Articles

Comparison of Lipid Binding and Transfer Properties of Two Lipid Transfer Proteins from Plants†

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ABSTRACT: Plant lipid transfer proteins (LTPs) are soluble proteins which are characterized by their in vitro ability to transfer phospholipids between two membranes. We have compared the functional properties of two LTPs purified from maize and wheat seeds knowing that, despite a high degree of sequence identity, the two proteins exhibit structural differences. It was found that wheat LTP had a lower transfer activity than the maize LTP, consistent with a lower kinetics of fatty acid binding. The lower affinity for the fatty acids of the wheat LTP could be explained by a narrowing occurring in the middle part of the binding site, as revealed by comparing the fluorescence spectra of various anthroyloxy-labeled fatty acids associated with the two LTPs. The affinity for some natural fatty acids was studied by competition with fluorescent fatty acids toward binding to the protein. Again, wheat LTP had a lower affinity for those molecules. All together, these observations reveal the complexity of the LTP family in plants, probably reflecting the multiple roles played by these proteins.

Nonspecific lipid transfer proteins (ns-LTPs)[1] represent a protein family which is ubiquitous in plants (*1*, *2*). They have been purified from a wide variety of plant seeds such as maize (*3*), wheat (*4*), barley (*5*), and rice (*6*) but also from plant leaves (*7*). Comparison of their biochemical properties shows several common characteristics. They are all soluble, and relatively small, containing from 91 to 95 amino acid residues, and their isoelectric point is basic (between 8.8 and 10.0). When the primary sequences are compared, differences appear leading to an identity of 40−50% and a similarity of approximately 70% between LTPs of various origins (*8*). However, they all possess eight cysteine residues located at conserved positions which form four disulfide bonds (*9*). Structural studies, by infrared or NMR spectroscopy or X-ray crystallography, have shown that these bridges connect the four helical fragments (*4*), which represent the major length of the protein, in a single compact domain continued by a long C-terminal tail (*10*−*16*). Comparison of nucleotide sequences deposited in gene banks indicates that now more than 100 potential LTPs have been identified on the basis of sequence homologies and conservation of the cysteins. Moreover, in a given plant, one can find one or several isoforms of ns-LTPs expressed in different tissues (*17*−*22*).

In vitro, these proteins are characterized by their ability to exchange glycerophospholipids or galactolipids between membranes (*1*, *9*). Several in vivo functions have been attributed to ns-LTPs, including transport of cuticular components (*20*, *23*), inhibition of the growth of bacterial or fungal pathogens (*24*−*26*), and participation in the oxidative metabolism of glyoxysomes (*21*). All these activities are related to the possibility that the proteins bind a hydrophobic molecule such as a fatty acid or a fatty acid derivative (fatty acid ester or phospholipid). Indeed, structural studies have shown that the four helices delineate a hydrophobic cavity which runs through the protein and where can be bound a fatty acid (*15*), an acyl-CoA (*27*), a lyso derivative (*11*), and even a phospholipid (*28*). To obtain better insight into the diversity of the LTP family, one needs to study the interaction of these proteins with their potential ligands. However, the isolation of a LTP−phospholipid complex from an incubation of the maize protein and phospholipid liposomes led to the conclusion that less than 1% of the protein could be recovered as a complexed form (*29*). Because of a low apparent affinity for a long-chain diacylphospholipid, the binding site is occupied by lipids only as long as membranes are present, which is similar to what was observed with an unrelated mammalian transfer protein, the sterol carrier protein (*30*). Alternatively, one can use fluorescent analogues of various lipid molecules, such as fatty acids (*31*) or phospholipids (*32*), which do not require physical separation for studying the interaction as spectral

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[1] Abbreviations: ns-LTP, nonspecific lipid transfer protein; EDTA, ethylenediaminetetraacetic acid; LUV, large unilamellar vesicles; rms, root-mean-square; SDS, sodium dodecyl sulfate; SUV, small unilamellar vesicles.

