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

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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

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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).