

Two-dimensional ^1H -NMR studies of maize lipid-transfer protein Sequence-specific assignment and secondary structure

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Correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY) and NOE spectroscopy (NOESY) experiments have been used to assign sequentially the ^1H 500-MHz NMR spectra of a non-specific (ns) lipid-transfer protein extracted from maize seeds. The spin-system identification and sequential assignment were combined with secondary-structure determination to identify most of the proton resonances of this 93-residue protein. From the sequential connectivities it was established that the secondary structure mainly involved four helical fragments: H1, H2, H3 and H4. This secondary structure was compared with that of wheat ns-lipid-transfer protein recently determined. The four helices are located in nearly the same regions, but helix H4 is appreciably longer in the maize protein than in the wheat protein. Comparison of the transfer activities reveals that the maize protein is more efficient than the wheat ns-lipid-transfer protein and that this difference is probably due to the affinity of the lipid for the binding site and not to the interfacial activation, i.e. adsorption of the ns-lipid-transfer protein to the membrane. From these results, it is suggested that helix H4 is a part of the lipid-binding site or contributes to the folding of this site. The present data define the basis for a further modelling of the three-dimensional structure of the maize ns-lipid-transfer protein which will be compared with that of the wheat ns-lipid-transfer protein in order to establish structure/activity relationships for this class of carriers by using natural ns-lipid-transfer protein mutants.

Lipid-transfer proteins form a broad family of proteins, present in micro-organisms, plants and animals. Their main characteristic property is to facilitate inter membrane transfer of lipids *in vitro*. Some lipid-transfer proteins, such as the phosphatidylinositol-phosphatidylcholine transfer protein from bovine liver, transfer more specifically phospholipids, whereas others are able to transfer phospholipids, glycolipids, fatty acids and sterols (see [1–3] for recent reviews). All the plant lipid-transfer proteins extracted from different sources and organs belong to the family of non-specific (ns) lipid-transfer proteins, since they are able to transfer various phospholipids as well as galactosyldiacylglycerols and they also bind free fatty acids [1].

More than 15 primary structures of plant ns-lipid-transfer proteins have been determined and a consensus sequence has been recently established [4]. The eight Cys residues present in these sequences are strictly conserved and the positions of the four disulfide bridges has been determined in the castor bean ns-lipid-transfer protein [5]. It can then be anticipated that these covalent links should play a similar role in the

overall folding of the polypeptide backbones. ns-lipid-transfer proteins have similar molecular masses (≈ 9 kDa) and are basic, with isoelectric points ranging from 8.8 to 10.5. Animal ns-lipid-transfer proteins are also basic, but they do not exhibit significant sequence similarities with plant ns-lipid-transfer protein [6]. Recently, we established the three-dimensional structure of a plant ns-lipid-transfer protein, extracted from wheat seeds (unpublished results), on the basis of two-dimensional and three-dimensional ^1H -NMR data [7]. This structure involves a right-handed bundle of four helices packed against a non-canonical structure composed of a series of turns defining what we called a saxophone shape. This is a first step in the understanding of the mechanism of lipid transfer, *in vitro*. The real biological function of plant ns-lipid-transfer proteins *in vivo*, has remained unknown. Considering their phospholipid-transfer capacity, it was initially speculated that these proteins play important functions in the biogenesis and renewal of biological membranes [1–3]. However, the discovery that they are synthesised as higher-molecular-mass precursors, composed of a peptide signal and a mature protein, suggests that these proteins pass through the secretory pathway [8, 9]. This has been definitely confirmed by recent studies showing that ns-lipid-transfer proteins are located in the cell wall of seeds [10–12]. Such a cell routing is obviously inconsistent with an intracellular lipid shuttle. From their location and their ability to bind different acyl chains, it has also been suggested that ns-lipid-transfer proteins have an activity in cutin

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Abbreviations. COSY, correlation spectroscopy; 2QF, double quantum filtered; 2Q, double quantum; 3QF, triple quantum filtered; TOCSY, total correlation spectroscopy; NOESY, NOE spectroscopy; ns, non-specific.

formation, by transferring hydrophobic cutin monomers [11]. More recently, the discovery of their antimicrobial activity, *in vitro*, has suggested that ns-lipid-transfer proteins might be involved in the plant defense against pathogens [13, 14]. This antimicrobial activity might be related to their interactions with membrane lipids as it has been shown for plant thionins, which are also basic and low-molecular-mass cysteine-rich proteins [15]. Thus, the biological function of plant ns-lipid-transfer proteins should probably be related to their affinity with amphiphilic lipids and the lipid transfer is certainly, in a first attempt, the mechanism to consider in structure/function studies. Indeed, it is a quite simple assay to appreciate differences in lipid-protein interactions within the lipid-transfer protein family and furthermore, this activity is susceptible to different biotechnological applications [Maureaud, T., Laruel, R. and Sabadie, M. (1992) European Patent Application 920311; Record, E., Lesage, L., Marion, D., Cahagnier, B., Richard-Molard, D. and Asther, M. (1993) French Patent Application 9301518].

To establish structure/lipid-transfer-activity relationships, we have undertaken the structural study by two-dimensional NMR of a second plant ns-lipid-transfer protein extracted from maize seeds. It displays about 60% sequence similarity with the wheat protein and a higher transfer activity. The assignment of ^1H -NMR spectra and the determination of secondary-structure elements deduced from the inter-residue NOE bring preliminary structural elements to explain these differences.

