Biochimica et Biophysica Acta 1808 (2011) 446–453

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

The intrinsically disordered late embryogenesis abundant protein LEA18 from *Arabidopsis thaliana* modulates membrane stability through binding and folding

Michaela Hundertmark [a,1], Rumiana Dimova [b], Jan Lengefeld [c], Robert Seckler [c], Dirk K. Hincha [a,*]

[a] Max-Planck-Institut für Molekulare Pflanzenphysiologie, Am Mühlenberg 1, D-14476 Potsdam, Germany
[b] Max-Planck-Institut für Kolloid- und Grenzflächenforschung, Am Mühlenberg 1, D-14476 Potsdam, Germany
[c] Institut für Physikalische Biochemie, Universität Potsdam, Karl-Liebknecht-Str. 24-25, D-14476 Potsdam, Germany

ARTICLE INFO

Article history:
Received 29 July 2010
Received in revised form 16 September 2010
Accepted 16 September 2010
Available online 25 September 2010

Keywords:
Intrinsically disordered protein
Late embryogenesis abundant protein
Membrane stability
Protein–membrane interaction
Protein folding

ABSTRACT

Intrinsically disordered proteins (IDPs) constitute a substantial part of cellular proteomes. Late embryogenesis abundant (LEA) proteins are mostly predicted to be IDPs associated with dehydration tolerance in many plant, animal and bacterial species. Their functions, however, are largely unexplored and also their structure and interactions with potential target molecules have only recently been experimentally investigated in a small number of proteins. Here, we report on the structure and interactions with membranes of the Pfam LEA_1 protein LEA18 from the higher plant *Arabidopsis thaliana*. This functionally uncharacterized positively charged protein specifically aggregated and destabilized negatively charged liposomes. Isothermal titration calorimetry showed binding of the protein to both charged and uncharged membranes. LEA18 alone was largely unstructured in solution. While uncharged membranes had no influence on the secondary structure of LEA18, the protein partially folded into β-sheet structure in the presence of negatively charged liposomes. These data suggest that LEA18 does not function as a membrane stabilizing protein, as suggested for other LEA proteins. Instead, a possible function of LEA18 could be the composition-dependent modulation of membrane stability, e.g., during signaling or vesicle-mediated transport.

1. Introduction

Late embryogenesis abundant (LEA) proteins were first described almost 30 years ago. They were found to be highly abundant during the late stages of cotton seed development, when the embryo becomes desiccation tolerant [1]. Subsequently, related proteins were found not only in the seeds of all other investigated plant species, but also in other plant tissues, in some bacterial species and in animals such as nematodes, rotifers and brine shrimp (see [2] for a review). In all these cases the occurrence of the proteins was related to environmental stress conditions such as freezing, drought, or desiccation. Numerous genetical investigations in a broad range of species have provided compelling evidence for a functional role of at least some LEA proteins in cellular stress tolerance [2]. However, the functional mechanisms of these proteins are still only poorly understood.

One of the problems in predicting LEA protein function has been the apparent lack of stable structure in most of the investigated proteins. For decades, the basic concept of structural biology has been the close relationship between stable three-dimensional structure and a resulting function, such as the catalytic activity of an enzyme. In recent years it has been recognized that a substantial part of all cellular proteomes is made up of proteins that either completely lack stable structure or that have large unstructured domains [3]. These proteins are now mainly referred to in the literature as intrinsically disordered proteins (IDPs). For many IDPs or IDP domains it has been shown that they perform essential functions, e.g. in cellular signal transduction and in the regulation of gene expression [4–6]. The ability of IDPs to bind various target molecules, such as RNA, DNA and other (structured) proteins [5,7] is of crucial importance for these functions. In many cases it has been observed that IDPs acquire increased secondary structure upon binding to their target molecules [8,9].

In the fully sequenced model plant species *Arabidopsis thaliana*, 51 genes encoding LEA proteins have been identified and the vast majority was predicted to be IDPs [10]. Experimental evidence for their lack of stable secondary structure has so far only been published for seven of these proteins [11–13] and in addition for a small number of LEA proteins from other plant and animal species [2,10]. Some LEA

Abbreviations: CD, circular dichroism; CF, carboxyfluorescein; EPE, egg phosphatidylethanolamine; EPG, egg phosphatidylglycerol; FRET, fluorescence resonance energy transfer; IDP, intrinsically disordered protein; ITC, isothermal titration calorimetry; LEA, late embryogenesis abundant; MBP, myelin basic protein; NBD-PE, N-(7-nitro-2,1,3-benzoxadiazol-4-yl) dipalmitoyl-phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine; Rh-PE, N-(lissamine Rhodamine B sulfonyl) dipalmitoyl-phosphatidylethanolamine

* Corresponding author. Tel.: +49 331 567 8253; fax: +49 331 567 8250.
E-mail address: hincha@mpimp-golm.mpg.de (D.K. Hincha).
[1] Present address: UMR 1191 Physiologie Moléculaire des Semences, 16 Bvd Lavoisier, 49045 Angers Cedex 1, France.

0005-2736/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2010.09.010

proteins acquire secondary structure during drying [14–19], indicating that they are able to fold under the appropriate conditions. Functionally, only a few LEA proteins have been investigated using different in-vitro assays that all assumed a direct protective effect for other biological structures or molecules. Such assays showed that some LEA proteins are able to stabilize sensitive enzymes during freezing and drying [2,20]. This has been related to their ability to prevent enzyme aggregation under stress conditions [21–24]. LEA proteins can also interact with model membranes in the dry state, a condition where the respective LEA proteins were shown to be folded [13,16,25]. In a few cases, binding of LEA proteins to membranes in solution was shown, sometimes accompanied by partial folding [11,26,27]. In these cases, however, no information on the influence of LEA protein binding on membrane stability was provided.

In *A. thaliana*, the 51 LEA proteins fall into nine groups that have been distinguished by amino acid sequence similarity [10,28]. One of these groups is characterized by the presence of the Pfam LEA_1 (PF03760) domain. These proteins were originally described as the D-113 group in cotton seeds [29] and later as group 4 [30]. Here, we provide a structural and functional characterization of LEA18, a functionally uncharacterized LEA_1 protein from *A. thaliana*. LEA18 is a small (10.5 kDa) seed specific, basic (pI = 9.7) and highly hydrophilic protein that has been predicted to be an IDP [10]. We show that LEA18 binds to negatively charged membranes. This binding induces partial folding of this otherwise largely unstructured protein, vesicle aggregation and leakage of soluble content from liposomes.

2. Materials and methods

2.1. Cloning

The gene *LEA18* (At2g35300) was cloned from the RIKEN Arabidopsis full-length cDNA clone pda07797 [31,32]. The coding sequence was PCR-amplified and inserted into the vector pENTR.SD. D-TOPO (Invitrogen, Karlsruhe, Germany). The identity of the insert was checked by sequencing. The gene was transferred to the pDEST17 expression vector (Invitrogen) for recombinant protein production under the control of the T7 expression system. The pDEST17 vector was used to express the protein with an N-terminal 6xHis-tag.

