

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

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Abstract

The structure of a nonspecific lipid transfer protein from barley (ns-LTP_{barley}) in complex with palmitate has been determined by NMR spectroscopy. The structure has been compared to the structure of ns-LTP_{barley} in the absence of palmitate, to the structure of ns-LTP_{barley} in complex with palmitoyl coenzyme A, to the structure of ns-LTP_{maize} in its free form, and to the maize protein complexed with palmitate. Binding of palmitate only affects the structure of ns-LTP_{barley} moderately in contrast to the binding of palmitoyl coenzyme A, which leads to a considerable expansion of the protein. The modes of binding palmitate to the maize and barley protein are different. Although in neither case there are major conformational changes in the protein, the orientation of the palmitate in the two proteins is exactly opposite.

Keywords: barley lipid transfer protein; binding; complex; cutin synthesis; NMR spectroscopy; solution structure

Plant lipid transfer proteins (LTPs) are a group of small, basic proteins. They exhibit a strong sequence as well as structural homology, and with a few exceptions they contain eight cysteines conserved in four disulfide bridges (Desormeaux et al., 1992). Plant LTPs show a very broad specificity; hence, they are prefixed nonspecific (ns).

In addition to being able to facilitate lipid transfer between membranes, a moderate binding of fatty acids, acyl-coenzyme A (CoA) and lyso-phosphatidylcholines (Lyso-PC) has been shown for a number of plant ns-LTPs (Rickers et al., 1985; Østergaard et al., 1993; Gomar et al., 1996). This dual ability has led to a proposed function of these proteins in membrane biogenesis (Aron del et al., 1990). However, a number of studies have shown ns-LTPs to be secreted, and this has led to the proposal of other hypotheses. Plant ns-LTPs are capable of inhibiting bacterial and fungal pathogens and are, therefore, thought to play a role in plant defense (Molina et al., 1993). Because of the location of ns-LTPs

in the cell wall, these proteins are also thought to transport cutin monomers required for the biosynthesis of surface wax (Sterk et al., 1991).

The three-dimensional structure of ns-LTPs are known for four different species including maize (Shin et al., 1995; Gomar et al., 1996), wheat (Gincel et al., 1994), barley (Heinemann et al., 1996), and rice (Lee et al., 1998), and a ns-LTP like structure of Ace-AMPI extracted from onion seeds (Tassin et al., 1998). These all show the plant ns-LTPs to be single compact domain proteins comprised of four α -helices and a long extended C-terminus with no regular secondary structure. In addition three structures of ns-LTPs in complex with ligands have been published. These are the crystal structure of ns-LTP_{maize} in complex with palmitate (Shin et al., 1995), the structure solved by NMR of ns-LTP_{barley} in complex with palmitoyl coenzyme A (PCoA) (Lerche et al., 1997), and the complex between ns-LTP_{wheat} and a diacyl phospholipid (DMPG) (Sodano et al., 1997). From these studies it is evident that although both the sequences and the three-dimensional structures are very similar among plant ns-LTPs, the binding modes of these proteins differ.

Here, the structure determination by NMR spectroscopy of ns-LTP_{barley} in complex with palmitate is being described. It is the aim of this work to understand the effect of the size of a ligand on the ligand binding-induced conformational changes in the protein structure, and to compare the binding modes of palmitate in two different ns-LTPs from barley and maize, respectively.

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Abbreviations: DMPG, dimyristoylphosphatidylglycerol; H_A, helix A; H_B, helix B; H_C, helix C; H_D, helix D; HSQC, heteronuclear single quantum coherence; Lyso-PC, lysophosphatidylcholine; ns-LTP, nonspecific lipid transfer protein; NOE, nuclear Overhauser effect; NOESY, NOE spectrometry; PCoA, palmitoyl coenzyme A; RMSD, root-mean-square deviation; TOCSY, total correlation spectroscopy.

Results

Assignments

The ^1H assignments of ns-LTP_{barley} in complex with palmitate were performed using the Wüthrich strategy (Wüthrich, 1986) combined with the PRONTO spin system identification tool (Kjær et al., 1994). A list of the assignments has been deposited in the BioMagRes Bank, with the accession code 4142.

An analysis of the three recorded NOESY spectra resulted in 743 nonredundant intraprotein NOE-derived distance restraints and 127 dihedral angle restraints. The distribution in sequence of the distance restraints is shown in Figures 1A and 1B, along with a summary of the dihedral angles restraints in Figure 1C. To assign the methylene groups of palmitate, ^{13}C -HSQC-NOE spectra of the complex with ^{13}C -labeled palmitate were recorded. This resulted in assignment of six methylene groups, and allowed 23 interprotein-ligand NOE-derived distance restraints. The ^{13}C -labeling of palmitate made it possible to distinguish the methyl group and three additional proton resonances in the alpha end of the palmitate and three proton resonances in the carboxyl end of the palmitate (Fig. 2). No restraints have been applied to the eight methylene groups in the middle of the chain. The protein residues close in space to the ligand are primarily hydrophobic residues in the core of the protein, but also the disulfide bridge, Cys13–Cys27, which links the second part of H_A with H_B is in the vicinity of the ligand.

The NMR data, including ^1H -NMR signals, coupling constants, stereospecific assignments, and the distribution of the sequential and medium range NOEs resemble the data of the free form of ns-LTP_{barley}. Thus, the secondary structure elements of the ns-LTP_{barley} in complex with palmitate are identical to the ones found in the free form. The four disulfide bridges were likewise identified by NOEs between β -protons in each pair, and Pro23 like in the structure of the free ns-LTP_{barley} forms a *cis*-peptide bond (Heinemann et al., 1996; Lerche et al., 1997).