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changes occur upon association of the probe with the protein. Using fluorescent derivatives of fatty acids and a maize ns-LTP, we were able to demonstrate that the association of an acyl chain with the proteins depends on both the chain length and its degree of unsaturation (*31*). Moreover, the hydrophobic pocket of the maize protein is wide enough to accommodate two acyl chains, inferring its ability to transport a phospholipid. Nonetheless, the cavity may adopt a variable size from one protein to another and even be interrupted, thus preventing any lipid binding as in the *Ace*-AMP1 protein from onion seeds (*32*). The overall structure of the protein should then be able to influence the interaction with the lipidic ligands.

It is thus very interesting to compare the structural and functional properties of LTPs from various sources. We have chosen proteins purified from wheat and maize, two monocotyledonous plants, because they exhibit marked differences in their three-dimensional structures. We report here on the association of wheat LTP with various lipids and compare it to maize LTP.

MATERIALS AND METHODS

LTP Purification. Maize LTP was purified from seeds (*Zea mays* L., cv. Mona) according to published methods (*3*).

Wheat LTP was prepared by a modified method. Seeds (*Triticum aestivum* L., cv. Camp Rémy) were soaked for 36 h at 4 °C in 100 mM KCl, 5 mM EDTA, 8 mM 2-mercaptoethanol, and 100 mM Tris-HCl buffer (pH 7.8). After being ground in the same buffer and filtered through two layers of gauze, the homogenate was centrifuged for 30 min at 5000*g*; the supernatant was collected, and its pH was adjusted to 5.0. After a 30 min centrifugation at 10000*g*, the pH of the supernatant was brought to 7.8 with 2 M Tris base, and ammonium sulfate was added to achieve 40% saturation. After 2 h at 4 °C, the suspension was centrifuged for 15 min at 5000*g* and the ammonium sulfate concentration in the supernatant was increased to 80% saturation. After 2 h at 4 °C, the mixture was centrifuged (5000*g* for 30 min), and the pellet was resuspended in 10 mM Tris-acetate buffer (pH 7.0) and dialyzed for 1 day against the same buffer. Then, the dialysate was chromatographed on G-75 Sephadex and then on CM-Sepharose as described for the maize protein. The presence and purity of the protein were assessed along with this procedure by polyacrylamide gel electrophoresis in the presence of SDS. Cross-reactivity with an anti-maize LTP antibody (*33*) was checked after blotting of the gel on a nitrocellulose sheet.

Prior to use in the assays with lipid molecules, the purified protein that eluted from the ion exchange chromatography column was dialyzed for 4 h against 8 mM 2-mercaptoethanol and 10 mM Tris-acetate buffer (pH 7.2). The protein concentration was assayed by the method of Lowry et al. (*34*) with bovine serum albumin as a standard.

Comparison of Three-Dimensional Structures. Three-dimensional structures of wheat LTP (*10*) and maize LTP (*11*) deduced from multidimensional NMR spectroscopy were obtained from the PDB data bank (file names 1LPT and 1AFH, respectively). Structure superimposition was carried out using RasMol, and the rms deviation for the C_α positions was computed using Swiss-Model and Swiss-PdbViewer (*35*).

Phospholipid Transfer Assays. Phospholipid transfer activity was determined using radiolabeled liposomes as phospholipid donor membranes and maize mitochondria as acceptor membranes (*3*). Liposomes contained [^{3}H]phosphatidylcholine (purified from potato tuber slices after incubation with tritiated precursors) as the exchangeable lipid and [^{14}C]cholesteryloleate as the nonexchangeable one (*29*). They were introduced in the assay either as small unilamellar vesicles (SUV; 25−30 nm in diameter) obtained by sonication (*3*) or as large unilamellar vesicles (LUV; 100−150 nm in diameter) obtained by extrusion according to the manufacturer's protocol (LiposoFast system, Milsch Equipment). In any case, the same quantity of phospholipids was used as either SUV or LUV. The lipid transfer reaction was allowed to proceed for 30 min at 30 °C, and then mitochondria were separated from liposomes by centrifugation. The extent of phosphatidylcholine transfer was estimated after subtraction from the total tritium radioactivity associated with mitochondria of the radioactivity due to liposome cosedimentation estimated by the ^{14}C radioactivity.