MATERIALS AND METHODS

Purification of ns-lipid-transfer proteins

5 kg maize seeds (*Zea mays* L., var. Mondain), kindly provided by M. Foret from INRA Agri-Obtention (La Minière, France) were ground to flour which was defatted at room temperature with 30 l pentane. After filtration on a buchner funnel, the maize flour was dried overnight under reduced pressure.

The dried flour was mixed for 2 h at 4°C with 10 l Tris/HCl 100 mM, pH 7.8, containing 100 mM KCl, 5 mM EDTA and 8 mM 2-mercaptoethanol. After centrifugation at 5000 g for 30 min, the supernatant was adjusted to pH 5. After centrifugation at 5000 g for 30 min, the resulting supernatant was brought to pH 7.8 with 2 M Tris, and ammonium sulfate was gradually added to 40% saturation. After centrifugation at 5000 g for 15 min, the supernatant was brought to 80% saturation with ammonium sulfate and centrifuged at 5000 g for 15 min. The protein pellet was suspended in 10 mM ammonium acetate, pH 6.8, and dialysed for at least two days into 10 mM ammonium acetate, pH 6.8, using Spectrapor dialysis tubing with an exclusion limit of 3 kDa (Spectrum). Proteins were separated by size-exclusion chromatography and fractions containing proteins with apparent molecular mass below 15 kDa in SDS/PAGE were pooled and freeze/dried as previously described [4]. 50 mg freeze/dried proteins were dissolved in 10 mM ammonium acetate, pH 6.8, and separated by cation-exchange chromatography on a CS Neo-bar column (1.3 cm $\times$ 3 cm; Dyno Particles). A gradient from 50 mM to 700 mM ammonium acetate, pH 6.8, was applied at a flow rate of 3 ml/min. Maize ns-lipid-transfer protein eluted at about 200 mM. The identity of the purified maize ns-lipid-transfer protein to the known sequence [8] was determined by amino acid analysis and ion-spray MS. Amino acid analysis and ion-spray MS were carried out as

previously described [4, 16]. The same procedure was used for the purification of wheat ns-lipid-transfer protein in order to compare their transfer activities.

Transfer assay

Transfer activity was determined by a fluorescence assay described by Van Paridon et al. [17] using acceptor vesicles composed of egg yolk phosphatidylcholine so that, in the assay, the concentration of phospholipids in acceptor vesicles was 30-times the concentration of phospholipids in donor vesicles. The donor vesicles were composed of Pyrene-PtdCho or of a mixture of Pyrene-PtdCho/Pyrene-PtdGro derivatives (Molecular Probes).

NMR studies

For NMR studies, the protein samples were dissolved in D_2O at a concentration of 4 mM, or in a 90% H_2O /10% D_2O mixture at a concentration of 4 mM or 8 mM. The solutions were buffered with 40 mM or 80 mM sodium acetate adjusted to pH 5.4. ^1H -NMR spectra were recorded on a Bruker AMX500 spectrometer, and data were processed on a Bruker X-32 station using the UXNMR program. All experiments were recorded at three temperatures: 283 K, 300 K and 308 K, and the chemical shifts were calibrated on the water signal as 4.92, 4.75 and 4.68 ppm, respectively. The mixing times were set to 120 ms and 200 ms for NOE spectroscopy (NOESY) experiments [18], and 60 ms for total correlation spectroscopy (TOCSY) experiments [19, 20]. To reduce the first-order phase correction along the first axis in NOESY and TOCSY experiments, the first 90°_x pulse was replaced by a 360°_x - 270°_x composite pulse [21]. In NOESY and TOCSY experiments, the water resonance was suppressed by combination of a low-power presaturation ($\gamma\text{B}_2 = 50\text{Hz}$) and a jump and return sequence [22]. The double-quantum-filtered correlation spectroscopy experiments (2QF-COSY) [23], double-quantum COSY experiments (2Q-COSY) [24] and triple-quantum-filtered COSY experiments (3QF-COSY) [25] were performed with a stronger water signal presaturation. In 2QF-COSY experiments we used a stimulated cross-peaks under bleached alphas (SCUBA) technique to restore the magnetisation at the bleached C_αH [26].

Exchange proton measurement experiments were performed at 283 K as follows. The protein sample was dissolved in D_2O at 283 K and placed in a preshimmed spectrometer 15 min after dissolution. Eight one-dimensional spectra were acquired at intervals of 10 min each time. Three short (4–7 h) NOESY spectra ($\tau_m = 120\text{ms}$) were performed.

The $^3J_{\alpha\text{NH}}$ coupling constants were measured on a high-resolution 2QF-COSY (acquired with 8096 and 800 points in the t_2 and t_1 directions, respectively) and verified in a high-resolution NOESY spectrum for large coupling constants.

Prior to the Fourier transformation, the free induction decays (FID) were zero-filled in the t_1 direction to 1024 real data points, and multiplied by phase-shifted sine bell in both directions. Base-line distortions were eliminated using a base-line correction with a fifth-order polynomial.