Structure determination

The structure calculations were carried out using the XPLOR 3.8 protocol (Brünger, 1992). An evaluation in terms of identity and quality of the 20 lowest energy structures was performed partly by comparing the coordinates of the individual structures to a calculated average structure and partly by calculating the average energies extracted from the force field used in the calculations (Table 1). When superimposed on an average structure the RMSD for the peptide backbone (N,C α ,C' and O) is 0.81 ± 0.15 Å, and for all nonhydrogen atoms 1.22 ± 0.20 Å. In Figure 1D the distribution of the RMSD per residue is presented. The RMSD for the two restrained ends of the ligand is 0.24 ± 0.01 for the three residues in the carboxyl end and 0.17 ± 0.1 for the four residues in the methyl group end, respectively. The nonrestrained middle part of the acyl chain has a slightly higher RMSD of 0.6 ± 0.12 , as seen in Figure 1E. The RMSD for the nonhydrogen atoms of the entire complex is 1.66 ± 0.79 Å (Fig. 3). Here, two significantly different conformations of the ligand are seen in the ensemble of 20 structures. Two of the 20 structures differ from the remaining 18 by placing four middle methylene groups of the acyl chain bent toward residues in helix H_B. In the majority of the structures, the orientation of these methylene groups is toward helix H_C.

The ligand is completely embedded within the hydrophobic pocket formed by residue V6, L14, and V17 in H_A; V31 and L34 in H_B;

V47, L51, and I54 in H_C; L61, L63, and I69 in H_D, and I81 and I85 in the C-terminus (Fig. 4). The methyl group end of the palmitate makes contacts to Tyr79 and Arg44, placing this end of the ligand near the loop between H_B and H_C. The other end of the palmitate, the carboxyl end, is situated near the loop between H_A and H_B close to the disulfide bridge, which keeps these two helices together. The H2 protons of the ligand make contacts to hydrophobic residues in the C-terminus and to Ile69 in H_D. The carboxylate group of the ligand is within electrostatic range of both the $-\text{NH}_3^+$ group in Lys9 in H_A and that of Lys72 in the far end of H_D. However, there is no experimental observation that could support either of these latter interactions.

Discussion

Binding of a small and large ligand to ns-LTP_{barley}

The arrangement of the four α -helices and the C-terminus is similar to the one seen in the structure of the free ns-LTP_{barley}. The RMSD between the backbone atoms (N,C α ,C',CO) is 1.33 Å for the two structures. When comparing the chemical shifts, the applied distance restraints and the C α –C α distances in the complex of ns-LTP_{barley} and palmitate with the similar parameters in the structure of the free ns-LTP_{barley} a uniform picture is formed. Overall, there are no significant changes in chemical shifts between the two forms of ns-LTP_{barley}, although a few residues, Val77–Ile81, have changed up to 1 ppm (Fig. 5). The perturbed residues are thus primarily in the C-terminal region, but also to a lesser extent in H_C and in the second half of H_A. The distance restraints applied in the structure calculation of the complex between ns-LTP_{barley} and palmitate are in most cases the same as those identified in the spectra of the free protein. Generally, the C α –C α distance differences in the two forms of ns-LTP_{barley} are small, and the differences are mostly seen in the C-terminus, which has moved closer in space toward the rest of the molecule (Fig. 6). This is opposite to the complex of ns-LTP_{barley} and the larger ligand PCoA. For this complex both chemical shifts and C α –C α distances significantly differs compared to the free form of ns-LTP_{barley}. It is noteworthy that the structural changes are recognized in the same regions in the two complexes compared to the free protein. The separation of H_B and H_C is in both complexes perturbed in order for the ligands to be accommodated in the protein structure. The position of the C-terminus in the complex with the larger ligand is pushed outward into the solvent, whereas in the complex between ns-LTP_{barley} and palmitate it has come closer in space to especially H_B than in the free protein. The different behavior of the C-terminus, when ns-LTP_{barley} binds the two different ligands, suggests that this part of the ns-LTP structure can be very flexible.

Binding of palmitate to barley and maize ns-LTPs

The complex between ns-LTP_{barley} and palmitate is the fourth ns-LTP structure in complex with a ligand to be reported. This work is a parallel study to the ns-LTP_{maize} complex with palmitate, and an extension of previous work with the complex of PCoA and ns-LTP_{barley}. In the case of palmitate binding, the three-dimensional structure undergoes only significant structural perturbation in the corresponding residues, 77 through 83 of the C-terminus. However, a comparison of the actual interactions between the ligand and the protein in the two complexes makes it clear that although