Binding Kinetics of Fluorescent Fatty Acids. Dialyzed LTP (8 μM) was mixed with 8 mM 2-mercaptoethanol and 10 mM Mops buffer (pH 7.2, final volume of 1.5 mL) in a fluorimeter cuvette thermostated at 25 °C. 1-Pyrenedodecanoic acid (0.2 μM, from a 0.5 mM stock solution in ethanol) was added while the mixture was being stirred and the fluorescence intensity recorded at 378 nm (excitation at 343 nm) on a Perkin-Elmer LS-5 spectrofluorimeter.

Saturation by Fluorescent Fatty Acids. Dialyzed LTP was prepared for fluorescence measurement as described in the previous paragraph. Increasing amounts of 1-pyrenedodecanoic acid were added, and the fluorescence intensity at 378 nm was recorded after binding equilibration was reached (stable fluorescence reading).

Fluorescence Spectra of Anthroyloxy Fatty Acids Bound to LTP. Dialyzed LTP (8 μM) was incubated in buffer as described above. (Anthroyloxy)stearic acid (2 μM) labeled at position 6, 9, or 12 or 16-(anthroyloxy)palmitic acid (from concentrated solutions in ethanol) was added to the cuvette. After binding was completed, fluorescence spectra were recorded from 400 to 490 nm (excitation at 368 nm). All the spectra were corrected by computerized subtraction of a baseline spectrum recorded in the absence of fluorescent fatty acids. No correction was made for eventual free fatty acid fluorescence as the actual amount was not known. In any case, the fluorescence intensity due to these species would be less than 10% of the total as estimated from spectra recorded in the absence of protein.

Quantum Yield Determination. Quantum yields of the various analogues associated with the proteins have been determined by comparison with standards with known quantum yields (*36*).

Competition Experiments. Unlabeled fatty acids (oleic, linoleic, and linolenic ones) were prepared at a concentration of 0.5 mM in ethanol. A mixture of LTP (5.4 μM) and 12-(anthroyloxy)stearic acid (4 μM) was prepared in 8 mM 2-mercaptoethanol and 10 mM Mops buffer (pH 7.2) and its fluorescence recorded at 444 nm. Increasing amounts of competitive, unlabeled fatty acids were added, and the fluorescence intensity was recorded after stabilization.

Chemicals. Anthroyloxy derivatives and pyrene derivatives were purchased from Molecular Probes. Unlabeled fatty acids

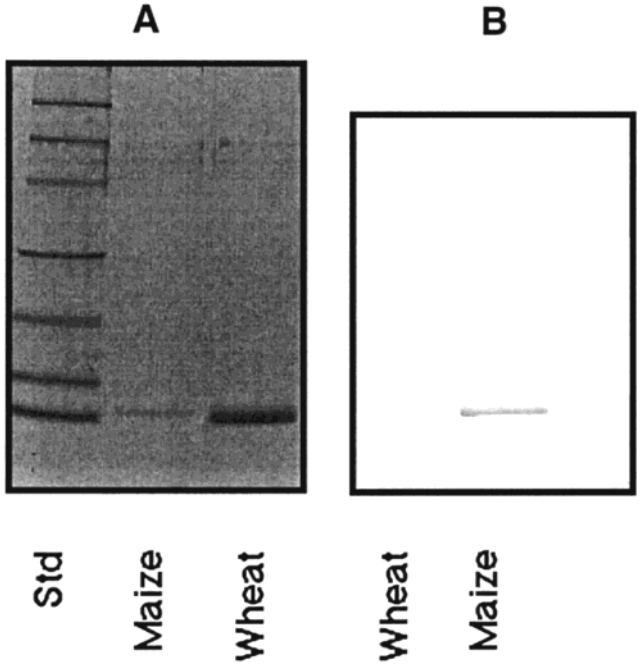

FIGURE 1: (A) SDS−polyacrylamide gel electrophoresis of purified LTPs: left lane, molecular mass standards; middle lane, purified maize LTP (10 μg); and right lane, wheat LTP (10 μg). (B) Western blot of the polyacrylamide gel revealed by an anti-maize LTP antibody and an anti-IgG labeled with peroxidase: left lane, wheat LTP; and right lane, maize LTP.

were purchased from Sigma Aldrich France. Gel chromatography resins were from Pharmacia Biotech (Uppsala, Sweden).

