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# Group 3 Late Embryogenesis Abundant Proteins from Embryos of *Artemia franciscana*: Structural Properties and Protective Abilities during Desiccation

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## ABSTRACT

Group 3 late embryogenesis abundant (LEA) proteins are highly hydrophilic, and their expression is associated with desiccation tolerance in both plants and animals. Here we show that two LEA proteins from embryos of *Artemia franciscana*, AfrLEA2 and AfrLEA3m, are intrinsically disordered in solution but upon desiccation gain secondary structure, as measured by circular dichroism. Trifluoroethanol and sodium dodecyl sulfate are both shown to induce  $\alpha$ -helical structure in AfrLEA2 and AfrLEA3m. Bioinformatic predictions of secondary-structure content for both proteins correspond most closely to conformations measured in the dry state. Because some LEA proteins afford protection to desiccation-sensitive proteins during drying and subsequent rehydration, we tested for this capacity in AfrLEA2 and AfrLEA3m. The protective capacities vary, depending on the target enzyme. For the cytoplasmic enzyme lactate dehydrogenase, neither AfrLEA2 nor AfrLEA3m, with or without trehalose present, was able to afford protection better than that provided by bovine serum albumin (BSA) under the same conditions. However, for another cytoplasmic enzyme, phosphofructokinase, both AfrLEA2 and AfrLEA3m in the presence of trehalose were able to afford protection far greater than that provided by BSA with trehalose. Finally, for the mitochondrial enzyme citrate synthase, 400- $\mu$ g/mL AfrLEA3m without trehalose provided significantly more protection than the same concentration of either AfrLEA2 or BSA.

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## Introduction

Group 3 late embryogenesis abundant (LEA) proteins are a family of proteins accumulated by organisms in relation to water stress (Tunnacliffe and Wise 2007; Tunnacliffe et al. 2010; Hand et al. 2011). Major features of LEA proteins include high hydrophilicity and low sequence complexity (Cumming 1999; Tunnacliffe and Wise 2007; Hand et al. 2011). One well-characterized function attributed to LEA proteins is their ability to protect the activity of desiccation-sensitive enzymes against multiple types of water stress (for recent reviews, see Tunnacliffe and Wise 2007; Tunnacliffe et al. 2010; Hand et al. 2011; Hinch and Thalhammer 2012). Investigations into the secondary structure of LEA proteins reveal that the majority of LEA proteins are predominantly disordered in solution (Wolkers et al. 2001; Goyal et al. 2003; Shih et al. 2004, 2012; Pouchkina-Stantcheva et al. 2007; Tolleter et al. 2007; Thalhammer et al. 2010; Popova et al. 2011; Hundertmark et al. 2012) and gain structure upon desiccation (e.g., Goyal et al. 2003). Gain of well-defined secondary structure by LEA proteins during dehydration has led to the hypothesis that LEA proteins may function specifically in the dry state (e.g., Li and He 2009). Alternatively, there is also evidence that LEA proteins might function as unstructured proteins in the hydrated state. Several studies have shown that LEA proteins are able to reduce the aggregation of polyglutamine (polyQ) or amyloid  $\beta$ -peptide when coexpressed in mammalian cells (Chakrabortee et al. 2007, 2012; Liu et al. 2011). Marunde et al. (2013) showed that a group 1 LEA protein can improve cell viability and mitochondrial function at very modest levels of water stress, which are unlikely to promote substantial coiling of LEA proteins. Regardless of whether LEA proteins function in both hydrated and dry states, structural characterization is an important step in a comprehensive assessment of individual LEA proteins. We investigate the secondary structure of two group 3 LEA proteins present in embryos of *Artemia franciscana*, AfrLEA2 and AfrLEA3m, dried and in solution, as well as their capacity to adopt secondary structure after the addition of sodium dodecyl sulfate (SDS) or trifluoroethanol (TFE). In addition to structural studies, we tested the ability of recombinant AfrLEA2 and AfrLEA3m, both alone and in concert with trehalose, to afford protection to three different target enzymes during desiccation and subsequent rehydration.