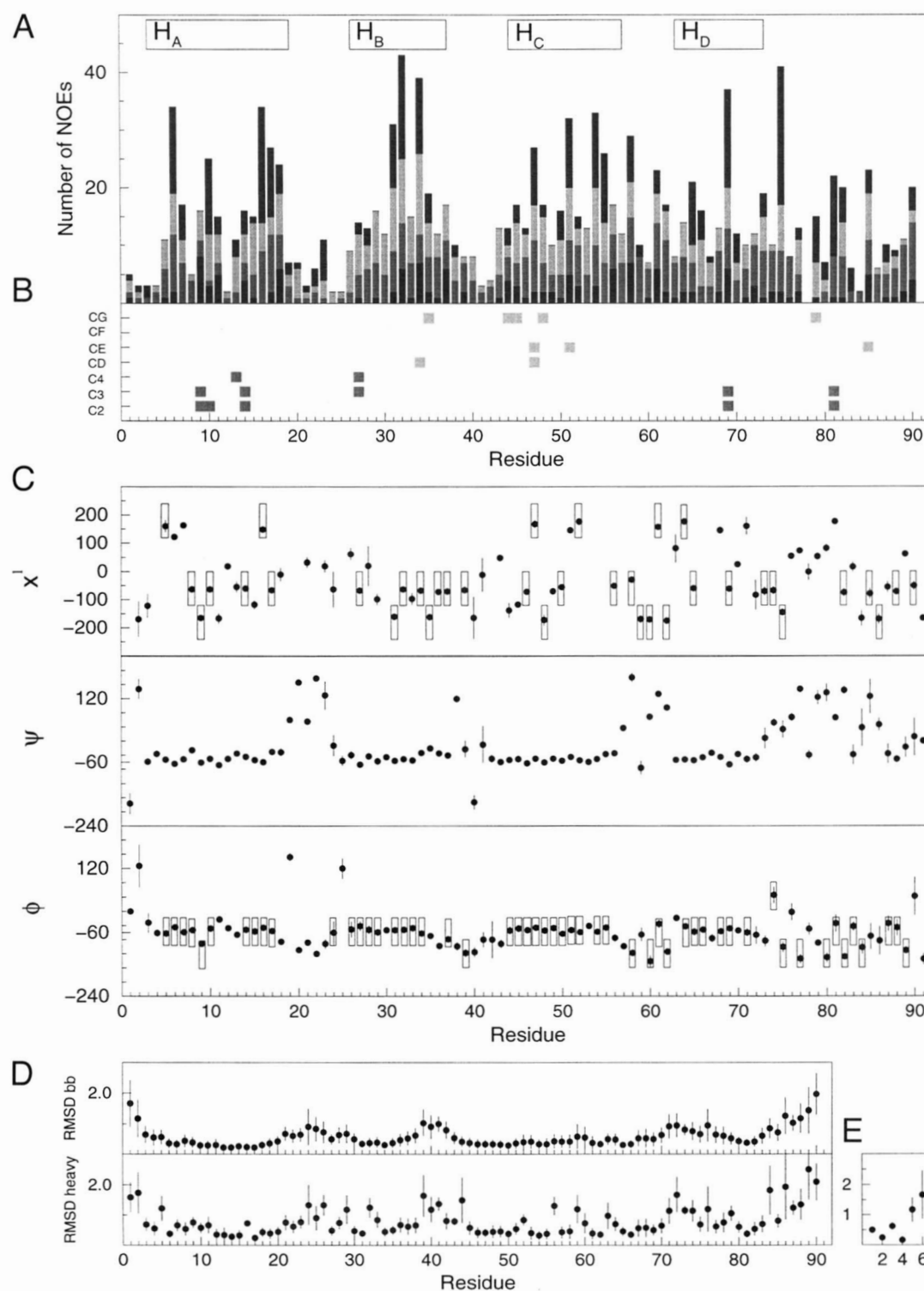


Fig. 1. Assessment and summary of structural restraints. **A:** The NOE distribution in complexed ns-LTP_{barley} shown in stacked bars. Intraresidual NOEs (bottom black), sequential NOEs (dark dots), medium range NOEs (light dots), and long range NOEs (top black). Boxes indicate the positions of α -helical structure. **B:** The NOE distribution of protein-ligand NOEs. Light squares represent NOEs between the protein and the methyl group or either of the three additional restrained methylenes in the omega end of the ligand. Dark squares represents NOEs between the protein and either of the three restrained methylenes in the carboxyl end of the ligand. **C:** Average ϕ , ψ , and χ^1 angles extracted from the ensemble of 20 model structures. Boxes indicate the restraints applied. **D:** The RMSD for the complexed protein. The top figure show the RMSD of the backbone atoms (N,C α ,C' and O). The bottom figure show the RMSD of all nonhydrogen atoms. **E:** The RMSD for the ligand. The ligand methylenes have been grouped according to the restraints applied: in order of appearance it is the carboxyl group, the first three methylene groups in the alpha end (C2, C3, C4), the middle nonrestrained eight methylenes (C5-CC), the methyl group (CG), together with the three last methylenes (CD, CE, CF), all nonhydrogen atoms, and finally, all ligand atoms.

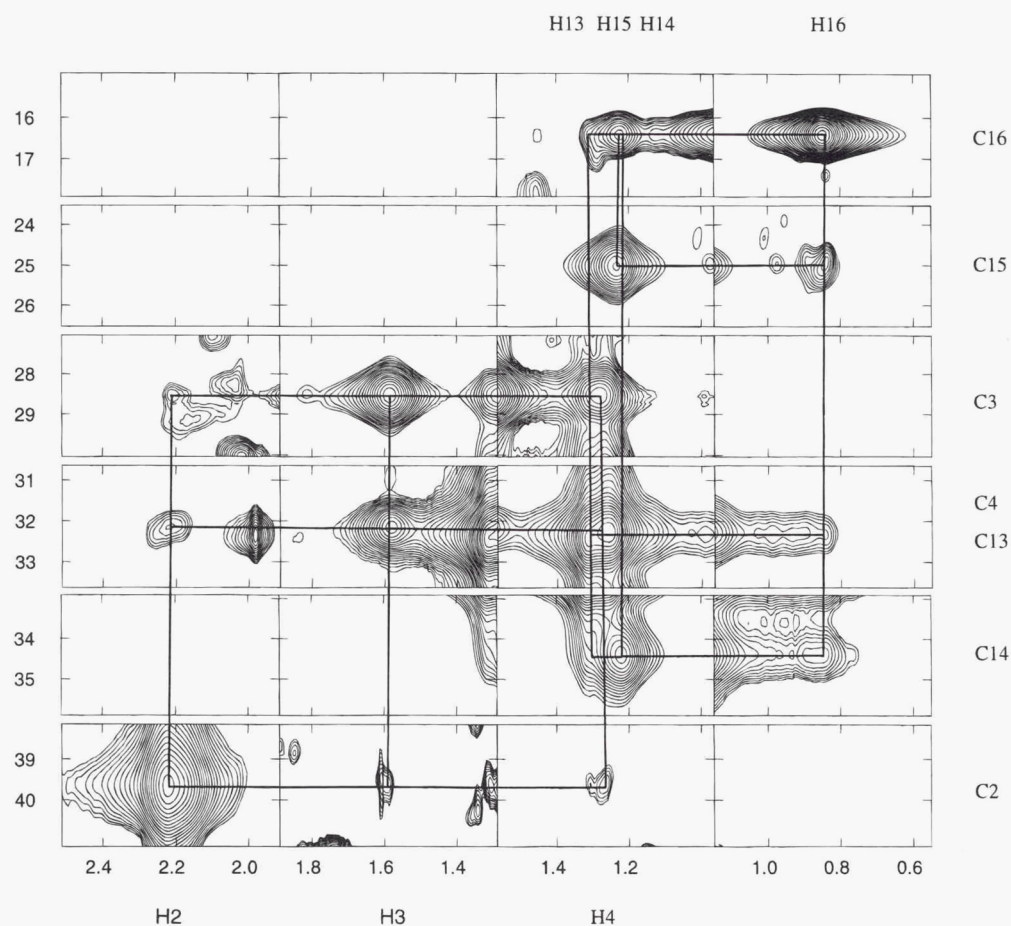


Fig. 2. HSQC-NOE spectrum. Diagnostic ligand NOEs. Lines indicating the NOE pattern of the assigned two ends of the palmitate.

