the non-specific lipid-transfer protein from maize seedlings. *Structure* **3**, 189–199.
- 42 Tassin-Moindrot S, Caille A, Douliez J-P & Vovelle F (2000) The wide binding properties of a wheat nonspecific lipid-transfer protein. Solution structure of a complex with prostaglandin B2. *Eur J Biochem* **267**, 1117–1124.
- 43 Lerche MH, Kragelund BB, Bech LM & Poulsen FM (1997) Barley lipid-transfer protein complexed with palmitoyl CoA: the structure reveals a hydrophobic binding site that can expand to fit both large and small lipid-like ligands. *Structure* **5**, 291–306.
- 44 Lerche MH & Poulsen FM (1998) Solution structure of barley lipid transfer protein complexed with palmitate. Two different binding modes of palmitate in the homologous maize and barley nonspecific lipid transfer proteins. *Protein Sci* **7**, 2490–2498.
- 45 Pasquato N, Berni R, Folli C, Folloni S, Cianci M, Pantano S, Helliwell J & Zanotti G (2006) Crystal structure of peach Pru p 3, the prototypic member of the family of plant non-specific lipid transfer protein pan-allergens. *J Mol Biol* **356**, 684–694.
- 46 Tsuboi S, Osafune T, Nishimura M & Yamada M (1992) Nonspecific lipid transfer protein in castor bean cotyledon cells: subcellular localization and a possible role in lipid metabolism. *J Biochem* **111**, 500–508.
- 47 Pyee J, Yu H & Kolattukudy PE (1994) Identification of a lipid transfer protein as the major protein in the surface wax of broccoli (*Brassica oleracea*) leaves. *Arch Biochem Biophys* **331**, 460–468.
- 48 Takishima K, Watanabe S, Yama da M, Suga T & Mamiya G (1988) Amino acid sequences of two nonspecific lipid-transferproteins from germinated castor bean. *Eur J Biochem* **177**, 241–249.
- 49 Hanada K, Kumagai K, Tomishige N & Kawano M (2007) CERT and intracellular trafficking of ceramide. *Biochim Biophys Acta* **1771**, 644–653.
- 50 D'Angelo G, Polishchuk E, Di Tullio G, Santoro M, Campli AD, Godi A, West G, Bielawski J, Chuang CC,

- van der Spoel AC *et al.* (2007) Glycosphingolipid synthesis requires FAPP2 transfer of glucosylceramide. *Nature* **449**, 62–67.
- 51 Halter D, Neumann S, van Dijk SM, Wolthoorn J, de Mazière AM, Vieira OV, Mattjus P, Klumperman J, van Meer G & Sprong H (2007) Pre- and post-Golgi translocation of glucosylceramide in glycosphingolipid synthesis. *J Cell Biol* **179**, 101–115.
- 52 Arilla MC, Gonzalez-Rioja R, Ibarrola I, Mir A, Monteseirin J, Conde J, Martínez A & Asturias JA (2006) A sensitive monoclonal antibody-based enzyme-linked immunosorbent assay to quantify *Parietaria judaica* major allergens, Par j 1 and Par j 2. *Clin Exp Allergy* **36**, 87–93.
- 53 González-Rioja R, Ferrer A, Arilla MC, Ibarrola I, Viguera AR, Andreu C, Martínez A & Asturias JA (2007) Diagnosis of *Parietaria judaica* pollen allergy using natural and recombinant Par j 1 and Par j 2 allergens. *Clin Exp Allergy* **37**, 243–250.
- 54 Gill SC & von Hippel PH (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal Biochem* **182**, 319–326.
- 55 Pastorello EA, Farioli L, Pravettoni V, Ortolani C, Hispano M, Monza M, Baroglio C, Scibola E, Ansaloni R, Incorvaia C *et al.* (1999) The major allergen of peach (*Prunus persica*) is a lipid transfer protein. *J Allergy Clin Immunol* **103**, 520–526.
- 56 Arnold K, Bordoli L, Kopp J & Schwede T (2006) The SWISS-MODEL Workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* **22**, 195–201.
- 57 Brünger AT, Adams PD, Clore GM, DeLano WL, Gros P, Grosse-Kunstleve RW, Jiang JS, Kuszewski J, Nilges M, Pannu NS *et al.* (1998) Crystallography and NMR system (CNS): a new software system for macromolecular structure determination. *Acta Crystallogr D* **54**, 905–921.
- 58 Kleywegt GJ & Jones TA (1994) Detection, delineation, measurement and display of cavities in macromolecular structures. *Acta Crystallogr D* **50**, 178–185.
- 59 Jones TA, Zou JY, Cowan SW & Kjeldgaard M (1991) Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr A* **47**, 110–119.
- 60 Mayer LD, Hope MJ & Cullis PR (1986) Vesicles of variable sizes produced by a rapid extrusion procedure. *Biochim Biophys Acta* **858**, 161–168.
- 61 Deleage G & Geourjon C (1993) An interactive graphic program for calculating the secondary structure content of proteins from circular dichroism spectrum. *Comput Appl Biosci* **9**, 197–199.