It is well documented that group 2 LEA proteins have the ability to protect proteins against freezing (for review, see Tunnacliffe and Wise 2007), and protection during freezing has also been reported for group 3 LEA proteins, although not as

extensively as for group 2 (Honjoh et al. 2000; Goyal et al. 2005). In addition to protection against water stress imposed by freezing, LEA proteins from groups 1–3 can afford protection to enzymes during desiccation (Sanchez-Ballesta et al. 2004; Goyal et al. 2005; Grelet et al. 2005; Reyes et al. 2005). The ability of LEA proteins to protect the activity of desiccation-sensitive enzymes from the deleterious effects of dehydration can, at least partially, be attributed to an ability to prevent enzyme aggregation (Goyal et al. 2005). Goyal et al. (2005) propose that the unordered, flexible structure of LEA proteins allows them to function as a physical barrier between aggregation-prone molecules, a function that they term “molecular shield” activity. Although protection against protein aggregation is imperative if an organism is to survive desiccation, prevention of protein denaturation must also be considered (Tompa and Kovacs 2010). Compared to those of classic chaperones, direct interactions between LEA proteins and target molecules are not as well characterized, but evidence for loose interaction has been reported (Nakayama et al. 2007).

In addition to protective macromolecules such as LEA proteins, anhydrobiotic organisms typically accumulate organic solutes such as trehalose, which aid in macromolecular protection during water stress (Yancey et al. 1982; Yancey 2005). Trehalose constitutes as much as 20% of the dry weight of *A. franciscana* embryos (Crowe et al. 1987). LEA proteins and trehalose in combination are capable of providing a synergistic protection to target molecules (Goyal et al. 2005). It is pertinent to note here that trehalose is not an absolute requirement for desiccation tolerance, because it is not accumulated by bdelloid rotifers (Lapinski and Tunnacliffe 2003; Caprioli et al. 2004) or various tardigrades (Hengherr et al. 2008); however, trehalose undoubtedly plays a role in the organisms in which it is accumulated. The importance of trehalose is exemplified in one organism that accumulates the sugar by the observation that *Aphelenchus avenae* is not able to survive desiccation unless ample time is provided for the conversion of glycogen to trehalose, as occurs during slow drying (Madin and Crowe 1975; Crowe et al. 1977). For AfrLEA2 and AfrLEA3m, our results demonstrate that these proteins are intrinsically disordered in solution, gain structure upon water restriction, and provide protection in a target-specific manner to proteins in the dried state; the effects are either additive or synergistic with the presence of trehalose.

## Methods

### Recombinant LEA Proteins from *Artemia franciscana*

As described in Hand et al. (2007) and Menze et al. (2009), AfrLEA2 (GenBank accession no. ACA47268.1) and AfrLEA3m (ACM16586.1) are designated group 3 LEA proteins on the basis of the classification scheme outlined by Wise (2003). Because the number of LEA proteins reported from animals continues to expand, a reclassification of groups could be warranted in the future. Preparation and purification of recombinant AfrLEA2 and AfrLEA3m were accomplished according to the procedures described in Boswell et al. (2014). Briefly, the orig-

inal nucleic acid sequences were amplified from our existing complementary DNA library from *A. franciscana* and ligated into expression vectors, and then competent bacterial cells were transformed with the genes. AfrLEA2 was expressed with an N-terminal 6X-His tag, and AfrLEA3m was expressed with a C-terminal 6X-His tag so as not to interfere with the mitochondrial localization sequence found at the N-terminus. Bacterial cells were chemically lysed with Bugbuster Protein Extraction Reagent (Novagen, Rockland, MA) in the presence of a protease inhibitor cocktail (P8849; Sigma-Aldrich, St. Louis, MO). Cellular debris was removed by centrifugation, and the resulting supernatant was subjected to affinity chromatography on a HisTrap FF crude column (GE Healthcare, Waukesha, WI) in a binding buffer composed of 20 mM sodium phosphate, 0.5 M NaCl, and 20 mM imidazole, pH 7.5. Fractions containing recombinant protein were heat-treated at 95°C for 20 min and centrifuged (20,000 g, 30 min) to separate the soluble