RESULTS

Purification of Wheat LTP. After the last step of chromatography, it was possible to obtain a fraction in which the only detectable band on SDS−polyacrylamide gel electrophoresis migrated at an apparent molecular mass of 9 kDa (Figure 1A), consistent with the known properties of this peptide (*4*). Even if the Coomassie blue staining was more intense with wheat LTP, the same amount of protein has been deposited in each case (10 μg), indicating a higher avidity for the dye of the wheat protein. It has to be noted that wheat LTP did not cross-react with an anti-maize LTP antibody in a Western blot system (Figure 1B).

Three-Dimensional Structure Comparison. Structures of maize LTP and wheat LTP, extracted from the three-dimensional PDB database, were coplotted (Figure 2). It is obvious that the two structures are not completely superimposable and that differences exist at the level of the helices or S−S bonds. One can calculate the rms deviation between corresponding C_α positions in the two proteins which is 3.49 Å. This value has to be compared to the value of 1.2 Å obtained when maize LTP was compared to rice LTP (*13*).

Phospholipid Transfer Ability. The efficiency of phosphatidylcholine transfer between artificial liposomes and mitochondria catalyzed by both wheat and maize LTP was compared, and the results are displayed in Table 1. With the two categories of liposomes that were used, the maize LTP was largely more active than the wheat one. One can see that SUV were better donors than LUV as more lipid could be transferred from the small vesicles. However, the ratio between the transfer efficiencies (maize vs wheat) decreased when LUV were used instead of SUV.

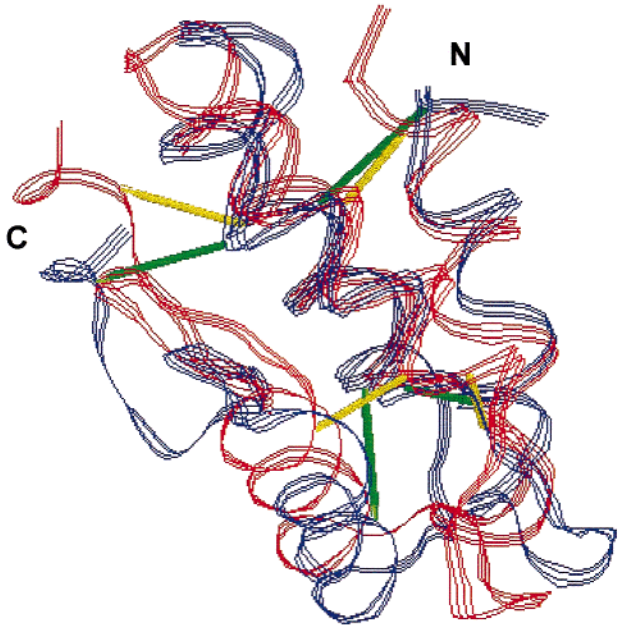

FIGURE 2: Superimposed three-dimensional structures of maize LTP (red strands and yellow S−S bonds) and wheat LTP (blue strands and green S−S bonds) as deposited in the PDB. Both structures were obtained by high-resolution NMR spectroscopy (*10*, *11*). This figure was drawn using RasMol. N and C represent the amino termini and the carboxy termini of the proteins.

Table 1: Intermembrane Phosphatidylcholine Transfer Catalyzed by either Wheat or Maize LTP[a]

donor membrane	LUV	SUV
maize LTP	0.85 ± 0.08	3.8 ± 0.3
wheat LTP	0.15 ± 0.03	0.48 ± 0.10

[a] Donor membranes were either small unilamellar vesicles (diameter of ~25 nm) or large unilamellar vesicles (diameter of ~150 nm), and acceptor membranes were maize mitochondria. Results are expressed as nanomoles of phosphatidylcholine transferred per minute per milligram of LTP and are the mean ± the standard error of values obtained in four to six independent assays.

Association of 1-Pyrenedodecanoic Acid with LTP. We first studied the kinetics of association of the fluorescent lipid with the two kinds of LTPs. The binding of the probe is accompanied by an increase in fluorescence intensity (Figure 3). However, the increase did not proceed at the same rate; the apparent half-time of binding completion was approximately 2.5 s for maize LTP and 8 s for wheat LTP. A similar type of result was obtained with other analogues [e.g., 12-(anthroyloxy)stearic acid] which associated at a higher rate with maize protein than with wheat protein (data not shown).