RESULTS

Lipid-transfer activities of maize and wheat ns-lipid-transfer proteins

The maize and wheat ns-lipid-transfer proteins have been purified by the same procedure and the transfer assays have

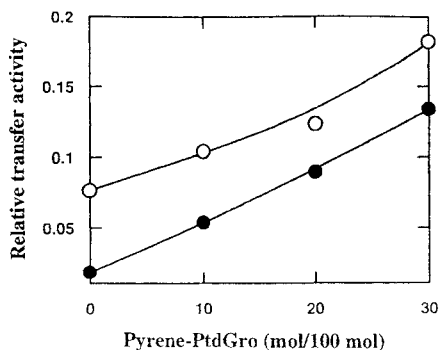


Fig. 1. Transfer activities of wheat (○) and maize (●) ns-lipid-transfer proteins determined by the fluorescence-transfer assay using different concentrations mol of Pyrene-PtdGro in Pyrene-PtdCho as donor vesicles and egg yolk PtdCho as acceptor vesicles (see Materials and Methods).

been carried out with the same liposome preparations so that the differences in lipid-transfer activities can be regarded as the result of their intrinsic properties and not as extrinsic effects due to the purification procedure. From the results presented in Fig. 1, it is obvious that the maize ns-lipid-transfer protein is more efficient than the wheat ns-lipid-transfer protein. This large difference is also observed when anionic phospholipids are added in the acceptor liposomes. Increasing surface negative charge of donor liposomes improves similarly the transfer activities of both ns-lipid-transfer proteins.

Primary structure

As seen in Fig. 4, a comparison between the sequences of wheat (90 residues) and maize (93 residues) ns-lipid-transfer proteins, after realignment to obtain a maximum of strict residue conservation, shows that the eight Cys residues are identically located in both proteins as they are in the whole family. Due to the lack of adequate protease sites, a chemical determination of disulfide bridges in the maize protein was unsuccessful. Therefore, we assume an organisation of S-S bonds in this protein similar to that found in the castor bean sequence [5]. Some differences between maize and wheat ns-lipid-transfer proteins appear in the number of Pro residues. The wheat protein contains an additional Pro residue in the N-terminal region, involved in a Gly-Pro-Gly-Pro sequence corresponding to a Gly-Ser-Gly-Pro (residues 22–25) sequence in the maize ns-lipid-transfer protein and another one forming a Pro-Pro doublet corresponding to a Pro-Ser (residues 72–73) doublet in the maize alignment (Fig. 4). As seen later, these differences should correspond to local conformational differences between the two proteins. In the maize ns-lipid-transfer protein, a high number of Ala and Ser residues (respectively, 17 and 14) should somewhat complicate the assignment of its ^1H -NMR spectra.

^1H -NMR spectra assignments

Sequence-specific ^1H -NMR assignments were achieved by combining two well-established methods: one developed by Wüthrich et al. [27], and based on spin-system identification, and the other, usually named the main-chain-directed method (MCD), developed by Englander and Wand [28], and based on secondary-structure identification. Our general strategy was as follow. In a first step, 2QF-COSY and

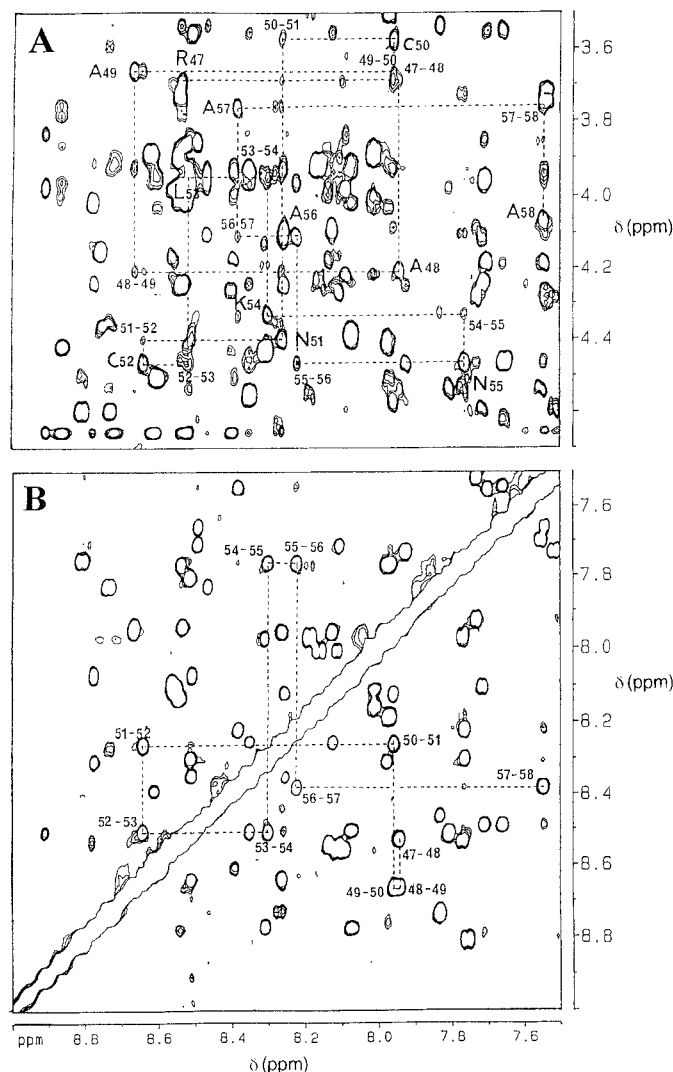


Fig. 2. Sequential assignment of segment 47–58 is shown on a NOESY spectra ($\tau_m = 120$ ms) of maize ns-lipid-transfer protein. (A) Fingerprint region: $\alpha_r\text{-NH}_{r+1}$ connectivities; (B) amide region; $\text{NH}_r\text{-NH}_{r+1}$ connectivities.