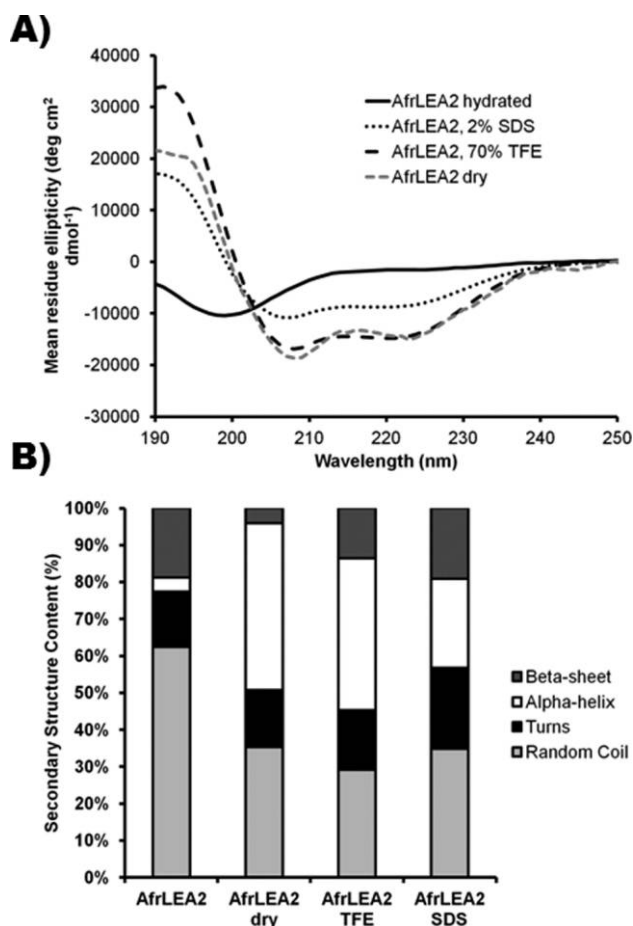


Figure 1. A, Circular dichroism (CD) spectra of recombinant AfrLEA2 in the hydrated state, in the presence of 2% sodium dodecyl sulfate (SDS) or 70% trifluoroethanol (TFE), and after desiccation. B, Structural composition of recombinant AfrLEA2, as calculated from the respective CD data for the hydrated protein, the protein exposed to 2% SDS or 70% TFE, and the dried protein. Algorithms used for the secondary-structure analyses are described in “Methods.”