2.2. Recombinant protein production and purification

The pDEST17.LEA18 construct was transformed into the BL21 Star (DE3) *Escherichia coli* strain for recombinant protein production. To purify the recombinant LEA18, the bacterial cell lysate was incubated in a boiling water bath for 10 min and precipitated proteins were removed by centrifugation at 4000*g* for 15 min at 4 °C. The supernatant was filtered through a 0.2 μm filter and applied to a 1 ml HisTrap HP column (GE Healthcare) equilibrated with 20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole (pH 7.4) with a flow rate of 1 ml/min. The column was washed with increasing concentrations of imidazole and LEA18 was eluted with 250 mM imidazole. Wash and elution fractions were analyzed by SDS-PAGE and the fractions containing LEA18 were pooled and dialyzed against ddH_2O. After dialysis, protein purity was estimated by SDS-PAGE and Coomassie blue staining.

2.3. Preparation of liposomes

EPC and POPC were obtained from Avanti Polar Lipids (Alabaster, AL). EPG was purchased from Sigma and EPE from Lipid Products (Redhill, Surrey, UK). Lipids were mixed in the appropriate mass fractions in chloroform, dried under a stream of N_2 and stored under vacuum for at least 2 h to remove traces of solvent. Liposomes for leakage studies were made as previously described [33]. Briefly, an appropriate amount of lipid was hydrated in 250 μl of 100 mM CF, 10 mM TES and 0.1 mM EDTA (pH 7.4) and extruded using a Liposofast hand-held extruder ([34]; Avestin, Ottawa, Canada) with 100 nm pore filters. To remove external CF, the liposomes were passed through a Sephadex G-25 column (NAP-5, GE Healthcare) in 10 mM TES, 0.1 mM EDTA and 50 mM NaCl (TEN buffer, pH 7.4). Liposomes containing an additional 1 mol% each of the fluorescent probe pair NBD-PE and Rh-PE for fusion assays by fluorescence resonance energy transfer [35] were made in TEN buffer as described [33,36]. Briefly, two liposome samples were prepared: one sample was labeled with 1 mol% of both NBD-PE and Rh-PE, while the other sample was unlabeled. The two samples were combined after extrusion in a 1:9 (labeled:unlabeled) ratio, resulting in a final lipid concentration of 10 mg/ml.

2.4. Leakage and liposome fusion experiments

Liposomes (10 mg/ml in TEN) were mixed with the same volume of LEA18 or one of the negative control proteins thaumatin (from *Thaumatococcus daniellii*; Sigma) or β-lactoglobulin (bovine; Sigma) at a concentration of 1 mg/ml in TEN or with pure buffer and were either incubated at 28 °C or frozen for 2 h at −20 °C in an ethylene glycol bath and thawed at room temperature. For leakage experiments, 12 μl samples were diluted with 300 μl TEN in the wells of black 96-well plates in three replica. CF fluorescence was measured with a Fluoroskan Ascent (Labsystems, Helsinki, Finland) fluorescence microplate reader at an excitation wavelength of 444 nm and an emission wavelength of 555 nm [37]. While fluorescence is strongly quenched at the high concentration inside the intact liposomes, it increases when CF is released into the surrounding buffer. The total CF fluorescence (i.e. 100% leakage) was determined after lysis of the liposomes with 5 μl of a 0.1% Triton X-100 solution. Membrane fusion was measured as a reduction of resonance energy transfer between Rh-PE and NBD-PE by measuring the increase in NBD fluorescence due to dilution of the probes after fusion of labeled and unlabeled membranes [35] with a Kontron SFM 25 fluorometer (Bio-Tek Instruments, Neufahrn, Germany) at excitation and emission wavelengths of 450 and 530 nm, respectively [33,36]. Maximal NBD fluorescence in each sample (i.e. 100% fusion) was determined after lysis of the liposomes with Triton X-100.

2.5. Particle size measurements

Liposomes (50 μl with 20 mg lipid/ml) were mixed with an equal volume of protein solutions to yield the appropriate lipid:protein mass ratios indicated in the figure and degassed under vacuum for 15 min. Particle size was measured in triplicate at 25 °C with a Zetasizer Nano (Malvern Instruments).

2.6. Isothermal titration calorimetry

ITC was carried out with a VP-ITC Microcalorimeter (MicroCal). Liposomes were prepared in TEN buffer as described above and diluted to a final concentration of 2 mg/ml. All solutions were degassed under vacuum for 10 min. The reference cell of the ITC instrument contained TEN buffer, the working cell was filled with liposomes in the same buffer. Injections of 20 μl of protein solution (0.4 mg/ml in TEN) into the working cell were performed with an injection time of 20 s and a lag time of 200 s between the injections. The minimum lipid:protein ratio reached with the titrations was approximately 400:1. The stirring speed was 309 rpm. The measurements were performed at 25 °C. The first injection with a volume of 2 μl was discarded in the analysis due to possible dilution during the pre-equilibration stage of the experiment. The measurements were analyzed using Origin 7 with the Microcal package (Originlab).

2.7. Circular dichroism spectroscopy

CD was performed with a Jasco-715 spectropolarimeter (Jasco Instruments) as described recently [13]. Solutions of approximately 2 mg/ml protein in H_2O were mixed with an equal amount of H_2O or of liposomes (5 mg/ml in H_2O) and measured in a 0.1-mm pathlength cuvette. The mean-residue circular dichroism was calculated as:

$$\Delta\varepsilon_{MR} = \frac{\Delta A \cdot mMW}{d \cdot c}$$

$\Delta\varepsilon_{MR}$ = mean-residue circular dichroism [M^{-1} cm^{-1}], ΔA = circular dichroism, mMW = mean molecular mass of an amino acid in LEA18 [g mol^{-1}], d = pathlength [cm], c = protein concentration [g l^{-1}].

Spectra were analyzed with the CDPro software [38] using three different algorithms: CONTINLL, CDSSTR and SELCON3. Sets of reference spectra containing denatured proteins were chosen for the analysis. The results for LEA18 were similar for the three parallel samples and all algorithms, thus averages are shown.

3. Results

3.1. LEA18 induces leakage of soluble content from liposomes

We obtained a full-length cDNA clone for the Arabidopsis gene LEA18 (At2g35300) from the RIKEN RAFL collection [31,32] and expressed the LEA protein in *E. coli*. The recombinant protein carried a 6xHis tag and was purified by heat treatment and metal chelate chromatography. The resulting protein was essentially pure as shown by SDS-PAGE and Coomassie blue staining (Fig. 1). Similar to other LEA proteins [11], LEA18 migrated at a higher apparent molecular mass (approximately 19 kDa) than expected (11.4 kDa including the 6xHis tag).

As outlined above, it is generally assumed that the role of LEA proteins is the stabilization of cellular structures under stress conditions. A simple and convenient model to study the influence of additives on membrane stability employs liposomes loaded with the fluorescent dye CF at a concentration where the dye is self-quenching. Leakage of the dye during a stress treatment can then be monitored as an increase in CF fluorescence due to dye leakage into the surrounding solution. We used this experimental approach to evaluate the effect of LEA18 on membrane stability. To ensure that the observed effects were specific to LEA18 and not the unspecific results of the presence of any protein, we also investigated the effects of two well-characterized, structurally unrelated proteins, thaumatin (22.3 kDa, structure stabilized by eight disulphide bridges) and β-lactoglobulin (18.4 kDa, mainly β-strand). These proteins were preferred to the more often used bovine serum albumin (66 kDa) because their molecular masses are closer to that of LEA18 (10.5 kDa).