TOCSY spectra recorded in D_2O solution were used to classify the non-aromatic individual spin systems into six classes, i.e., Gly, Ala-Thr, Val-Leu, Ile, AMX spin systems and all others corresponding to residues carrying long side chains. These spin systems were completed and further distinctions within each class were introduced by establishing complete scalar connectivities including NH protons, from TOCSY spectra in H_2O , and by combining the aromatic system with their own AMX systems using NOESY spectra in D_2O . Sequential NOE corresponding to d_{NN} , $d_{\alpha\text{N}}$ and $d_{\beta\text{N}}$ were used to establish sequential connectivities between two or several residues. Due to the redundancy of some residues in the sequence (10 Gly, 17 Ala, 14 Ser, 6 Ile), it was impossible to identify unambiguously all the protons of several of these residues in the first step of the assignment. Severe overlaps arising from Ser residues, and $\text{C}_\alpha\text{H}/\text{C}_\beta\text{H}$ connectivities of Thr residues prevented us assigning Gly residues in the D_2O spectrum, even by considering their typical large active coupling constants. This problem was partly circumvented by analysing first the fingerprint region of a 2QF-COSY spectrum recorded in H_2O . The assignment was completed

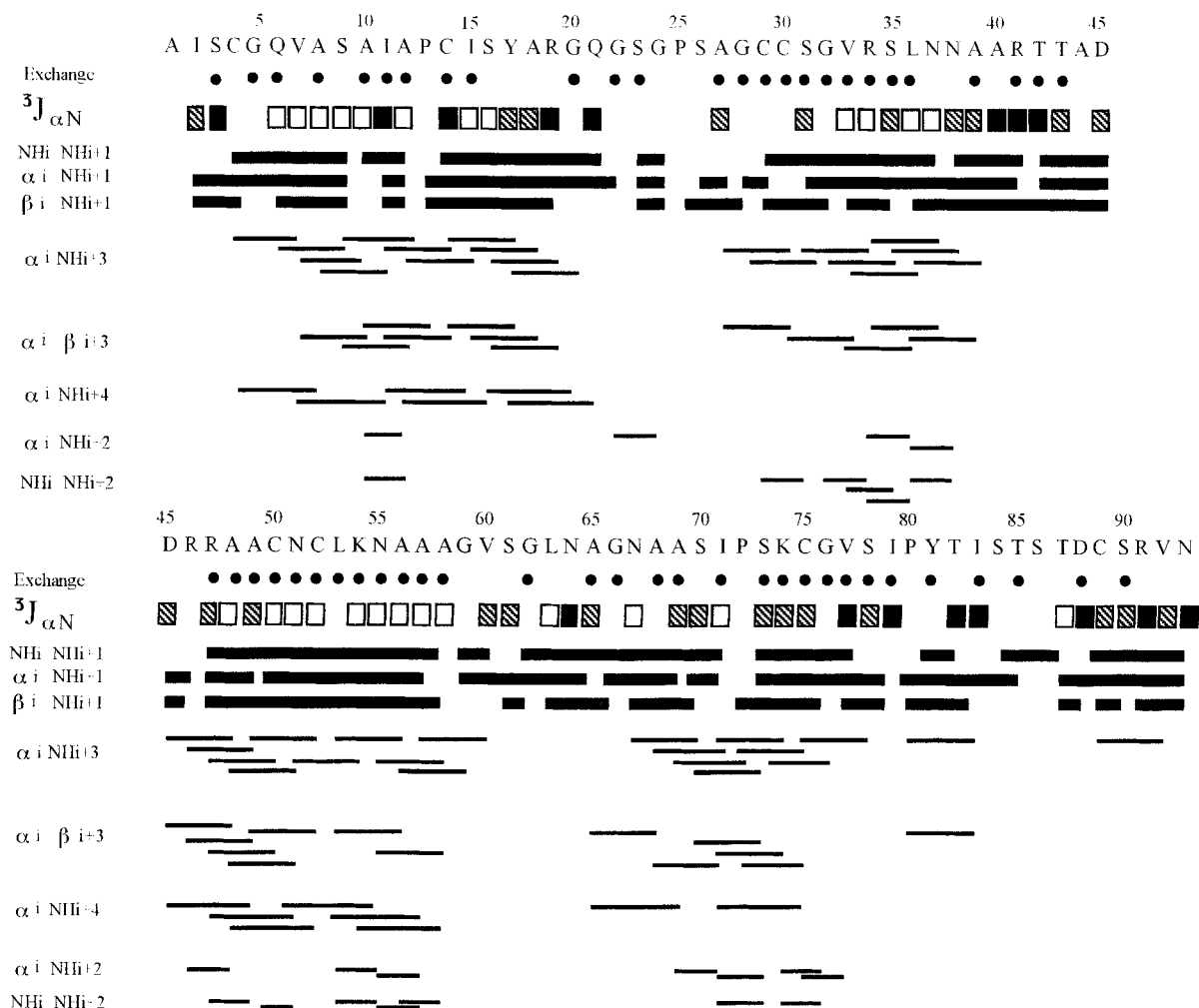


Fig. 3. Sequence of maize ns-lipid-transfer protein with a schematic summary of sequential connectivities which support the resonance assignment. Definite evidence of the helical regions is given by stretches of $d_{NN}(i, i+1)$, $d_{\alpha N}(i, i+3)$, $d_{\alpha\beta}(i, i+3)$, $d_{\alpha N}(i, i+4)$ and $d_{\alpha N}(i, i+2)$ connectivities read on NOESY spectra ($\tau_m = 120$ ms). Line thickness is not proportional to NOE intensity. Thick lines are used for sequential connectivities and thin lines are used for medium range connectivities. Values of $^3J_{\alpha NH}$ coupling constants are classified as small ($\square$; ≤ 6 Hz), medium ($\boxtimes$; > 6 Hz and ≤ 8 Hz) and large ($\blacksquare$; > 8 Hz). ($\bullet$), Residues that contain slowly exchanging amide protons.