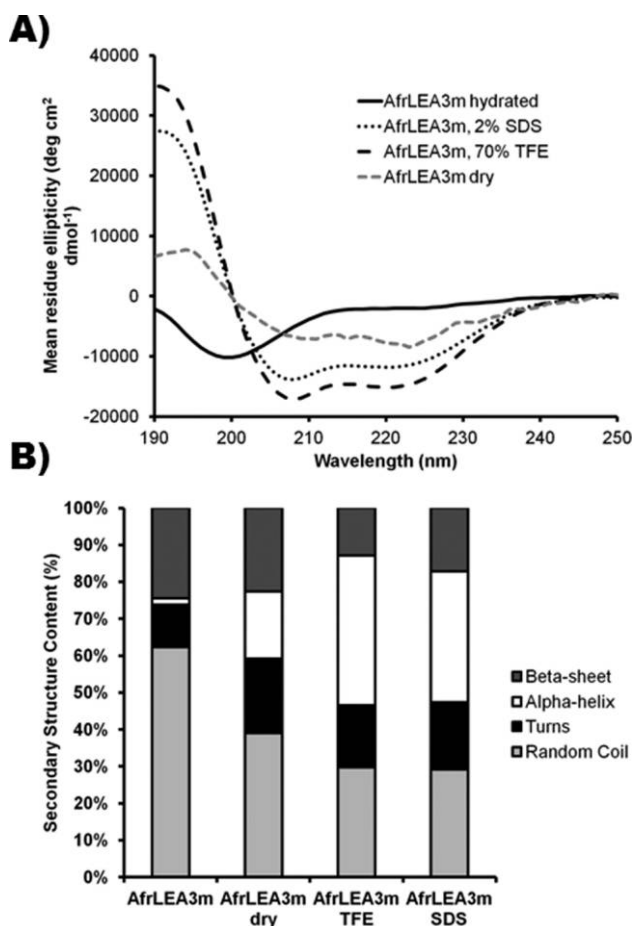


Figure 2. A, Circular dichroism (CD) spectra of recombinant AfrLEA3m in the hydrated state, in the presence of 2% sodium dodecyl sulfate (SDS) or 70% trifluoroethanol (TFE), and after desiccation. B, Structural composition of recombinant AfrLEA3m, as calculated from the respective CD data for the hydrated protein, the protein exposed to 2% SDS or 70% TFE, and the dried protein. Algorithms used for the secondary-structure analyses are described in "Methods."

fraction. The protein samples were then applied to an anion exchange column (HiTrap Q FF; GE Healthcare) in a starting buffer composed of 20 mM triethanolamine and 10 mM NaCl, pH 7.0. After elution, the fractions containing pure recombinant protein were exchanged into LEA storage buffer (20 mM HEPES, 50 mM NaCl, pH 7.5) and concentrated using Amicon Ultra Centrifugal filters (Ultracel-10K; Millipore, Billerica, MA).

Recombinant AfrLEA2 has a total molecular mass of 43.1 kDa (38.9 kDa plus 4.2 kDa for a 6X-His tag and associated vector sequence). AfrLEA3m is a mitochondrial LEA protein with a deduced molecular mass of 34.1 kDa, which includes the 3.2-kDa mitochondrial targeting sequence.

#### Far-Ultraviolet Circular Dichroism Spectroscopy

Circular dichroism (CD) spectra were recorded on a Jasco J-815 spectropolarimeter (Jasco Analytical Instruments, Easton,

MD). The path length was 0.1 cm, and measurements were taken for wavelengths from 190 to 250 nm. Spectra were measured in a 10 mM potassium phosphate buffer at protein concentrations of 0.14 mg/mL for recombinant AfrLEA2, 0.16 mg/mL for recombinant AfrLEA3m, and 0.16 mg/mL for bovine serum albumin (BSA; Sigma Aldrich, product code A6003). The buffer (blank) spectrum was subtracted from each sample spectrum. After blank subtraction, each spectrum was converted to mean residue ellipticity and smoothed via the Savitzky-Golay method (Savitzky and Golay 1964), with a convolution width of 9. Buffer subtraction, conversion to mean residue ellipticity, and smoothing were performed with the Spectra Manager software (Jasco Analytical Instruments). For measurements of dried proteins, 50  $\mu$ L of protein solution (at the respective concentrations above) were dried on one side of a demountable cuvette overnight in a dry box containing the desiccant Drierite (W. A. Hammond Drierite, Xenia, OH). A