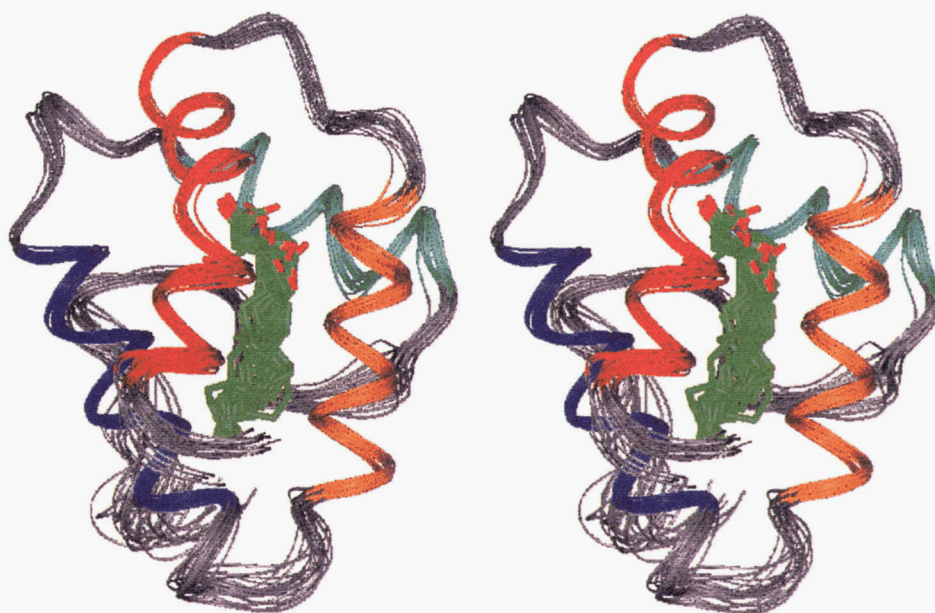


Fig. 3. A stereo view of an NMR bundle of the complex between ns-LTP_{barley} and palmitate. The 20 structures are shown as a superposition of the peptide backbone. H_A (red), H_B (orange), H_C (blue), H_D (cyan), and the ligand is colored according to atoms, carbon (green), and oxygen (red).

Table 1. Structural statistics of the family of 20 structures representing the complex between ns-LTP_{barley} and palmitate

Distance restraints			
Intraresidual			152
Sequential ($ i - j = 1$)			218
Medium range ($ i - j < 5$)			199
Long range ($ i - j > 5$)			174
Intraligand			11
Protein–ligand			
Hydrogen bonds			35
Dihedral angle restraints			
ϕ			53
χ^1			37
χ^2			10
Deviations from experimentally derived restraints (per structure)			
NOE violations > 0.3 Å			0.1
RMSD			0.029 ± 0.002
Angle violations $> 5^\circ$			0.05
RMSD			0.41 ± 0.086
Hydrogen bonds > 0.3 Å			0.4
Deviations from ideal geometry (RMSD)			
Impropers (Å)			0.42 ± 0.02
Bonds (Å)			0.013 ± 0.0003
Angles ($^\circ$)			3.07 ± 0.046
Xplor38 energies (kcal mol ⁻¹) ^a			
Bond			45.76 ± 1.61
Angle			378.48 ± 10.57
Dihedral angle			487.21 ± 10.24
Impropers			10.16 ± 1.024
Van der Waals			-299.24 ± 9.93
Electrostatics			$-1,456.36 \pm 29.85$
Hydrogen bonds			-137.64 ± 8.9
NOE			28.26 ± 3.19
Dihedral angle restraints			0.0068 ± 0.0029
Procheck (RMSD) ^b			
ϕ and ψ angles lying in the core region of the Ramachandran plot (%)	71.1	Equiv. res. 2.8 Å	
H-bond energy SD (kcal/mol)	0.9	Equiv. res. 2.2 Å	
χ^1 pooled SD ($^\circ$)	24.6	Equiv. res. 2.8 Å	
χ^2 trans SD ($^\circ$)	22.9	Equiv. res. 2.5 Å	
RMSD with respect to the mean structure (Å) ^c			
H _A			0.24 ± 0.06
H _B			0.24 ± 0.06
H _C			0.19 ± 0.05
H _D			0.29 ± 0.07
Backbone atoms (N, C α , C', and O) of protein			0.81 ± 0.15
All nonhydrogen atoms of protein			1.27 ± 0.20
All nonhydrogen atoms ligand			1.65 ± 0.79
All nonhydrogen atoms complex			1.46 ± 0.49

^aThese energy terms are calculated using the full CHARMM force field implemented in XPLOR.

^bCalculated using PROCHECK_NMR (Laskowski et al., 1993).

^cThe mean structure was calculated by the fitting of N, C α , C', and O for all residues.

corresponding hydrophobic residues make contact, the contacts are different. In the maize complex, the carboxyl end of the palmitate is in close vicinity to Arg44 and Tyr79, and the methyl group makes contacts to hydrophobic residues in the second

half of H_A and in H_D. This is the complete opposite in the barley complex.

It has previously been suggested that the binding site of ns-LTP_{maize} is a hydrophobic groove in the globular structure, which is covered by the C-terminal peptide to form a tunnel with a wide opening in one end and a narrow opening in the other end (Shin et al., 1995). The wide opening is considered to be the entrance. This is where the carboxylate binds. The narrow opening is considered a closed exit. This is where the methyl group binds. The present structure of ns-LTP_{barley} in complex with palmitate, as well as the structure of the complex with the larger and acylated ligand palmitoyl coenzyme A, both have the methyl end of the fatty acid at the site that corresponds to the carboxylate site in ns-LTP_{maize}. In ns-LTP_{barley} it seems unlikely that the larger acylated ligand could have entered at the site corresponding to the proposed entrance in the maize protein. This difference in binding mode would suggest that, in terms of the narrowing tunnel model, the corresponding entrance and exit in the maize and barley proteins have been interchanged.