by considering a 2Q-COSY spectrum recorded in H_2O in which Gly residues were the only ones giving remote connectivities $\omega_{\alpha 1} + \omega_{\alpha 2}$ (F_1) on its own amide proton ω_{NH} (F_2). In this way, nine out of ten Gly residues could be assigned. The last one was assigned by using a NOESY spectrum recorded at a lower temperature in which the cross-peaks were sufficiently separated.

For most of the AMX spin systems, only one $C_{\alpha}H-C_{\beta}H$ cross-peak could be identified in the 2QF-COSY spectrum. TOCSY and 3QF-COSY spectra are required to complete their assignments. Partial overlaps between Ser, Gly and $C_{\alpha}H-C_{\beta}H$ Thr connectivities were overcome by the use of 3QF-COSY where these residues were filtered out. The final assignment of redundant Ser residues was completed in the course of the sequential assignment procedure since their amide protons were spread all over the amide resonance region. Only two of the four Pro residues were completely identified during the sequential assignment procedure. The starting points were the observation of strong $d_{\alpha N}$ connectivities. Pro13, Pro25 and Pro72 were found in the *trans* conformation on the basis of strong $d_{\alpha NH_{i-1}}$ sequential NOE. Proton resonances of Pro72 and Pro80 were partially determined.

The phenyl-ring protons of the two aromatic residues, Tyr17 and Tyr81 were identified by their typical COSY connectivities while the remainder of the side-chain protons were assigned by observation of strong NOESY intra-residual cross-peaks.

The $NH/C_{\alpha}H$ connectivities were established in the COSY spectrum and relayed connectivities observed in the TOCSY spectrum. All the amide protons have been identified.

Sequential resonance assignments

Sequential assignments were established from NOESY spectra recorded at three different temperatures: 283 K ($\tau_m = 120$ ms), 300 K ($\tau_m = 200$ ms) and 308 K ($\tau_m = 120$ ms and 200 ms). Comparison of these NOESY data resolved ambiguities that arose from overlapping connectivities. The usual procedures were followed. Once the intra-residual cross-peaks were identified, a search was made for sequential connectivities $d_{\alpha N}$, d_{NN} and $d_{\beta N}$. Fig. 2 illustrates the assignment pathways for the segment 47–58. Subsequently, medium-range connectivities, used to identify helical secondary struc-

Table 1. ^1H chemical shifts and $^3J_{\alpha\text{NH}}$ coupling constant values for maize ns-lipid-transfer protein at pH 5.4 and 308 K.