Both LTPs were also titrated by 1-pyrenedodecanoic acid (Figure 4). Overall, the two curves were similar. After a first phase where the fluorescence intensity increased with increasing amounts of added fatty acid, the fluorescence leveled off and then decreased. We have previously shown (*31*) that this reflects the colocalization of two molecules of analogues in the binding site, inducing a fluorescence quenching. However, the turning point was obtained for a higher fatty acid-to-protein ratio in the case of wheat LTP. Accordingly, the fluorescence increase in the first phase was

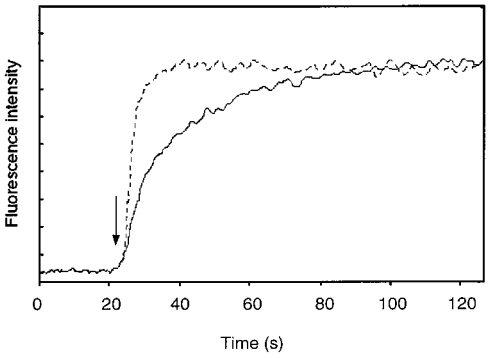

FIGURE 3: Kinetics of binding of 1-pyrenedodecanoic acid to maize LTP (···) or wheat LTP (—). At time indicated by the arrow, 0.2 μM fatty acid was added to either one of the LTPs (8 μM) in 8 mM 2-mercaptoethanol and 10 mM Mops buffer (pH 7.2). The fluorescence intensity was recorded at 378 nm, the excitation wavelength being set at 343 nm.

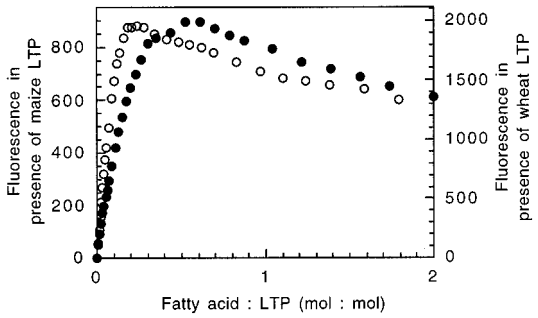

FIGURE 4: Association of 1-pyrenedodecanoic acid with LTP. Increasing amounts of the fluorescent fatty acid were added to a LTP solution under the conditions described in the legend of Figure 3. After binding equilibration was reached, the fluorescence intensity was recorded. The fluorescence obtained with maize LTP (white symbols and left scale) was compared to that obtained with wheat LTP (black symbols and right scale) as a function of the molar ratio between the fatty acid added and the protein. The titration curves are representative of at least three independent experiments.

steepest when the maize LTP was titrated. The occurrence of the binding of a second fatty acid, accompanied by fluorescence quenching, prevented the curve from reaching an apparent plateau indicative of a saturation of the binding site. As a consequence, it was not possible to derive binding constants from the titration data. It also must be noted that the intensity level recorded with wheat LTP was much higher than that obtained with maize LTP, even at low ligand-to-protein ratios. Accordingly, the quantum yield of the analogue (0.2−0.3 μM) was higher when bound to wheat LTP (0.516 ± 0.010) than when bound to maize LTP (0.276 ± 0.031).

Fluorescence of Anthroyloxy Analogues Bound to LTP. Wheat or maize LTPs were incubated with fatty acid derivatives labeled at four different positions along the acyl chain. For each compound, the fluorescence spectrum was recorded and pairwise comparisons between the two proteins were established (Figure 5). The emitted light was found at shorter wavelengths when the probe was associated with wheat LTP, the difference being maximum at median (ninth carbon) or lower median (twelfth carbon) positions. Comparison of the four spectra recorded with the wheat protein showed that the emission maximum depended on the analogue that was added. Labeling at position 6 or 16 was