Instead, the different binding modes seen in the complexes of the maize and barley proteins may suggest that the binding is accomplished after a relocation of the C-terminal peptide that expose the hydrophobic cavity and make an attachment of the hydrophobic ligand possible. A repositioning of the C-terminal peptide over the ligand may then exclude the hydrophobic ligand from nonfavorable contacts with water. This model would explain the properties of both proteins for encapsulating a large hydrophobic ligand. Closing over a larger ligand will require a curl-up of the

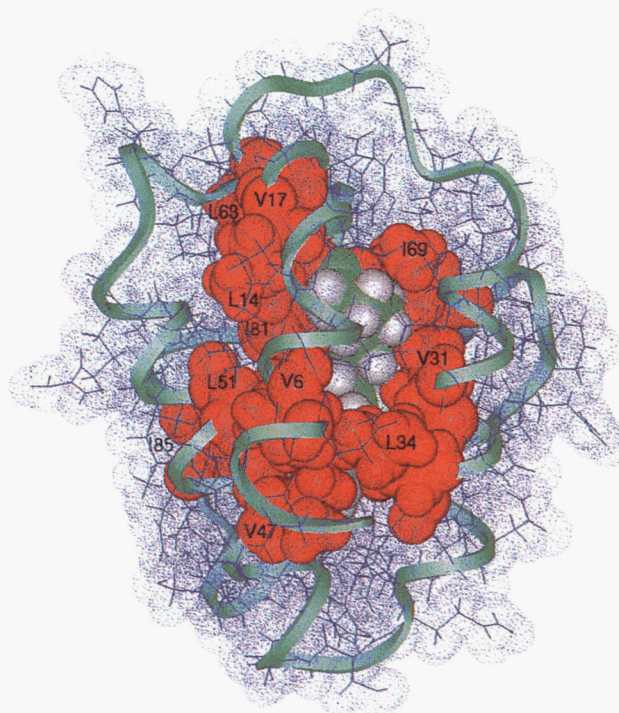


Fig. 4. The complex between ns-LTP_{barley} and palmitate is shown as van der Waals surface. ns-LTP_{barley} is shown as ribbon structure. Palmitate is colored according to atoms, carbon (green), hydrogen (white), and oxygen (red) and shown in CPK presentation. The hydrophobic residues constituting the hydrophobic pocket are colored red and presented in CPK presentation.

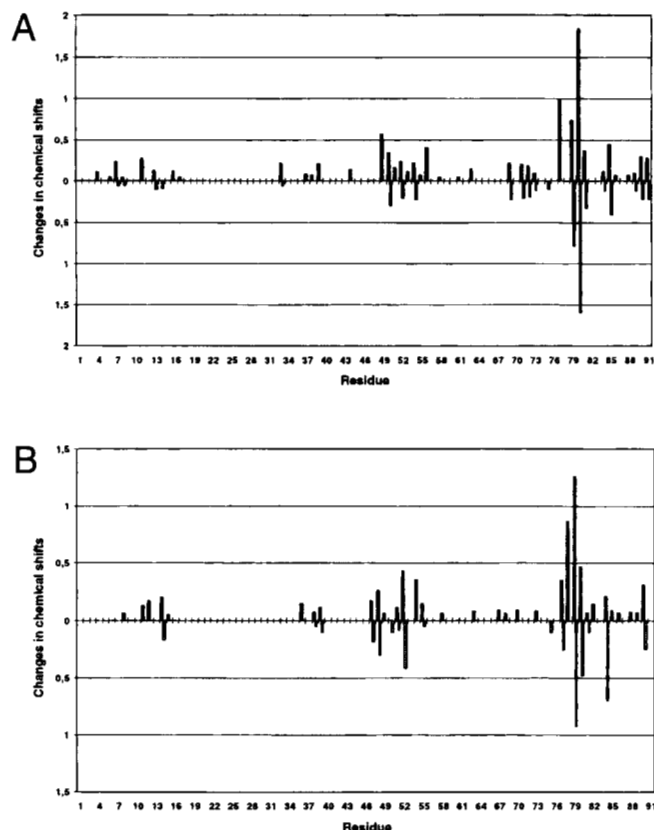


Fig. 5. Differences in chemical shift of backbone H^N and H^α in liganded and unliganded ns-LTP_{barley}, determined at pH 7.2. (A) The differences in H^N ; (B) the differences in H^α . Bars above the centered zero line represent the comparison of the free form of ns-LTP_{barley} and the complex with PCoA. Bars below the centered zero line represents the comparison of the free form of ns-LTP_{barley} and the complex with palmitate. Changes in chemical shifts are primarily identified in the C-terminal region of especially residue Val77–Ile81.

ligand and reposition the lid further apart from the rest of the protein than position in the native form of the protein, as seen for the binding of PCoA.

A polar residue attached to the hydrophobic ligand will have to reside outside the binding site at a position determined by the orientation of the ligand in the binding site.

Binding of larger ligands to ns-LTP

The reported complex between DMPG and ns-LTP_{wheat} differ to some extent from the ns-LTP_{maize} and ns-LTP_{barley} complexed with palmitate. The RMSD between the free ns-LTP_{wheat} structure and the structure of ns-LTP_{wheat} in complex with DMPG is slightly larger, suggesting that the much larger ligand alters the protein structure to a greater extent than for the complexes with palmitate (Table 2). From the kinemage of the two forms of ns-LTP_{wheat} in the report on the complexed ns-LTP_{wheat} structure (Sodano et al., 1997), it is evident that the perturbed residues in the C-terminus of the complexed ns-LTP_{wheat} are pushed outward into the solution. In the structures of maize and barley LTP in complex with palmitate, the C-terminus is closer to its positions in the respective free forms. In this respect the ns-LTP_{wheat} complex resembles the complex between ns-LTP_{barley} and PCoA.