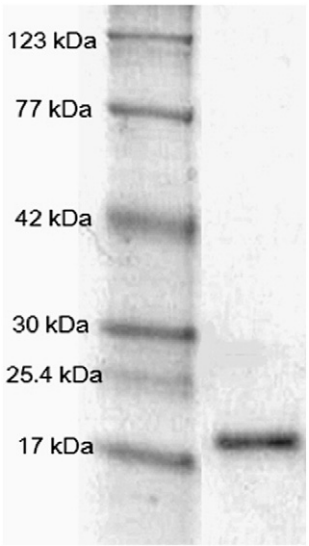

Fig. 1. SDS-PAGE analysis of purified recombinant LEA18. Molecular masses of standard proteins are indicated on the left.

Liposomes prepared from pure EPC showed only a low degree of leakage after freezing and thawing (Fig. 2). This was further reduced by the incorporation of the negatively charged lipid EPG into the membranes, in agreement with previous observations [39]. Incorporation of up to 40% of the nonbilayer lipid EPE did not destabilize the liposomes during freezing, also in agreement with previous reports [33]. However, enhanced leakage was observed when LEA18 was added to the liposomes, while thaumatin did not influence membrane stability under these conditions (Fig. 2). Increased CF leakage in the presence of LEA18 was evident with all investigated lipid compositions, but was strongest when EPG was present in the membranes. LEA18 induced complete CF leakage from liposomes containing 10% EPG, but this effect decreased with increasing amounts of EPG. The presence of EPE had no influence on the CF leakage induced by LEA18 during freezing when compared to pure EPC membranes.

To test whether the membrane destabilization by LEA18 was specific to the freezing treatment, liposomes made from 90% EPC and 10% EPG were incubated in the absence or presence of LEA18 at 28 °C (Fig. 3). While liposomes showed high stability in the absence of protein, LEA18 induced time dependent leakage, indicating membrane destabilization also under nonfreezing conditions. This destabilization was not observed in the presence of thaumatin or for liposomes containing only EPC, indicating the specificity of this effect under nonfreezing conditions for LEA18 and membranes carrying a negative surface charge.

Leakage of soluble content from liposomes is often accompanied by membrane fusion [40]. We used a FRET assay [35] to estimate membrane fusion from the dilution of a pair of fluorescently labeled lipids in liposomes due to mixing with unlabeled lipids from other liposomes resulting in reduced FRET. Fig. 4 shows that LEA18 only induced a small degree of fusion at 28 °C and that fusion was only slightly enhanced by the protein in membranes containing 10% EPG.

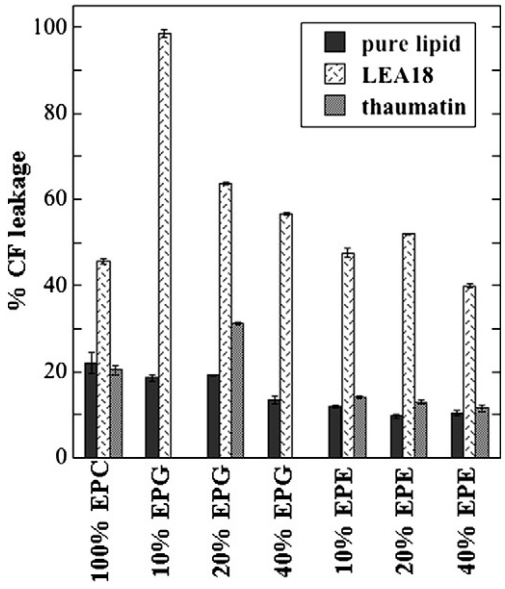

Fig. 2. Influence of LEA18 on CF leakage from liposomes after freezing and thawing. Liposomes composed of EPC and increasing fractions of the negatively charged phospholipid EPG, or the nonbilayer lipid EPE were frozen for 2 h at −20 °C in the absence or presence of LEA18 or thaumatin as a negative control protein at a mass ratio of lipid:protein of 10:1. CF leakage was measured after thawing. Data are averages ± SE from three parallel samples.

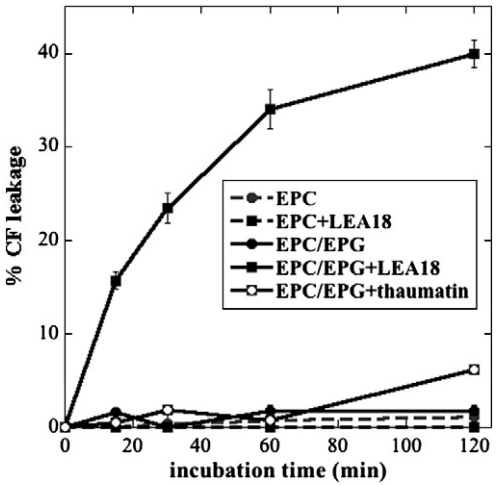

Fig. 3. Influence of LEA18 on CF leakage from liposomes during incubation at 28 °C. Liposomes containing either 100% EPC, or 90% EPC and 10% EPG were stored at 28 °C in the absence or presence of LEA18 or thaumatin as a negative control protein at a mass ratio of lipid:protein of 10:1. At the indicated time points CF leakage was measured. Data are averages ± SE from three parallel samples.

After freezing and thawing membrane fusion was minimal, irrespective of lipid composition or the presence of LEA18 or the control protein β-lactoglobulin. This clearly indicates that the massive leakage induced by LEA18 in liposomes containing 10% EPG was not related to membrane fusion events. The same effects on leakage and fusion were also observed when 90% POPC was used instead of 90% EPC in mixtures with 10% EPG. Since EPC consists mainly of POPC, this shows that there were no other components in EPC that were responsible for the observed effects of LEA18.

3.2. LEA18 specifically aggregates negatively charged vesicles

We observed that samples containing phosphatidylcholine (EPC or POPC) liposomes with 10% or 20% EPG became cloudy after the addition of LEA18, suggesting aggregation of the liposomes. Therefore, we estimated the amount and size of aggregates induced by LEA18 using light scattering measurements. In the absence of protein, both liposomes made from pure POPC or from 80% POPC/20% EPG showed a unimodal size distribution centered at around 100 nm (Fig. 5), which is the nominal size expected for liposomes extruded through 100 nm pore size membranes. This size distribution remained unchanged after the addition of LEA18 to liposomes made from pure POPC. With POPC/EPG liposomes, on the other hand, aggregates were observed in the presence of LEA18. At high lipid:protein mass ratios (250:1 and 100:1) no aggregates were observed, but at a mass ratio of 25:1 aggregates with a size of around 500 nm were detected. At the highest protein concentration (lipid:protein mass ratio 12.5:1) a large part of the liposomes was present in these aggregates. No aggregation was observed when β-lactoglobulin was added to POPC/EPG liposomes at a lipid:protein mass ratio of 25:1, indicating that liposome aggregation was also a specific effect of LEA18.

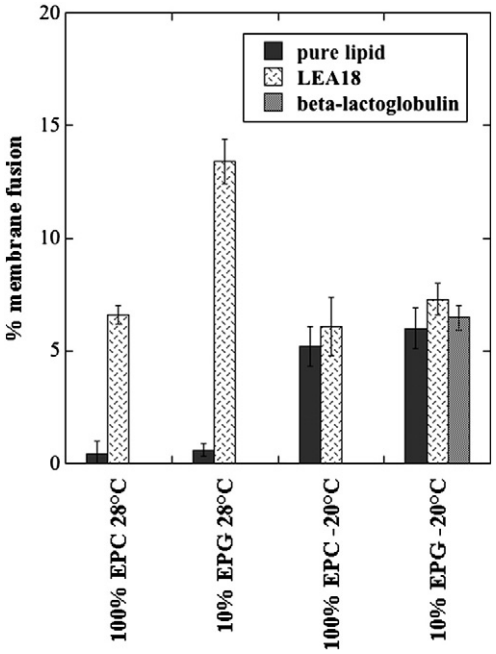

Fig. 4. Effect of LEA18 on membrane fusion. Liposomes containing either pure EPC, or 90% EPC and 10% EPG were incubated for 2 h at either 28 °C or −20 °C in the absence or presence of LEA18 or β-lactoglobulin as a negative control protein at a lipid:protein mass ratio of 10:1. Membrane fusion was determined by a FRET assay. Data are averages ± SE from three parallel samples.