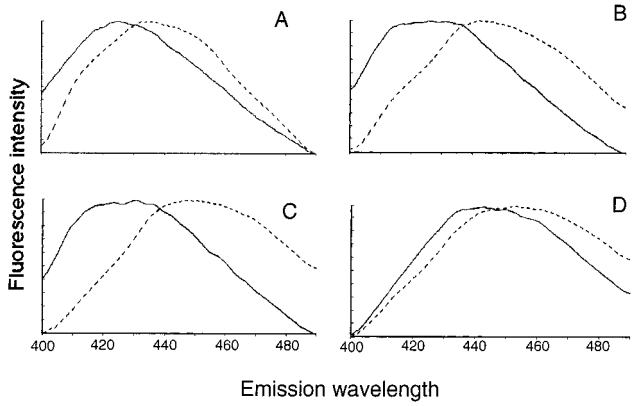

FIGURE 5: Fluorescence emission spectra of 6-, 9-, or 12-(anthroyloxy)stearic acid (spectra A−C, respectively) or 16-(anthroyloxy)palmitic acid (spectrum D) bound to maize LTP (···) or wheat LTP (—). LTP (8 μM) in the 2-mercaptoethanol and Mops buffer was incubated with 2 μM fluorescent fatty acid. After binding was complete, spectra were recorded with an excitation wavelength set at 368 nm. All the spectra were corrected for the spectrum obtained in the absence of the fluorophore.

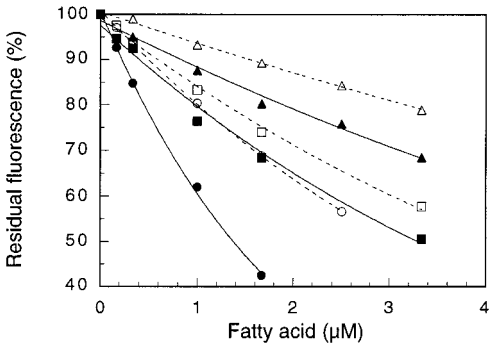

FIGURE 6: Competition between 12-(anthroyloxy)stearic acid and various unlabeled fatty acids. Purified LTP (5.4 μM) from maize (black symbols) or wheat (white symbols) was incubated with 4 μM fluorescent fatty acid. Various amounts of the unlabeled fatty acids [oleic (circles), linoleic (squares), and linolenic (triangles)] were added from concentrated solutions in ethanol. The fluorescence intensity (emission at 444 nm and excitation at 368 nm) was recorded after stabilization. The abscissa shows the unlabeled fatty acid concentration; the ordinate shows the percentage of the original fluorescence recorded after fatty acid addition.

accompanied by spectra exhibiting one maximum, slightly more blue-shifted for the 6-labeled derivative (~425 nm) than for the 16-labeled one (~440 nm). For both the 9- and 12-labeled derivatives, spectra were composite, indicating that a substantial amount of light was emitted at a low wavelength (~410 nm). These differences were also found when quantum yields of the analogues were compared. At positions 6, 9, 12, and 16, the values were 0.197, 0.219, 0.258, and 0.208 with maize LTP and 0.202, 0.257, 0.265, and 0.252 with wheat LTP, respectively (with a deviation of 0.006 over three independent assays).

Binding of Various Fatty Acids. Association of unlabeled fatty acids with LTP was studied by following the displacement of 12-(anthroyloxy)stearic acid from the protein upon addition of C18 fatty acids with one, two, or three unsaturations. Here again, wheat LTP and maize LTP did not adopt the same behavior (Figure 6). As a general rule, it required a higher amount of fatty acid to displace the probe from the wheat LTP than from the maize LTP. Also, the more unsaturated the fatty acid, the smaller the displacement

for an equivalent amount.