Residue	Chemical shift for				$^3J_{\alpha\text{N}}$
	NH	H α	H β	others	
	ppm				Hz
Ala1		4.28	1.50		
Ile2	8.41	4.44	1.79	γH_2 1.54, 1.31; γCH_3 0.75; δCH_3 0.73	7.4
Ser3	8.33	4.85	4.14, 3.95		9.3
Cys4	9.46	^a	^a		
Gly5	8.77	3.97 ^b			
Gln6	7.56	4.19	2.23, 2.30	γH_2 2.34, 2.44	5.5
Val7	7.67	3.56	2.24	γCH_3 1.07; 0.88	5.5
Ala8	8.51	3.87	1.49		4.9
Ser9	7.73	4.24	4.03, 3.98		5.5
Ala10	7.72	4.22	1.56		4.9
Ile11	7.57	4.55	1.93	γH_2 1.44, 1.32; γCH_3 0.86; δCH_3 0.71	8.6
Ala12	7.47	4.33	1.63		4.3
Pro13		3.70	2.53, 1.37	γH_2 1.99, 1.84; δH_2 3.77, 3.61	
Cys14	8.75	4.76	3.32		9.3
Ile15	7.85	3.55	2.20	γH_2 1.65, 1.57; δCH_3 0.91; γCH_3 0.72	4.9
Ser16	8.48	4.11	3.99, 3.94		5.5
Tyr17	7.17	4.95	3.37, 2.76	δH_2 6.78; ϵH_2 6.56	6.1
Ala18	8.55	3.74	1.44		6.2
Arg19	7.79	4.55	2.11, 1.79	γH_2 1.46; δH_2 3.18, 3.07; ϵNH 7.09	8.6
Gly20	7.99	3.50, 4.42			
Gln21	8.21	4.56	1.91, 1.97	γH_2 1.96, 2.27	9.2
Gly22	8.37	3.96, 4.47			
Ser23	8.63	4.58	3.98, 3.90		
Gly24	7.36	3.69, 3.15			
Pro25		4.18	1.87, 1.78	γH_2 1.69; δH_2 3.32, 2.45	
Ser26	8.77	4.37	4.17, 4.37		
Ala27	9.06	4.14	1.47		6.1
Gly28	8.76	3.95 ^b			
Cys29	7.99	4.41	3.46, 2.83		
Cys30	8.33	4.69	3.14, 2.83		
Ser31	8.79	4.26	4.03		6.8
Gly32	8.09	4.40, 3.94			
Val33	8.52	3.56	2.16	γCH_3 0.88, 1.05	5.6
Arg34	8.37	3.94	1.93	γH_2 1.78, 1.62; δH_2 3.31, 3.18; ϵNH 7.28	5.6
Ser35	8.27	4.60	4.25, 4.12		8.0
Leu36	8.14	4.10	1.83, 1.70	γH 1.55; δCH_3 0.96, 0.90	5.6
Asn37	7.98	4.59	2.93, 2.51	γNH_2 7.78, 6.77	5.5
Asn38	7.94	4.48	2.86	γNH_2 7.61, 6.90	6.8
Ala39	7.75	4.31	1.47		6.8
Ala40	7.53	4.61	1.24		9.3
Arg41	8.83	4.51	2.05, 1.84	γH_2 1.66; δH_2 3.27; ϵNH 7.18	8.7
Thr42	7.77	4.82	4.68	1.30	8.6
Thr43	8.93	4.00	3.87	1.30	6.7
Ala44	8.53	3.98	1.42		
Asp45	7.82	4.55	2.78, 2.55		6.1
Arg46	8.54	3.94	2.02	γH_2 1.87, 1.65; δH_2 3.06, 3.38; ϵNH 8.37	
Arg47	8.54	3.70	1.77	γH_2 1.88; δH_2 2.98, 3.10; ϵNH 7.43	6.2
Ala48	7.96	4.22	1.54		5.6
Ala49	8.68	3.68	1.37		6.2
Cys50	7.97	3.59	2.06, 3.29		4.9
Asn51	8.28	4.42	2.80, 2.71	γNH_2 7.12, 7.20	5.5
Cys52	8.66	4.46	3.09, 2.94		5.6
Leu53	8.53	3.98	2.05, 1.75	γH 1.55; δH_3 0.89, 0.76	
Lys54	8.32	4.36	2.03	γH_2 1.47; δH_2 1.75; ϵH_2 3.08, 3.01	5.5
Asn55	7.78	4.48	2.89, 2.84	γNH_2 6.89, 7.47	5.6
Ala56	8.24	4.12	1.42		5.6
Ala57	8.40	3.78	1.31		4.9
Ala58	7.56	4.08	1.55		4.9
Gly59	7.55	3.74, 4.29			
Val60	7.23	4.02	2.17	γCH_3 0.95, 0.75	8.0
Ser61	8.52	4.42	3.87, 4.01		6.8
Gly62	8.88	3.78, 3.98			
Leu63	7.39	3.74	1.66	γH 1.39; δCH_3 0.90, 0.86	5.6

Table 1. (Continued).

Residue	Chemical shift for				$^3J_{\alpha N}$
	NH	H α	H β	others	
	ppm				Hz
Asn64	9.30	4.80	2.76, 2.40	γ NH ₂ 7.62, 6.76	10.4
Ala65	8.80	4.02	1.48		6.1
Gly66	8.56	4.00, 3.91			
Asn67	8.15	4.18	2.51, 2.63	γ NH ₂ 6.97, 6.29	5.5
Ala68	8.56	3.92	1.54		
Ala69	8.12	4.30	1.58		6.2
Ser70	7.73	4.61	^c		8.0
Ile71	7.34	3.71	2.13	γ H ₂ 1.04, 2.60; γ CH ₃ 0.96; δ H ₃ 1.18	4.3
Pro72		4.60	2.37, 2.14	γ H ₂ 1.63, 2.36; δ H ₂ 3.63, 3.85	
Ser73	8.11	4.23	3.99, 3.85		6.8
Lys74	8.58	4.18	1.70, 1.84	γ H ₂ 1.84, 2.21; δ H ₂ 3.38, 3.82	7.4
Cys75	8.13	4.80	3.03, 2.85		8.0
Gly76	8.02	3.90, 4.01			
Val77	8.18	4.26	1.72	γ CH ₃ 0.81, 0.77	9.3
Ser78	8.55	4.46	3.75, 3.81		7.4
Ile79	7.67	4.62	1.24	γ H ₂ 1.39, 0.73; γ CH ₃ 0.62; δ H ₃ 0.53	9.2
Pro80		^c	^c	γ H ₂ 2.4, 2.17; δ H ₂ 3.98 ^b	
Tyr81	6.70	4.79	3.01, 3.10	δ H ₂ 6.69; ϵ H ₂ 6.54	
Thr82	7.99	4.52	4.04	γ CH ₃ 1.12	9.2
Ile83	8.62	3.96	1.93	γ H ₂ 1.70, 1.00; γ CH ₃ 0.77; δ H ₃ 0.87	9.9
Ser84	7.24	4.60	3.54, 3.96		
Thr85	8.75	4.37	4.53	γ CH ₃ 1.23	
Ser86	8.29	^c	3.77, 3.53		
Thr87	7.18	3.90	3.93	γ CH ₃ 1.24	4.3
Asp88	8.64	4.86	2.93, 2.64		9.2
Cys89	8.63	4.52	3.08, 2.89		7.4
Ser90	8.41	4.18	4.01, 3.91		7.4
Arg91	7.15	4.39	2.11, 1.69	γ H ₂ 1.68, 1.55; δ H ₂ 3.20; ϵ NH 7.13	8.6
Val92	7.06	3.93	2.19	γ CH ₃ 1.12, 1.01	7.4
Asn93	8.18	4.77	2.78, 2.64	γ NH ₂ 6.74, 7.41	9.2

^a Do not appear at 308 K.^b Degenerated resonance lines.^c Not defined or overlaps.

tures, provided further support for the sequential assignments.