3.3. LEA18 partially folds upon binding to PG-containing vesicles

The secondary structure of LEA18 was investigated using CD spectroscopy. In pure water, LEA18 exhibited far-UV CD spectra typical for unstructured proteins with a minimum around 200 nm (Fig. 6). Secondary structure estimates using different CD spectra analysis programs resulted in approximately 20% regular secondary structure for LEA18 under these conditions. In the presence of liposomes containing 80% POPC/20% EPG the CD spectrum of LEA18 in solution was clearly different from that of the protein in pure water, indicating partial folding. This was dependent on the presence of negative charges on the membrane surface, as pure POPC liposomes did not induce any changes in the CD spectrum of LEA18. A quantitative analysis indicated that in the presence of POPC/EPG liposomes the β-sheet content of LEA18 increased from less than 15% in water or in the presence of pure POPC liposomes, to approximately 35%, with an accompanying decrease in the disorder content (from about 70% to 40%).

3.4. LEA18 binds to both charged and uncharged lipid membranes

The thermodynamics of the interaction of LEA18 with phospholipid vesicles was characterized by ITC. Fig. 7A shows the heat flow resulting from titration of LEA18 into buffer with or without liposomes made either from pure POPC or from 80% POPC and 20% EPG. The observed negative heat flow in the presence of membranes (Fig. 7A and B) indicates that binding of LEA18 is an exothermic process and the reduced heat per injection with increasing number of injections indicates a tendency towards saturation of binding sites for the protein on the membranes (see [41,42] for reviews of the methodology). The released heat upon addition of LEA18 was more than twice as big in the presence of POPC vesicles as in pure buffer. It increased another tenfold in the presence of EPG-containing liposomes compared to pure POPC vesicles (Fig. 7A and B).

We obtained further thermodynamic information about the binding of LEA18 to lipid membranes by fitting a Langmuir adsorption isotherm to the ITC data [42]. For the binding to pure POPC membranes, we obtained an excellent fit (Fig. 7C), yielding a binding enthalpy of $\Delta H = -147 \pm 1$ cal/mol lipid (≈ -0.615 kJ/mol) and a binding constant $K = (1.39 \pm 0.02) \times 10^5\ M^{-1}$. Note that the enthalpy change ΔH is defined in this case per mole lipid. In order to evaluate the binding enthalpy per mole protein, independent knowledge about the number of bound lipids per protein is required. Alternatively, the data can be fitted using the one-set-of-binding-sites model with three fitting parameters: ΔH_p, K and n, where ΔH_p is the enthalpy change per mole protein and n reflects the reaction stoichiometry so that 1/n is the number of lipids bound to one protein molecule. However, because the data do not cover the necessary region of saturation,

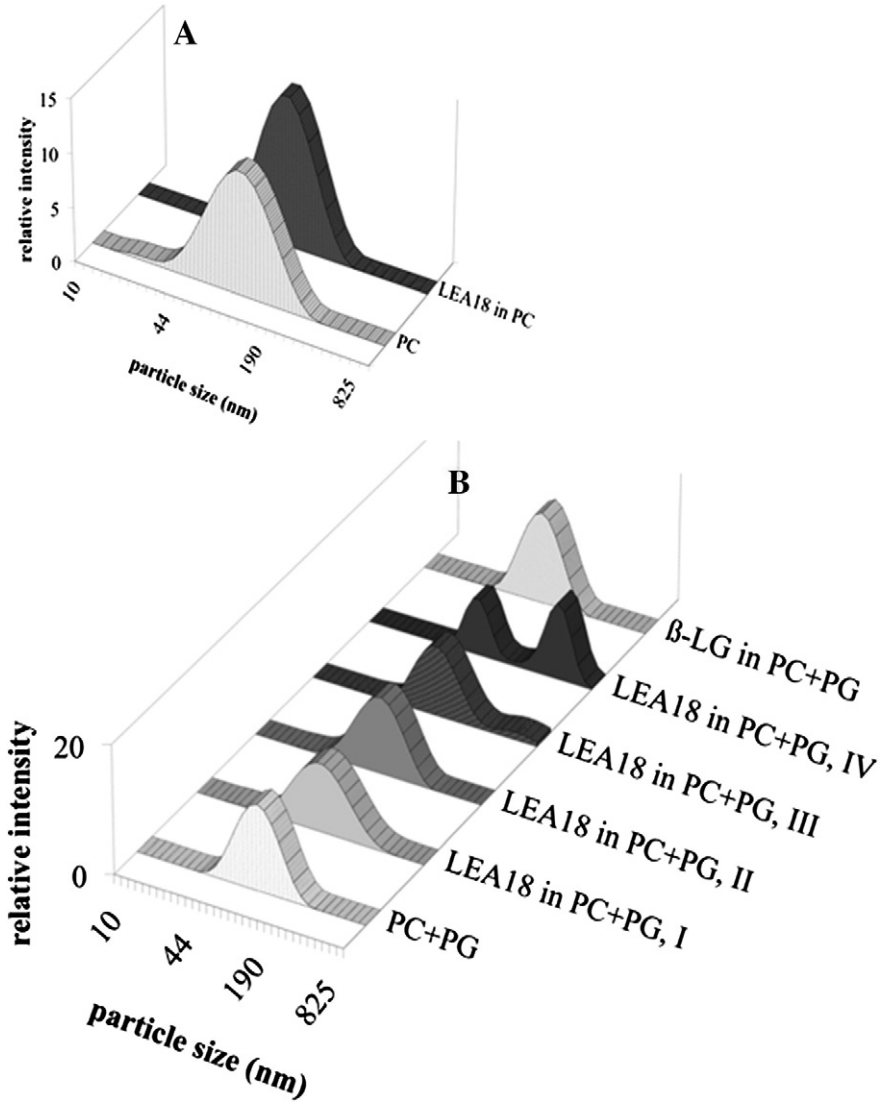

Fig. 5. Size distribution of liposomes incubated with different amounts of LEA18. Liposomes (10 mg lipid/ml) made from pure POPC (top panel) or 80% POPC/20% EPG (bottom panel) were incubated with LEA18. The mass ratio of pure POPC to LEA18 was 25:1, while different amounts of LEA18 were incubated with EPG-containing vesicles resulting in lipid:protein mass ratios of I = 250:1, II = 100:1, III = 25:1 and VI = 12.5:1. Particle size was measured after 15 min incubation at room temperature. In addition, EPG-containing liposomes were incubated with β-lactoglobulin (β-LG) as a negative control protein at a lipid:protein mass ratio of 25:1.

fitting this model is difficult due to the highly interdependent fitting parameters and the low sensitivity of the fit to the parameters. Assuming that the number of lipid molecules per bound protein is between a few hundred and 10,000, a suitable fit can be found for $n = 1.4 \times 10^{-3}$, $K = 7.8 \times 10^4\ M^{-1}$ and $\Delta H_p = -172$ kcal/mol protein. Obviously, the enthalpy ΔH determined per mole of lipid according to the Langmuir adsorption isotherm is on the order of n times the enthalpy per mole protein, $\Delta H \sim n\Delta H_p$. The obtained binding constants yield a Gibbs free energy change ΔG^0 between −26 kJ/mol and −29.3 kJ/mol (defined for standard state M^{-1}).