DISCUSSION

Plant ns-LTPs were defined by their in vitro ability to exchange phospholipids between two bilayers. They share a similar overall three-dimensional structure, with four α-helices stabilized by four disulfide bonds surrounding a hydrophobic cavity. However, as illustrated by the comparison between wheat LTP and maize LTP, the spatial conformation can be different enough to prevent an immunological cross-reactivity. Some of these LTP proteins exhibit a high transfer capability, as is the case for maize LTP. On the other hand, the antimicrobial protein (*Ace*-AMP1) extracted from onion seeds appears to adopt the LTP structure (*8*, *32*) while being unable to transfer a lipid (*24*). The phospholipid transfer catalyzed by the wheat LTP appeared to be intermediate. It was quite able to move lipids between two membranes; however, it did it at a 5−8-fold lower rate than the maize LTP, according to the donor membrane presentation. A priori, two explanations could be put forward to explain the data. (i) The LTP has a low level of interaction with the membranes, or (ii) the LTP did not efficiently load and unload a phosphatidylcholine molecule. The interaction between a membrane and a LTP has been attributed to electrostatic interactions between some positive charges on the proteins and negatives charges borne by the phospholipids (*37*). It has been shown that the positive electrostatic potential at the wheat LTP surface was close to that at the maize LTP surface (*8*). Thus, both LTPs should be able to equally approach the membrane. Nonetheless, the physical state of the membrane obviously played a role in the protein activity, as the transfer was different depending on whether the donor vesicles had a low radius of curvature (SUV) or an intermediate one (LUV). Indeed, it was shown that a LTP has to partially penetrate between lipid molecules (*37*). The higher level of lipid packing which exists in LUV membranes, compared to that in SUV membranes, would result in a more difficult association with the LTP. In fact, Sodano et al. (*28*) have measured a 5-fold lower affinity of wheat LTP for multilamellar vesicles than for SUV. The higher level of lipid packing in the former vesicle category was suggested to be responsible for it. Similarly, we could hypothesize a lower affinity of maize and wheat LTP for a packed membrane to explain our data. However, it has to be noted that the two LTPs did not exhibit the same response to lipid lateral pressure, with the maize LTP appearing to be more sensitive to this parameter. This sensitivity of intermembrane exchange toward bilayer curvature seems to be a general property. This physical parameter affects lipid intervesicular transfer catalyzed by phosphatidylcholine exchange protein (*38*), glycolipid transfer protein (*39*), or plasma phospholipid transfer protein (*40*) and also spontaneous cholesterol transfer (*41*).

When the binding of a lipid molecule to LTP is being studied, striking differences appeared between the two proteins. First, kinetic studies showed that the association between a fluorescent fatty acid analogue and the protein occurred more rapidly when the LTP was from maize seeds. This result resembles those reported by Tassin et al. (*32*). They studied the association of a fluorescent analogue of either phosphatidylcholine or phosphatidylglycerol with various LTPs and found that the binding to maize LTP was faster than that to wheat LTP. However, in these latter experiments, the analogues were introduced as liposomes and their association with the proteins required first their extraction from the membrane. In our experiments, the fatty acid analogues were in the solvent as monomers or micelles, two forms in rapid exchange, and thus readily available for binding to LTP. It is most probable that the insertion of the fatty acid in the hydrophobic wheat cavity was slower than in the maize one. This different behavior was also apparent when the titration curves of the two LTPs for titration by 1-pyrenedodecanoic acid were coplotted. The first step of the curves (where fluorescence increased) arose from the binding of the first fatty acid to the hydrophobic cavity. But it was possible for a second molecule to enter the cavity (*31*), and the fluorescence of these molecules was lost (either by quenching or by excimer formation). This led to the appearance of a pseudomaximum followed by a fluorescence decrease as more analogue was added. The curves indicated that more fluorescent fatty acid had to be present with wheat LTP to obtain the fluorescence decrease, an argument for a lower affinity of the wheat LTP for the compound (at least for the binding of the first fatty acid). Surprisingly, the fluorescence intensity of the analogue present in wheat LTP was higher than that of the analogue associated with maize LTP. This is an indication that the polarity of the protein at the level of the fluorophore was lower in the wheat LTP as revealed by a higher quantum yield of the probe complexed with the wheat protein.