The starting points for the sequential assignment were the unique and easily identifiable sequences Ala56 Ala57 Ala58 and Ser16 Tyr17. From there, elongation of the assignment along the polypeptide chain could be followed in both directions.

Identification of secondary structures

The presence of helical structures was implied by the observation of numerous typical d_{NN} connectivities. These observations were further supported by medium range NOE, especially those arising from spatial proximity between pairs of residues in positions (*i*) and (*i*+3). From the patterns of connectivities represented in Fig. 3 one can assume the existence of four helices. Qualitatively, such an assumption was confirmed by the presence of small $^3J_{NH-C\alpha H}$ coupling constants and slowly exchanging amide protons in these fragments.

The helical regions 4–20, 27–39, 45–60 and 65–79 indicated in Fig. 4 were then defined on NMR criteria. It is clear that the length of these helices will be defined more precisely in the modelling and the refinement of the complete three-dimensional structure of the protein. Dynamic effects

may occur at the ends of such structures as found in the H3 helix of the wheat ns-lipid-transfer protein (unpublished results).

DISCUSSION

The assignment of the two-dimensional ^1H -NMR spectra of the 93 amino acid residues of the maize ns-lipid-transfer protein is presented in Table 1. In addition to TOCSY, NOESY and 2QF-COSY experiments, 2Q-COSY and 3QF-COSY experiments proved to be extremely useful for overcoming difficulties arising from redundancy of Gly and Ser residues and for Ile residue identification.

Overall, regular secondary-structure elements of the protein (Fig. 4) were located according to NMR criteria (medium range NOE, $^3J_{NH-C\alpha H}$ coupling constants, NH exchange rates) within the sequence which was nearly completely assigned.

A complete modelling of the three-dimensional structure of the protein is now being undertaken, but several interesting conformational features emerge from the present data. The three-dimensional structure of the wheat ns-lipid-transfer protein was determined by Gincel, E., Simorre, J. P., Caille, A., Marion, D., Ptak, M. and Vovelle, F. (unpublished results) on the basis of two-dimensional and three-dimensional NMR

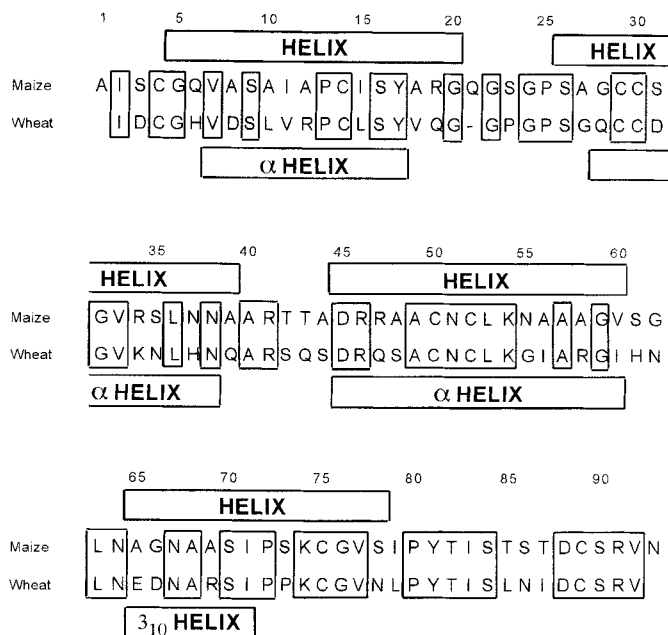


Fig. 4. Sequences of maize and wheat ns-lipid-transfer proteins are aligned. Residues strictly conserved in both proteins are squared. Helices defined on NMR criteria and by modelling in wheat ns-lipid-transfer protein and NMR on criteria in maize ns-lipid-transfer protein are shown below and above the sequences, respectively.

experiments [7]. As these two proteins exhibit about 60% sequence similarity, as it can be deduced from the alignment represented in Fig. 4, it can be expected that the two proteins should share some common conformational folds. Based on NMR data, the similarity extends to the details of the secondary structure, since the sequence locations of the four helices are identical in both proteins (Fig. 4).

Subject to the definition of length of helices, the comparison between wheat and maize ns-lipid-transfer proteins should help us to elucidate the molecular basis of lipid transfer since we show here that the maize ns-lipid-transfer protein is considerably more efficient than the wheat protein for transfer phospholipids. Therefore, it is tempting to relate the structural differences between these proteins to their transfer activities. The discussion will be based on the presence of secondary-structure elements and on the location of hydrophobic clusters in both proteins. In the solution structure of wheat ns-lipid-transfer protein, H1, H2 and H3 fragments were found to be α helices, while H4 showed characteristics of an irregular helix intermediate between α_R and 3_{10} forms (unpublished results). In both proteins, the first three helices, differ by one or two residues, i.e., the N-terminal Cys residue (Cys3) is not included in the wheat protein, whereas the corresponding residue in the maize ns-lipid-transfer protein (Cys4) is clearly helical, since characteristic NOE connectivities such as (Cys4) $C_\alpha H$ -(Val7)NH and (Cys4)- $C_\alpha H$ -(Ala8)NH were detected. A more drastic difference is observed when considering the last helix H4. In spite of the existence of some $C_\alpha H$ -NH $_{i+2}$ connectivities found in maize ns-lipid-transfer protein spectra, we cannot define in which conformation helix H4 is. However, it is approximately two-times longer than in wheat ns-lipid-transfer protein. Careful examination of the two sequences shows that in wheat ns-lipid-transfer protein, H4 is interrupted by two consecutive Pro residues and is followed by a non-canonical loop which

is strengthened by a (i)/(i+3) hydrogen bond between the residues 69 and 72 (unpublished results). In the maize protein, the unique Pro residue (Pro73) in this region does not seem to induce such a break since the helix extends five residues beyond.