In the case of membranes containing 20% of the negatively charged lipid EPG, the dependence of the released heat on protein concentration was more featureless and did not show a tendency of saturation in the binding sites (Fig. 7D). Thus, the simple fit following Langmuir adsorption is not suitable. Presumably, in this case the interactions of LEA18 with the membrane should be described by two sets of fitting parameters corresponding to each of the lipid types. The local enrichment of the positively charged protein in the vicinity of the negatively charged lipids due to electrostatic attraction should also be accounted for. While, at lipid:protein mass ratios above approximately 400:1 in these measurements, liposome aggregation should not be a factor, we expect that protein folding, as demonstrated by the CD spectra in Fig. 6, also contributed to the measured heat release. Fitting the data with several fitting parameters describing binding and protein folding is futile. Therefore, no reliable thermodynamic parameters could be extracted in this case. On a more qualitative basis, it can be concluded that the binding enthalpy in the case of LEA18 binding to POPC/EPG vesicles is about an order of magnitude higher than that of LEA18 binding to pure POPC vesicles as can be estimated from extrapolation of the ITC data in Fig. 7C and D to zero protein concentration.

4. Discussion

LEA18 is an IDP, with only about 20% secondary structure (α-helix, β-sheet) in solution. LEA18 interacted with membranes both in the frozen state and in solution and the interactions influenced both membranes and protein. The interactions were much stronger when the membranes contained negatively charged lipids and an effect on protein secondary structure was only observed in this case where the β-sheet content clearly increased. The fact that this partial folding and binding led to massive vesicle aggregation suggests that at least two

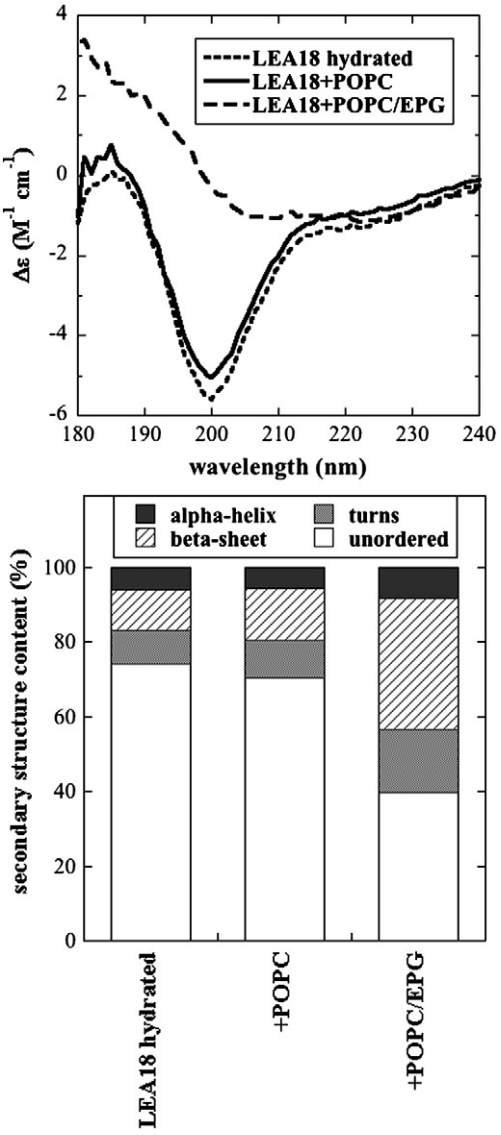

Fig. 6. Secondary structure analysis of LEA18. The top panel shows CD spectra of LEA18 in water (hydrated) or in water in the presence of liposomes made either from pure POPC or from 80% POPC/20% EPG with a lipid:protein mass ratio of 2.5:1. The bottom panel shows the corresponding quantification of secondary structure elements in LEA18.

separate positively charged residues of LEA18 were able to bind to different vesicles, with the rest of the protein functioning as a spacer. Alternatively, LEA18 could form dimers with the membrane binding residues at opposite ends, as suggested for myelin basic protein, an IDP that partially folds into an α-helical structure upon membrane binding [43]. An increase in β-sheet content at the expense of unordered conformations upon binding to PG membranes has also been reported for the membrane destabilizing snake venom peptide cardiotoxin [44]. Both LEA18 and cardiotoxin are strongly basic proteins.

In the presence of uncharged (100% POPC) membranes, clear evidence of membrane binding, presumably through hydrophobic interactions between the membrane lipids and the hydrophobic amino acids in LEA18 was obtained through ITC measurements. Compared to other peptides, the partitioning of LEA18 in neutral membranes is significant as indicated by the relatively high binding constant compared to magainin [45]. The interaction of LEA18 with both neutral and charged membranes was exothermic, i.e. enthalpy driven. In the case of POPC, binding had no measurable influence on protein secondary structure, vesicle aggregation, or membrane stability in solution.

Under the additional stresses associated with freezing, however, LEA18 increased CF leakage also from uncharged liposomes, albeit to a lesser degree than with EPG-containing liposomes. Increasing amounts of EPG in the vesicle membranes reduced leakage during freezing in the presence of LEA18, presumably through direct effects of EPG on membrane structure, such as increased inter-headgroup hydrogen-bonding [46,47]. Leakage during freezing was not associated with membrane fusion in either EPC or EPC/EPG liposomes. Also at 28 °C only little fusion occurred in the EPC/EPG vesicles compared to the massive leakage, indicating that a different mechanism must be responsible for the leakage induced by the positively charged LEA18 in negatively charged liposomes.

We suggest lipid demixing and the insertion of hydrophobic amino acids into the bilayer as possible mechanisms. It has been shown in previous studies that depending on fatty acyl chain structure PC/PG systems have a tendency for demixing even in the liquid-crystalline state and in the absence of proteins [48]. Addition of positively charged synthetic peptides [49], cytochrome c [50], lysozyme [51] or cardiotoxin [52] leads to the segregation of PG molecules that preferentially bind to the proteins. This segregation leads to PG-rich and PC-rich membrane domains that induce leakage, e.g. in the presence of lysozyme [53] and cytochrome c [54], through nonideal packing of the lipid molecules at the domain borders. Interestingly, it has recently been shown that this lipid demixing by cytochrome c is abolished with increasing fractions of PG in PC membranes [55]. This could be an additional factor to account for the reduced CF leakage during freezing from liposomes with higher PG content.

The destabilization can be enhanced by effects of the protein on the order of the hydrocarbon chains of the bound lipids through additional hydrophobic interactions [49,56]. In a synthetic basic peptide the presence of only two hydrophobic amino acids is sufficient to shift the position of the peptide backbone from the aqueous layer of a negatively charged membrane to a position below the lipid phosphate groups [57]. LEA18 contains one Ile and two Leu residues adjacent to Lys residues [10], making these hydrophobic amino acids good candidates for such an electrostatic/hydrophobic anchoring. In addition, in magainin and some of its synthetic variants hydrophobic amino acids are also involved in the binding of this positively charged lytic peptide to pure POPC membranes [45] and the hydrophobic LEA protein PM25 from *Medicago truncatula* induces CF leakage from pure POPC liposomes already in the absence of freezing [21]. Site-directed mutagenesis studies will be necessary to clarify the functional role of specific amino acids in LEA18 for membrane binding and destabilization.