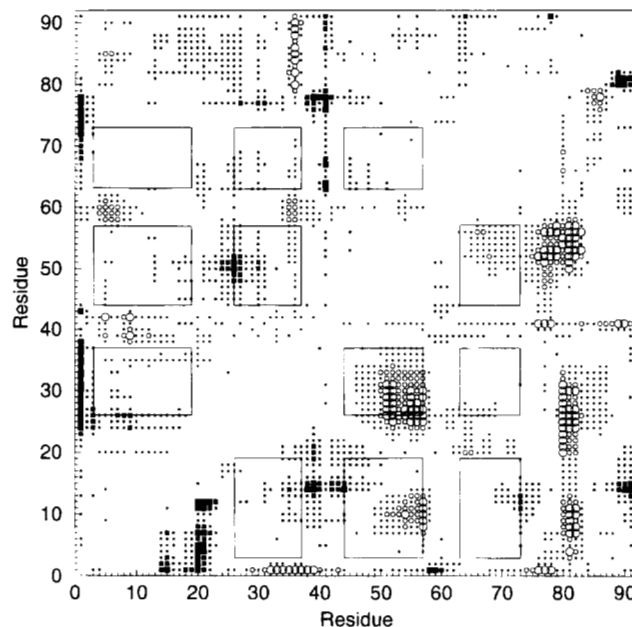


Fig. 6. α - α distance differences between the liganded and unliganded ns-LTP_{barley} structures. Above diagonal: α - α distance differences between the free ns-LTP_{barley} and the palmitate bound ns-LTP_{barley}. Below diagonal: α - α distance differences between the free ns-LTP_{barley} and the PCoA bound ns-LTP_{barley}. The comparison was performed using the minimized average structures, calculated from the three sets of structures. Open circles indicate that the complex has expanded compared to the free form and filled squares indicate a compression of the complex structure relative to the free protein. The circles and squares are increasing in size representing differences in distances from ± 2 to ± 8 Å. Boxes indicate sites for potential helix-helix interactions.

Binding modes of ns-LTPs

A comparison of the binding modes of different ns-LTPs suggests that these proteins have the capability of expansion or contraction. The result also suggests that the extent of expansion may depend on the volume of the lipophilic part of the ligand molecule. The analysis suggests that the C-terminal peptide of ns-LTP may play an important role in the interaction between these proteins and their ligands. From the known structures of ns-LTPs in a protein-ligand complex, it is clear that the C-terminal peptide is capable of contracting the protein structure as well as expanding the structure, depending on the volume of the ligand (Fig. 7). It is, therefore, possible that the ligand binding and release of ns-LTPs are controlled by the C-terminal peptide.

In the complex between ns-LTP_{barley} and PCoA, both the acyl chain and the hydrophobic parts of the CoA molecule are accom-

Table 2. RMS differences between ns-LTP structures

ns-LTP _{maize} -Pal/ns-LTP _{maize}	0.53 Å
ns-LTP _{barley} -PCoA/ns-LTP _{barley}	2.26 Å
ns-LTP _{barley} -Pal/ns-LTP _{barley}	1.33 Å
ns-LTP _{wheat} -DMPG/ns-LTP _{wheat}	1.40 Å ^a

^aOnly helical areas superimposed.

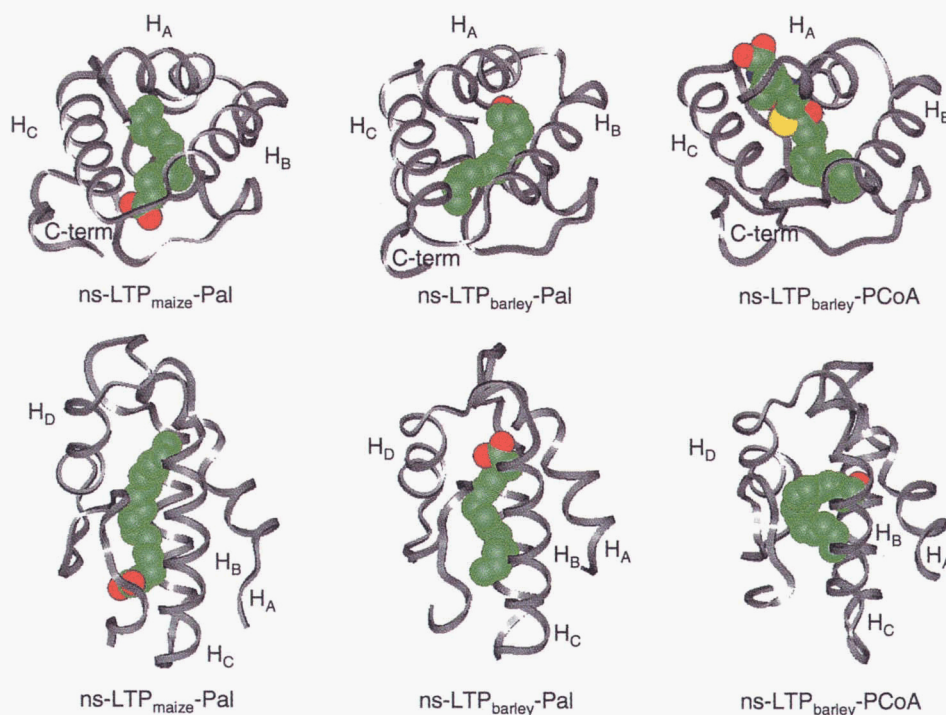


Fig. 7. A comparison of the two ns-LTP_{barley} complexes and the ns-LTP_{maize} complex. All protein structures are shown as ribbon structures, and the ligands are in CPK presentation and colored according to atoms, carbon (green), cysteine (yellow), oxygen (red), and nitrogen (blue). The helices are labeled in each structure. In the upper picture of ns-LTP_{barley} in complex with PCoA, all the hydrophobic parts of the ligand is shown (palmitoyl, cysteamine, and pantothenic acid), whereas only palmitate is shown in the lower picture of the same complex.

modated in the hydrophobic pocket of the protein. This large hydrophobic ligand causes a major conformational change in the ns-LTP compared to the minor structural perturbations seen in the complexes with palmitate (Fig. 7). Binding studies have shown that acyl-CoA esters bind more tightly to ns-LTPs than the corresponding fatty acids, though none of the measured binding constants suggest the binding to be highly specific. The hydrophobic parts of the PCoA ligand matches an *n*-alkanoic acid of a homologue range of 26 carbon atoms. This is one of the most common plant epicuticular wax constituents. From the location of ns-LTPs in cell walls (Thoma et al., 1994), and from the detection of high concentrations of these proteins in the surface wax (Pyee & Kolatukudy, 1994) along with the reported binding abilities, it is possible that the biological importance of these proteins may be to contain these very long acyl monomers necessary for the biosynthesis of cutin.