This difference at the level of fatty acid binding should be related to a different structure of the hydrophobic cavity which runs through the proteins (*8*, *10*, *12*, *15*, *27*). Estimation of the cavity volume in the unliganded state varies according to the computer program that was utilized to determine it; maize LTP and wheat LTP cavities have a respective volumes of 280 and 380 Å^3 (*8*) or of 408 and 360 Å^3 (*13*). Moreover, it has been shown that the cavity size was modified to some extent upon insertion of a ligand (*15*, *27*, *28*). We obtained insight into the cavity geometry by recording the emission spectra of anthroyloxy derivatives bound to the proteins. Their emission maxima are only weakly affected by the surrounding polarity but are very sensitive to steric hindrance around the anthroyloxy moiety (*42*, *43*). In the initial excited state, the anthracene ring is perpendicular to the carbonyl group and relaxation tends to make it coplanar, to the extent that rotational motion is not hindered. This orientation change is accompanied by a red shift of the fluorescence spectrum. Comparison of the spectra recorded with maize LTP showed that there was no strong steric hindrance of the probe motion along the binding site (*31*). In the case of wheat LTP, results were strikingly different. At the level of the ninth and twelfth carbon of the fatty acid, a very drastic hindrance should exist as a very important blue-shifted component appeared in the spectrum. This steric hindrance was less important at the level of the sixth carbon and still less at the sixteenth position. When the two proteins were compared, it appeared that rotational motion was always easier in the maize LTP than in the wheat LTP, especially between the sixth and the twelfth carbon positions. This (local) narrowing of the free cavity space could explain why the insertion of a fatty acid into the wheat protein was more difficult as reflected by the slower kinetics and the lower binding affinity. Emission spectra of anthroy-

loxy derivatives exhibiting a blue shift similar to the one obtained with wheat LTP, indicative of a steric hindrance in the middle region of the hydrocarbon chain, have been recorded for liver fatty acid binding protein (*36*). On the other hand, isoforms of this protein found in adipocytes or heart cells behave more like maize LTP with emission maxima in the 440−450 nm range (*44*). Differences could also be found in quantum yields of the probes complexed to the proteins. In the case of LTPs, yields were higher at positions 9, 12, and 16 of the acyl chain when the wheat protein was compared to the maize one. This indicates a less polar environment at these levels (*42*). Moreover, no striking difference appeared between the twelfth and the sixteenth position of a probe associated with wheat LTP, while a decrease occurred at the deeper position with the maize protein. This shows again that the two cavities are not equivalent. When compared to published data, the yields are overall much lower than the ones recorded with the same analogues and a liver fatty acid binding protein (*36*) but somewhat higher than the ones obtained with isoforms from adipocytes or heart (*44*). In fact, it is when the analogues are bound to retinoid binding protein that the yields are close to those obtained with the LTPs (*45*). It is thus difficult to find a common behavior with these proteins which all bind fatty acids.

To establish a relationship between the binding ability of the LTP toward the fluorescent analogues and the phospholipid transfer activity, it was necessary to estimate the binding capacity of natural, unlabeled fatty acids. We chose to study this property by a competitive assay between a given analogue [12-(anthroyloxy)stearic acid] and a series of C18 fatty acids. The LTP was first loaded with the fluorescent molecule; then increasing amounts of the competitor were added, and the displacement of the probe was followed by the decrease in fluorescence activity. Again, wheat LTP exhibited a general lower affinity for the molecules that were used. In all cases, increasing the number of double bonds in the fatty acid chain led to a lower displacement power of the molecule. It has to be noted that saturated species (16:0 or 18:0) exhibited properties very comparable to those of oleic acid with wheat (not shown) or maize LTP (*31*), showing that the presence of a single unsaturation did not affect the affinity of the fatty acid for the protein. Considering these data, one could assume that the lower ability to transfer phospholipids borne by wheat LTP was due (mainly) to its lower ability to bind the lipids as the phospholipids used in the transfer assays contain polyunsaturated fatty acyl chains. This property could, in turn, be attributed to the structure of the hydrophobic pocket where the lipids are positioned and which exhibited more steric constraints in the wheat protein. Such a difference in affinities for lipid molecules could be the major reason for a lesser ability of wheat LTP to transfer phospholipids since a lower fraction of the protein will be in a complexed state active in transfer reactions. However, the extracytoplasmic location of the known LTP, as assessed from the signal peptide present in their gene (*46*, *47*), precludes the possibility that transporting a phospholipid molecule is their unique role. When an implication in surface wax and cutin biosynthesis was examined (*20*, *48*), both maize and wheat LTP were capable of binding hydroxylated acyl chain precursors in this metabolism, but with a low affinity (not shown). Knowledge of the relationship between the cavity structure and the affinity for various lipidic ligands, as well as between protein structure and membrane interaction, would help in defining the role(s) of the various isoforms of LTP. As an example, it was shown that a low-temperature-inducible barley LTP differs in its structural and functional properties from maize LTP (*49*). This could be related to potential roles of LTP in frost acclimatization in plants.

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