The destabilizing effect of LEA18 on membranes observed in the present study is in disagreement with the "classical" role proposed for LEA proteins as cellular stabilizers, but the expression of the gene encoding LEA18 in seeds [10] indicates a potential role of this protein during the late seed maturation phase when desiccation tolerance is established. However, the present investigation was only concerned with hydrated systems, as even at −20 °C there is still liquid water present in equilibrium with ice due to the freezing point depression affected by the solutes (buffer, salts) that are excluded from the ice crystals and therefore accumulate in a residual highly concentrated solution. Furthermore, the secondary structure of LEA18 is highly flexible, the protein being largely disordered in pure water and in the presence of uncharged membranes and partially β-sheet in the presence of negatively charged membranes. While the unstructured protein had no influence on the stability of EPC liposomes in solution, the β-sheet containing protein destabilized EPG-containing liposomes. It can not be excluded that during desiccation LEA18 becomes mainly α-helical and is able to stabilize either membranes or proteins, as previously shown for other LEA_1 proteins (see Section 1 for details). It might even be envisaged that such protective interactions

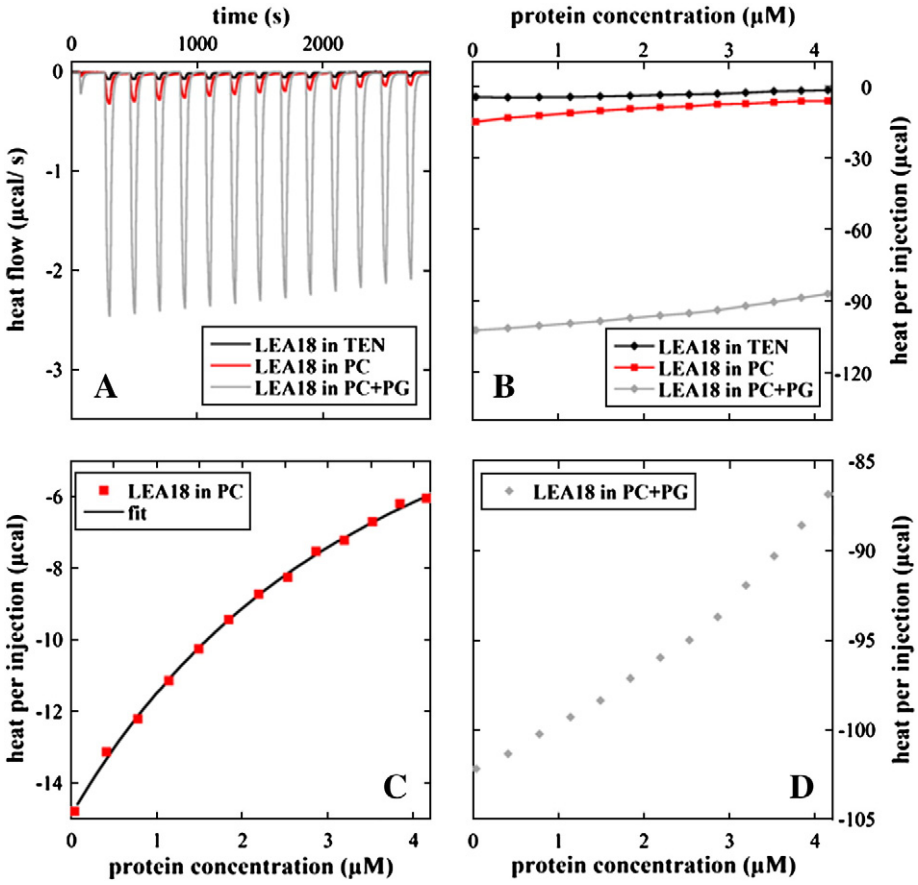

Fig. 7. ITC analysis of membrane binding by LEA18. (A) LEA18 was injected into buffer or liposome solution and the resulting heat flow was monitored. The liposomes were made either from pure POPC or from 80% POPC/20% EPG. (B) The area under each peak was integrated to obtain the heat per injection in microcalories per mole protein. The result was plotted as a function of the micromolar concentration of LEA18. (C) A model based on a Langmuir adsorption isotherm was fitted to the ITC data from the titration of LEA18 into a solution containing POPC liposomes, assuming that the heat of dilution was constant at −3 µcal per injection as suggested by the titration of LEA18 into buffer (A and B). From this model the thermodynamic characteristics of membrane binding were calculated, as detailed in the text. (D) Applying the same model to the ITC data from the titration of LEA18 into a solution containing POPC/EPG liposomes was not possible (see text for details).

with other proteins could prevent LEA18 from its destabilizing interactions with membranes.

Nevertheless, the properties of LEA18 described in the present study are compatible with the hypothesis that the protein may play a role in vesicle transport or signaling during development, while seeds attain desiccation tolerance, but before maturation drying. Through its ability to bind to particular membrane lipids and to lead to localized vesicle docking, limited membrane fusion and release of vesicle content, LEA18 may play a role in vesicle unloading. Also, similar to MBP, it may interact with other proteins in addition to cellular membranes and thereby function in signal transduction from the plasma membrane to internal membrane systems or the cytoskeleton [43]. MBP is an IDP that is able to adjust its secondary structure to various binding partners [58], thereby transmitting signals from the plasma membrane to actin filaments. An involvement of LEA proteins in cellular signaling as part of the adaptation to water loss has not been previously discussed, although a loss-of-function mutant in the LEA protein EM6 of *A. thaliana* showed aberrant seed development [59]. Further genetical and mutational studies will be required to define the function of LEA18 in seed development and desiccation tolerance.

5. Conclusions

LEA proteins comprise several phylogenetically unrelated families [10]. Since some families have members in bacterial, plant and animal species, it can be assumed that these proteins constitute an evolutionary old molecular adaptation to dehydration stress. This can in its extreme form result in anhydrobiosis, i.e. the survival in the desiccated state [60] as exemplified in mature orthodox plant seeds. While there is general agreement in the field that LEA proteins must play important roles in cellular dehydration tolerance, no comprehensive functional in-vitro and in-vivo analysis has been published for any LEA protein. The main emphasis of in-vitro studies has been on the ability of LEA proteins to stabilize membranes or enzymes during freezing and drying [2,20]. The possibility of other more indirect functions has been largely neglected. This seems surprising, given the extensive evidence for the involvement of various IDPs in signal transduction and the regulation of gene expression [4–6].

The present paper provides a comprehensive analysis of the interactions of a LEA protein with membranes in solution and of the effects of these interactions on the structure of the protein and the stability of the target membranes. The data strongly suggest that direct membrane stabilization is not the primary function of LEA18, at least in the hydrated state. Instead, the results indicate a lipid composition-dependent binding and folding interaction that leads to membrane domain formation inducing leakage and limited fusion that may play a role in cellular vesicle transport. In addition, it has been shown that lipid domain formation can increase the activity of calcium channels [61] and presumably other membrane channels and transporters.

Acknowledgments

M.H. acknowledges financial support through a PhD fellowship from the University of Potsdam.

References

[1] L. Dure III, S.C. Greenway, G.A. Galau, Developmental biochemistry of cottonseed embryogenesis and germination: changing messenger ribonucleic acid populations as shown by in vitro and in vivo protein synthesis, Biochemistry 20 (1981) 4162–4168.