In both proteins, a Pro residue is present in H1 helices and should induce a bend of their axes [29]. Another Pro residue is included in the H4 helical stretch in the maize protein. Distortions induced by these residues lead to abnormally high values of the $^3J_{C_\alpha H-NH}$ coupling constants in neighbouring residues. Such values were found in wheat ns-lipid-transfer protein for residues (i-2) (Val10) and (i+1) (Cys13) in H1 helix as well as in maize protein for residues (i-2) (Ile11) and (i+1) (Cys14) in H1 helix and (i-2) (Ser70) residue in H4 helix.

The high coupling-constant values also observed for residues located at the end of helices H1 (Arg19) and H4 (Cys75, Val77), are still compatible with distorted helical conformations located in broad area of Ramachandran plots [30, 31].

The C-terminal part of the wheat ns-lipid-transfer protein does not exhibit any regular structure but a well-defined saxophone shape (unpublished results), involving several turns, namely a γ -turn stabilised by an hydrogen bond (Ser82)CO...HN(Asn84) and a β -turn stabilised by a salt bridge. We cannot predict the precise conformation of the C-terminus of the maize protein. Nevertheless, the existence of turns is strongly supported by a medium-range NOE connectivity between (Cys89) $C_\alpha H$ and (Val92)NH, strong NH $_i$ -NH $_{i+1}$ connectivities and concomitant weak sequential $C_\alpha H$ -NH $_{i+1}$ interactions. Similar considerations led us to assume the existence of a turn involving the Pro80-Ile83 sequence.

As shown in Fig. 4, the hydrophobic residues are not strictly conserved, but each is replaced by another which shares almost the same hydrophobicity, i.e., Val→Ile, Leu→Ala, Leu→Ile or Val→Ala. This supports the idea that hydrophobic clusters which actually compact the protein, are conserved.

Qualitatively, we can then assume that the global folding of the maize and wheat ns-lipid-transfer protein should be rather similar. A precise comparison will be made when the modelling of the maize ns-lipid-transfer protein structure on the basis on NMR constraints that we have now undertaken, is completed. Nevertheless, we can indicate some possible relationships between the structural features revealed by NMR and the capacity of ns-lipid-transfer proteins to interact with lipids. We have previously demonstrated by infrared spectroscopy that wheat ns-lipid-transfer protein is able to bind lyso-palmitoyl-sn-glycerophosphocholine (lyso-Pam-GroPCho) [4] and that this complexation facilitates the crystallization of the wheat ns-lipid-transfer protein [32]. An hydrophobic cleft was observed in the wheat protein involving hydrophobic residues located in H1, H2 and H3 helices, and in the C-terminal saxophone structure (unpublished results). Finally, in the experimental conditions required for two-dimensional ^1H -NMR experiments, when a lyso-Pam-GroPCho-protein complex is formed, the largest changes of chemical shifts are observed in the second half of this protein [33]. Considering the difference in lipid-transfer activities of the two lipid-transfer proteins, it is remarkable to discover differences in their secondary structures, especially in the length of the H4 helices. If we consider that lipid transfer involves two basic phenomena, adsorption to the membrane interface and lipid binding, two sites should occur in the protein to ensure lipid transfer. The results of the transfer assays show that the presence of phosphatidylglycerol (PtdGro) in

the donor vesicles stimulates lipid transfer, probably by improving interfacial electrostatic interactions between the lipid phosphate head group of the anionic PtdGro and the basic residues of ns-lipid-transfer proteins. Furthermore, the similar stimulating effects of this anionic lipid on the transfer activity of maize and wheat protein suggest that the adsorption is not critical to explain their difference of activity. Therefore, the elongation of helix H4 would play a role in the ligand-complexation mechanism and not in the interfacial activation. From the three-dimensional model of wheat ns-lipid-transfer protein, it could be proposed that the polar part of the lipid-binding site would involve the polar residues located on the loop L2 connecting H2 and H3 helices (unpublished results). In the maize ns-lipid-transfer protein, the helix H4 could contribute to define a binding site for the polar head of lipids. This obviously would lead to a quite different orientation of the lipid molecule in the hydrophobic cleft. Another possibility is that the long helix H4 in the maize protein induces a slight modification of the orientation of the C-terminus saxophone structure and therefore of its packing in the hydrophobic cleft which could change the lipid-protein hydrophobic interactions in the site.

An interesting comparison will be also made with the structure of the hydrophobic protein from soybean [34], recently determined by X-ray crystallography [35]. The biological activity of this 8.3-kDa protein is not clearly established. Although it does not exhibit transfer activity (Kader, J. C., personal communication), its three-dimensional structure reveals remarkable similarities with the wheat ns-lipid-transfer protein one [35]. Such a comparison should also extend to a lipid-binding protein recently extracted from wheat seeds and named puroindoline, which shares sequence similarities with ns-lipid-transfer proteins [16, 36].

From the analysis of the structures and the activities of these different proteins, we should be able to construct a model of lipid binding and possibly of lipid transfer by structures involving principally a right-handed bundle of four helices.

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