The different ns-LTPs also seem to use the hydrophobic binding pocket differently. A sequence comparison of the three species for which their ligand binding has been examined reveals a single mutation in the binding site of the barley protein. In ns-LTP_{barley} the charged Lys9 replaces the corresponding conserved hydrophobic position of the other ns-LTPs. This lysine residue facilitates the formation of a hydrogen bond or salt bridge between the carboxylate of the fatty acid and the amino group of the lysine. The comparison of the maize and barley ns-LTP in complex with palmitate suggests that this mutation may be responsible for the inversion of the coordination of the ligand.

The difference in binding mode between maize and barley LTP may suggest that ns-LTPs in different species could serve slightly different biological functions.

Materials and methods

Sample preparation

ns-LTP_{barley} was isolated and purified as described previously (Sørensen et al., 1993; Lerche et al., 1997). ¹³C-Labeled palmitate was purchased from Cambridge Isotope Laboratories, Inc. (Cambridge, Massachusetts). Two different samples were prepared from lyophilized material: [bLTP]/[C₁₆]:[2 mM]/[6 mM] and [bLTP]/[¹³C₁₆]:[1.8 mM]/[6.2 mM] in 600 μ L solutions. The pH was in both samples adjusted to 7.2.

Recording and processing of NMR data

All NMR spectra were recorded at 310 K on a Bruker AMX600 spectrometer operating at a frequency of 600 MHz for ¹H. A series of homonuclear spectra were recorded including DQF-COSY (Piantini et al., 1982), TOCSY (Braunschweiler & Ernst, 1983), and NOESY (Kumar et al., 1981). All spectra were recorded with 512 and 2,048 complex points in the *t*₁ and the *t*₂ dimensions, and the spectral width was 7,246 Hz. The distance restraints have been derived from NOESY spectra recorded with mixing times of 80, 120, and 200 ms. The free induction decays were zero filled to 1,024 and 4,096 complex points, respectively, in the *t*₁ and *t*₂ dimensions and multiplied with a phase-shifted sine-bell function in the *t*₁ dimension and a sine function in the *t*₂ dimension. To determine the ³J_{H^NH ^{α}} coupling constants (Ludvigsen et al., 1991) both the DQF-COSY and the 80 ms NOESY spectra were multiplied with a π /five phase-shifted sine-bell in both dimensions. Two TOCSY spectra were recorded one with a mixing time of

44 ms and one with 30 ms. The TOCSY spectra were likewise zero filled to 1,024, and 4,096 complex points in the t_1 and t_2 dimensions and subsequently multiplied with a phase-shifted sine-bell function in the t_1 dimension and an exponential function in the t_2 dimension. Two types of heteronuclear spectra were recorded, an HSQC (Bodenhausen & Ruben, 1980) and an HSQC-NOE (Müller et al., 1987) experiment. In these experiments 256 complex points were recorded with 512 transients in the t_1 dimension and 2,048 complex points in the t_2 dimension. The spectral width in the ^{13}C -dimension was 4,448 Hz. Two HSQC-NOE spectra were recorded with a mixing time of 120 and 200 ms, respectively. To obtain quadrature detection in t_1 and pure line shapes, the method of States-TPPI was used in all recordings (States et al., 1982). The recorded data were processed using the MNMR (Kjær et al., 1994) or the XWINNMR software packages (Bruker, Karlsruhe, Germany).

Structure determination

The assigned NOE cross peaks were divided into three classes according to the intensities, strong, medium, and weak. These were translated to distance restraints, 1.8–2.8, 1.8–3.7, and 1.8–5 Å, respectively. In case of methyl groups, the upper limit distance was increased by 0.5 Å (Clare et al., 1986). Secondary structure hydrogen bonds were initially identified in the calculated structures and subsequently included as restraints in the final structure calculations. For N–O distances 2.9 ± 0.2 Å, and in the case of H^{N} –O distances 1.95 ± 0.15 Å. The ϕ angles were derived from the measured $^3J_{\text{H}^{\text{N}}\text{H}^{\alpha}}$ coupling constants, and the measured intensity of the corresponding H^{N} – H^{α} NOE cross peak (Andersen & Poulsen, 1992). The ϕ angles were restrained by $\pm 40^\circ$ to the rotamer at either -120 , -57 , or 60 . The side-chain dihedral angles were restrained by $\pm 60^\circ$ to the determined rotamer at -60 , 60 , and 180 .

Initially, redundant distance information in the NOE-derived distance restraints were removed using the DIANA program (Güntert et al., 1991). Subsequently, the standard protocol and standard parameters of XPLOR version 3.8 were used to calculate structures of the complex. Forty protein structures of the ns-LTP_{barley} complex with palmitate were calculated in the absence of the ligand. None of these structures had distance violations exceeding 0.3 Å and angle violations beyond 5° . These structures were combined one by one with calculated random structures of the ligand using the XPLOR 3.8 protocol. The 40 structures of the complex were further refined in a restrained dynamic step making use of the full CHARMM potential. All calculations were performed on an Origin 200 (SGI). The structures were visualized on an Octane (SGI) and analyzed using the XPLOR 3.8 program, the WHAT_CHECK program (Hoofst et al., 1996), and the computer program PROCHECK_NMR (Laskowski et al., 1993). The coordinates of an ensemble of the 10 lowest energy structures of the complex have been deposited in PDB, and are accessible by the accession code 1be2.