[2] A. Tunnacliffe, M.J. Wise, The continuing conundrum of LEA proteins, Naturwissenschaften 94 (2007) 791–812.

[3] C.J. Oldfield, Y. Cheng, M.S. Cortese, C.J. Brown, V.N. Uversky, A.K. Dunker, Comparing and combining predictors of mostly disordered proteins, Biochemistry 44 (2005) 1989–2000.

[4] H.J. Dyson, P.E. Wright, Intrinsically unstructured proteins and their functions, Nat. Rev. Mol. Cell Biol. 6 (2005) 197–208.

[5] J. Liu, N.B. Perumal, C.J. Oldfield, E.W. Su, V.N. Uversky, A.K. Dunker, Intrinsic disorder in transcription factors, Biochemistry 45 (2006) 6873–6888.

[6] P. Tompa, The interplay between structure and function in intrinsically unstructured proteins, FEBS Lett. 579 (2005) 3346–3354.

[7] P. Tompa, P. Csermely, The role of structural disorder in the function of RNA and protein chaperones, FASEB J. 18 (2004) 1169–1175.

[8] C.J. Oldfield, Y. Cheng, M.S. Cortese, P. Romero, V.N. Uversky, A.K. Dunker, Coupled folding and binding with α-helix-forming molecular recognition elements, Biochemistry 44 (2005) 12454–12470.

[9] P.E. Wright, H.J. Dyson, Linking folding and binding, Curr. Opin. Struct. Biol. 19 (2009) 31–38.

[10] M. Hundertmark, D.K. Hincha, LEA (late embryogenesis abundant) proteins and their encoding genes in *Arabidopsis thaliana*, BMC Genomics 9 (2008) 118.

[11] D. Kovacs, E. Kalmar, Z. Torok, P. Tompa, Chaperone activity of ERD10 and ERD14, two disordered stress-related plant proteins, Plant Physiol. 147 (2008) 381–390.

[12] J.-M. Mouillon, P. Gustafsson, P. Harryson, Structural investigation of disordered stress proteins. Comparison of full-length dehydrins with isolated peptides of their conserved segments, Plant Physiol. 141 (2006) 638–650.

[13] A. Thalhammer, M. Hundertmark, A.V. Popova, R. Seckler, D.K. Hincha, Interaction of two intrinsically disordered plant stress proteins (COR15A and COR15B) with lipid membranes in the dry state, Biochim. Biophys. Acta 1798 (2010) 1812–1820.

[14] J. Boudet, J. Buitink, F.A. Hoekstra, H. Rogniaux, C. Larre, P. Satour, O. Leprince, Comparative analysis of the heat stable proteome of radicles of *Medicago truncatula* seeds during germination identifies late embryogenesis abundant proteins associated with desiccation tolerance, Plant Physiol. 140 (2006) 1418–1436.

[15] K. Goyal, L. Tisi, A. Basran, J. Browne, A. Burnell, J. Zurdo, A. Tunnacliffe, Transition from natively unfolded to folded state induced by desiccation in an anhydrobiotic nematode protein, J. Biol. Chem. 278 (2003) 12977–12984.

[16] N.N. Pouchkina-Stantcheva, B.M. McGee, C. Boschetti, D. Tolleter, S. Chakrabortee, A.V. Popova, F. Meersman, D. Macherel, D.K. Hincha, A. Tunnacliffe, Functional divergence of former alleles in an ancient asexual invertebrate, Science 318 (2007) 268–271.

[17] M. Shih, S. Lin, J. Hsieh, C. Tsou, T. Chow, T. Lin, Y. Hsing, Gene cloning and characterization of a soybean (*Glycine max* L.) LEA protein, GmPM16, Plant Mol. Biol. 56 (2004) 689–703.

[18] D. Tolleter, M. Jaquinod, C. Mangavel, C. Passirani, P. Saulnier, S. Manon, E. Teyssier, N. Payet, M.-H. Avelange-Macherel, D. Macherel, Structure and function of a mitochondrial late embryogenesis abundant protein are revealed by desiccation, Plant Cell 19 (2007) 1580–1589.

[19] W.F. Wolkers, S. McCready, W.F. Brandt, G.G. Lindsey, F.A. Hoekstra, Isolation and characterization of a D-7 LEA protein from pollen that stabilizes glasses in vitro, Biochim. Biophys. Acta 1544 (2001) 196–206.

[20] M. Battaglia, Y. Olvera-Carrillo, A. Garciarrubio, F. Campos, A.A. Covarrubias, The enigmatic LEA proteins and other hydrophilins, Plant Physiol. 148 (2008) 6–24.

[21] V. Boucher, J. Buitink, X. Lin, J. Boudet, F.A. Hoekstra, M. Hundertmark, D. Renard, O. Leprince, MtPM25 is an atypical hydrophobic late embryogenesis-abundant protein that dissociates cold and desiccation-aggregated proteins, Plant Cell Environ. 33 (2010) 418–430.

[22] S. Chakrabortee, C. Boschetti, L.J. Walton, S. Sarkar, D.C. Rubensztein, A. Tunnacliffe, Hydrophilic protein associated with desiccation tolerance exhibits broad protein stabilization function, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 18073–18078.

[23] K. Goyal, L.J. Walton, A. Tunnacliffe, LEA proteins prevent protein aggregation due to water stress, Biochem. J. 388 (2005) 151–157.

[24] K. Nakayama, K. Okawa, T. Kakizaki, T. Inaba, Evaluation of the protective activities of a late embryogenesis abundant (LEA) related protein, Cor15am, during various stresses *in vitro*, Biosci. Biotechnol. Biochem. 72 (2008) 1642–1645.

[25] D. Tolleter, D.K. Hincha, D. Macherel, A mitochondrial late embryogenesis abundant protein stabilizes model membranes in the dry state, Biochim. Biophys. Acta 1798 (2010) 1926–1933.

[26] M.-C. Koag, R.D. Fenton, S. Wilkens, T.J. Close, The binding of maize DHN1 to lipid vesicles. Gain of structure and lipid specificity, Plant Physiol. 131 (2003) 309–316.

[27] M.-C. Koag, S. Wilkens, R.D. Fenton, J. Resnik, E. Vo, T.J. Close, The K-segment of maize DHN1 mediates binding to anionic phospholipid vesicles and concomitant structural changes, Plant Physiol. 150 (2009) 1503–1514.

[28] N. Bies-Etheve, P. Gaubier-Comella, A. Debures, E. Lasserre, E. Jobet, M. Raynal, R. Cooke, M. Delseny, Inventory, evolution and expression profiling diversity of the LEA (late embryogenesis abundant) protein gene family in *Arabidopsis thaliana*, Plant Mol. Biol. 67 (2008) 107–124.

[29] L. Dure III, M. Crouch, J. Harada, T.-H.D. Ho, J. Mundy, R. Quatrano, T. Thomas, Z.R. Sung, Common amino acid sequence domains among the LEA proteins of higher plants, Plant Mol. Biol. 12 (1989) 475–486.

[30] E.A. Bray, Molecular responses to water deficit, Plant Physiol. 103 (1993) 1035–1040.

[31] T. Sakurai, M. Satou, K. Akiyama, K. Iida, M. Seki, T. Kuromori, M. Itoh, A. Konagaya, T. Toyoda, K. Shinozaki, RARGE: a large-scale database of RIKEN Arabidopsis resources ranging from transcriptome to phenome, Nucleic Acids Res. 33 (2005) D647–D650.