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References

- Andersen KV, Poulsen FM. 1992. Three-dimensional structure in solution of acyl-coenzyme A binding protein from bovine liver. *J Mol Biol* 226:1131–1141.
- Arondel V, Vergnolle C, Tchong F, Kader JC. 1990. Bifunctional lipid-transfer: Fatty acid-binding proteins in plants. *Mol Cell Biochem* 98:49–56.
- Bodenhausen G, Ruben DJ. 1980. Natural abundance nitrogen 15 NMR by enhanced heteronuclear spectroscopy. *Chem Phys Lett* 69:185–189.
- Braunschweiler L, Ernst RR. 1983. Coherence transfer by isotropic mixing: Application to proton correlation spectroscopy. *J Magn Reson* 53:521–528.
- Brünger AT. 1992. *X-PLOR Version 3.1. A system for X-ray crystallography and NMR*. New Haven, Connecticut: Yale University.
- Clare GM, Brünger AT, Karplus M, Gronenborn AM. 1986. Application of molecular dynamics with interproton distance restraints to three-dimensional protein structure determination. A model study of crambin. *J Mol Biol* 191:523–551.
- Desormeaux A, Blochet JE, Pezolet M, Marion D. 1992. Amino acid sequence of a nonspecific wheat phospholipid transfer protein and its conformation as revealed by infrared and Raman spectroscopy. Role of disulfide bridges and phospholipids in the stabilization of α -helix structure. *Biochim Biophys Acta* 1121:137–152.
- Gincel E, Simorre J, Caille A, Marion D, Ptak M, Vovelle F. 1994. Three-dimensional structure in solution of a wheat lipid-transfer protein from multidimensional ^1H -NMR data: A new folding from lipid carriers. *Eur J Biochem* 226:413–422.
- Gomar J, Petit MC, Sodano P, 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.
- Güntert P, Braun W, Wüthrich K. 1991. Efficient computation of three-dimensional protein structures in solution from nuclear magnetic resonance data using the program DIANA and the supporting programs CALIBA, HABAS and GLOMSA. *J Mol Biol* 217:517–530.
- Heinemann B, Andersen KV, Nielsen PR, Bech LM, Poulsen FM. 1996. Structure in solution of a four-helix lipid binding protein. *Protein Sci* 5:13–23.
- Hoofst RWW, Vriend G, Sander C, Abolay EE. 1996. Errors in protein structures. *Nature* 381:272.
- Kjær M, Andersen KV, Poulsen FM. 1994. Automated and semiautomated analysis of homo- and heteronuclear multidimensional nuclear magnetic resonance spectra of proteins: The program PRONTO. *Methods Enzymol* 239:288–307.
- Kumar A, Wagner G, Ernst RR, Wüthrich K. 1981. Buildup rates of the nuclear Overhauser effect measured by two-dimensional proton magnetic resonance spectroscopy: Implications for studies of protein conformation. *J Am Chem Soc* 103:3654–3658.
- Laskowski RA, MacArthur MW, Moss DS, Thornton JM. 1993. PROCHECK: A program to check the stereochemical quality of protein structures. *J Appl Crystallogr* 26:283–291.
- Lee JY, Min K, Cha H, Shin DH, Hwang KY, Suh SW. 1998. Rice nonspecific lipid transfer protein: The 1.6 Å crystal structure in the unliganded state reveals a small hydrophobic cavity. *J Mol Biol* 276:437–448.
- 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.
- Ludvigsen S, Andersen KV, Poulsen FM. 1991. Accurate measurements of coupling constants from two-dimensional nuclear magnetic resonance spectra of proteins and determination of ϕ -angles. *J Mol Biol* 217:731–736.
- Molina A, Segura A, Garcia-Olmedo F. 1993. Lipid transfer proteins (nsLTPs) from barley and maize leaves are potent inhibitors of bacterial and fungal plant pathogens. *FEBS Lett* 316:119–122.
- Müller N, Ernst RR, Wüthrich K. 1987. Sensitivity enhanced detection of weak nuclei using heteronuclear multiple quantum coherence. *J Am Chem Soc* 108:6482–6492.
- Østergaard J, Vergnolle C, Schoentgen F, Kader J. 1993. Acyl-binding/lipid transfer proteins from rape seedlings, a novel category of proteins interacting with lipids. *Biochim Biophys Acta* 1170:109–117.
- Piantini U, Sørensen OW, Ernst RR. 1982. Multiple quantum filters for elucidation of NMR coupling networks. *J Am Chem Soc* 104:6800–6801.
- 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* 311:460–468.
- Rickers J, Spener F, Kader J. 1985. A phospholipid transfer protein that binds long-chain fatty acids. *FEBS Lett* 180:29–32.
- Shin DH, Lee JY, Hwang KY, Kim KK, Huh SW. 1995. High-resolution crystal structure of the nonspecific lipid-transfer protein from maize seedlings. *Structure* 3:189–199.

- 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 nonspecific lipid transfer protein. Global fold of the complex. *FEBS Lett* 416:130–134.
- Sørensen SB, Bech LM, Muldbjerg M, Beenfeldt T, Breddam K. 1993. Barley lipid transfer protein 1 is involved in beer foam formation. *MBAA Tech Q* 30:136–145.
- States DJ, Haberkorn RA, Ruben DJ. 1982. A two-dimensional nuclear Overhauser experiment with pure absorption phase in four quadrants. *J Magn Reson* 48:286–292.
- Sterk P, Booij H, Scellekens GA, van Kammen A, de Vries SG. 1991. Cell-specific expression of the carrot EP2 lipid transfer protein gene. *Plant Cell* 3:907–921.
- Tassin S, Broekaret WF, Marion D, Acland DP, Ptak M, Vovelle F, Sodano P. 1998. Solution structure of Ace-AMP1, a potent antimicrobial protein extracted from onion seeds. Structural analogies with plant nonspecific lipid transfer proteins. *Biochemistry* 37:3623–3637.
- Thoma S, Hecht U, Kippers A, Botella J, de Vries S, Somerville C. 1994. Tissue specific expression of a gene encoding a cell wall-localized lipid transfer from *Arabidopsis*. *Plant Physiol* 105:35–45.
- Wüthrich K. 1986. *NMR of proteins and nucleic acids*. New York: Wiley.