[32] M. Seki, M. Narusaka, A. Kamiya, J. Ishida, M. Satou, T. Sakurai, M. Nakajima, A. Enju, K. Akiyama, Y. Oono, M. Muramatsu, Y. Hayashizaki, J. Kawai, P. Carninci, M. Itoh, Y. Ishii, T. Arakawa, K. Shibata, A. Shinagawa, K. Shinozaki, Functional annotation of a full-length Arabidopsis cDNA collection, Science 296 (2002) 141–145.

[33] D.K. Hincha, A.E. Oliver, J.H. Crowe, The effects of chloroplast lipids on the stability of liposomes during freezing and drying, Biochim. Biophys. Acta 1368 (1998) 150–160.

[34] R.C. MacDonald, R.I. MacDonald, B.P.M. Menco, K. Takeshita, N.K. Subbarao, L. Hu, Small-volume extrusion apparatus for preparation of large, unilamellar vesicles, Biochim. Biophys. Acta 1061 (1991) 297–303.

[35] D.K. Struck, D. Hoekstra, R.E. Pagano, Use of resonance energy transfer to monitor membrane fusion, Biochemistry 20 (1981) 4093–4099.

[36] A.E. Oliver, D.K. Hincha, L.M. Crowe, J.H. Crowe, Interactions of arbutin with dry and hydrated bilayers, Biochim. Biophys. Acta 1370 (1998) 87–97.

[37] D.K. Hincha, E. Zuther, E.M. Hellwege, A.G. Heyer, Specific effects of fructo- and gluco-oligosaccharides in the preservation of liposomes during drying, Glycobiology 12 (2002) 103–110.

[38] N. Sreerama, R.W. Woody, Estimation of protein secondary structure from circular dichroism spectra: inclusion of denatured proteins with native proteins in the analysis, Anal. Biochem. 287 (2000) 243–251.

[39] J.H. Crowe, L.M. Crowe, Factors affecting the stability of dry liposomes, Biochim. Biophys. Acta 939 (1988) 327–334.

[40] A.E. Oliver, D.K. Hincha, J.H. Crowe, Looking beyond sugars: the role of amphiphilic solutes in preventing adventitious reactions in anhydrobiotes at low water contents, Comp. Biochem. Physiol. 131A (2002) 515–525.

[41] J.E. Ladbury, B.Z. Chowdhry, Sensing the heat: the application of isothermal titration calorimetry to thermodynamic studies of biomolecular interactions, Chem. Biol. 3 (1996) 791–801.

[42] J. Seelig, Titration calorimetry of lipid-peptide interactions, Biochim. Biophys. Acta 1331 (1997) 103–116.

[43] J.M. Boggs, Myelin basic protein: a multifunctional protein, Cell. Mol. Life Sci. 63 (2006) 1945–1961.

[44] W.K. Surewicz, T.M. Stepanik, A.G. Szabo, H.H. Mantsch, Lipid-induced changes in the secondary structure of snake venom cardiotoxins, J. Biol. Chem. 263 (1988) 786–790.

[45] T. Wieprecht, M. Beyermann, J. Seelig, Binding of antibacterial magainin peptides to electrically neutral membranes: thermodynamics and structure, Biochemistry 38 (1999) 10377–10387.

[46] W. Hübner, A. Blume, Interactions at the lipid–water interface, Chem. Phys. Lipids 96 (1998) 99–123.

[47] E.C. Mushayakarara, P.T.T. Wong, H.H. Mantsch, The effect of pressure on the hydrogen bonding between carbonyl and hydroxyl moieties in 1, 2-dipalmitoylglycerol: a Fourier-transform infrared spectroscopy study, Biochim. Biophys. Acta 857 (1986) 259–264.

[48] P. Garidel, C. Johann, L. Mennicke, A. Blume, The mixing behavior of pseudobinary phosphatidylcholine-phosphatidylglycerol mixtures as a function of pH and chain length, Eur. Biophys. J. 26 (1997) 447–459.

[49] K. Gawrisch, J.A. Barry, L.L. Holte, T. Sinnwell, L.D. Bergelson, J.A. Ferretti, Role of interaction at the lipid-water interface for domain formation, Mol. Membr. Biol. 12 (1995) 83–88.

[50] T. Heimburg, B. Angerstein, D. Marsh, Binding of peripheral proteins to mixed lipid membranes: effect of lipid demixing upon binding, Biophys. J. 76 (1999) 2575–2586.

[51] G.P. Gorbenko, V.M. Ioffe, J.G. Molotkovsky, P.K.J. Kinnunen, Resonance energy transfer study of lysozyme–lipid interactions, Biochim. Biophys. Acta 1778 (2008) 1213–1221.

[52] M.A. Carbone, P.M. Macdonald, Cardiotoxin II segregates phosphatidylglycerol from mixtures with phosphatidylcholine: ^{31}P and ^{2}H NMR spectroscopic evidence, Biochemistry 35 (1996) 3368–3378.

[53] E. Posse, B.F. de Arcuri, R.D. Morero, Lysozyme interactions with phospholipid vesicles: relationships with fusion and release of aqueous content, Biochim. Biophys. Acta 1193 (1994) 101–106.

[54] A.E. Gad, B.L. Silver, G.D. Eytan, Polycation-induced fusion of negatively-charged vesicles, Biochim. Biophys. Acta 690 (1982) 124–132.

[55] G.P. Gorbenko, V.M. Trusova, J.G. Molotkovsky, P.K.J. Kinnunen, Cytochrome c induces lipid demixing in weakly charged phosphatidylcholine/phosphatidylglycerol model membranes as evidenced by resonance energy transfer, Biochim. Biophys. Acta 1788 (2009) 1358–1365.

[56] A. Mulgrew-Nesbitt, K. Diraviyam, J. Wang, S. Singh, P. Murray, Z. Li, L. Rogers, N. Mirkovic, D. Murray, The role of electrostatics in protein–membrane interactions, Biochim. Biophys. Acta 1761 (2006) 812–826.

[57] K.G. Victor, D.S. Cafiso, Location and dynamics of basic peptides at the membrane interface: electron paramagnetic resonance spectroscopy of tetramethylpiperidine-N-oxyl-4-amino-4-carboxylic acid-labeled peptides, Biophys. J. 81 (2001) 2241–2250.

[58] M.A. Ahmed, V.V. Bamm, L. Shi, M. Steiner-Mosonyi, J.F. Dawson, L. Brown, G. Harauz, V. Ladizhansky, Induced secondary structure and polymorphism in an intrinsically disordered structural linker of the CNS: solid-state NMR and FTIR spectroscopy of myelin basic protein bound to actin, Biophys. J. 96 (2009) 180–191.

[59] A.J. Manfre, L.M. Lanni, W.R.J. Marcotte, The Arabidopsis group 1 LATE EMBRYOGENESIS ABUNDANT protein ATEM6 is required for normal seed development, Plant Physiol. 140 (2006) 140–149.

[60] J.H. Crowe, F.A. Hoekstra, L.M. Crowe, Anhydrobiosis, Annu. Rev. Physiol. 54 (1992) 579–599.

[61] B. Cannon, M. Hermansson, S. Györke, P. Somerharju, J.A. Virtanen, K.H. Cheng, Regulation of calcium channel activity by lipid domain formation in planar lipid bilayers, Biophys. J. 85 (2003) 933–942.