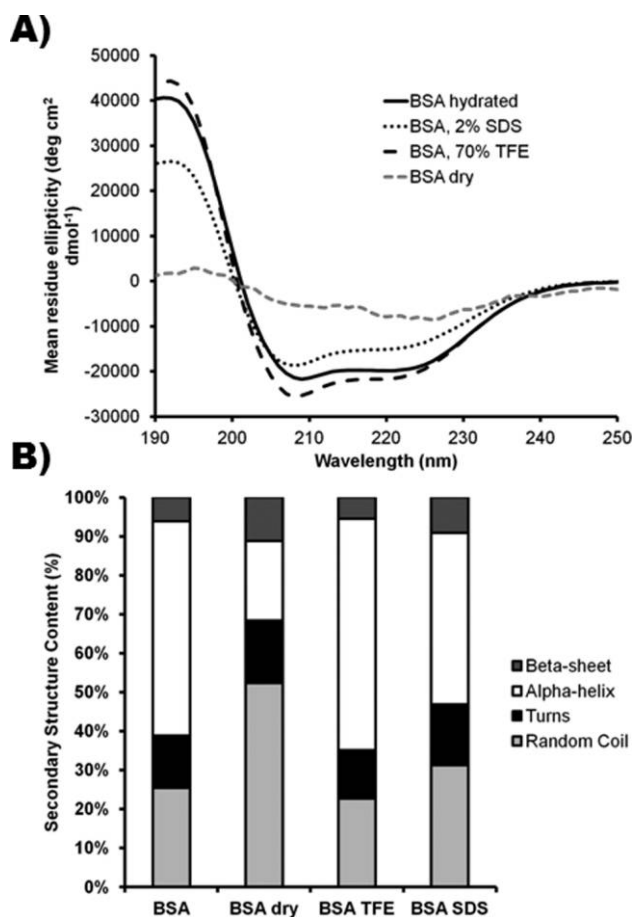


Figure 3. A, Circular dichroism (CD) analysis of bovine serum albumin (BSA) in the hydrated state, in the presence of 2% sodium dodecyl sulfate (SDS) or 70% trifluoroethanol (TFE), and after desiccation. B, Structural composition of BSA as calculated from the respective CD data for the hydrated protein, the protein exposed to 2% SDS or 70% TFE, and the dried protein. Algorithms used for the secondary-structure analyses are described in "Methods."



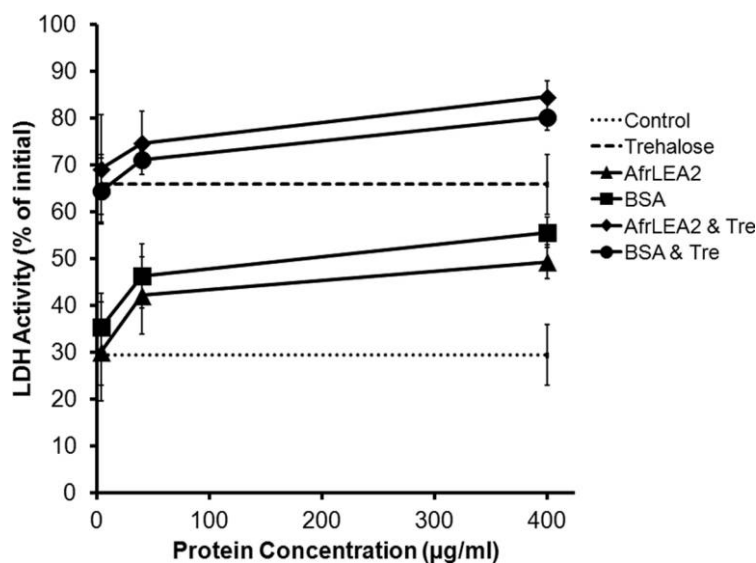


Figure 4. Residual lactate dehydrogenase (LDH) activity after desiccation for 1 wk without additives (control) or in the presence of different protectants. Late embryogenesis abundant (LEA) protein concentrations were 4, 40, and 400  $\mu\text{g}/\text{mL}$ . The protective ability of AfrLEA2 both with and without 100 mM trehalose (Tre) is compared to those of equivalent bovine serum albumin (BSA) solutions and 100 mM trehalose alone. Values are reported as percent of the initial LDH activity measured before desiccation (mean  $\pm$  SD,  $n = 9$ ). A significant level of protection was provided by 100 mM trehalose alone, compared to control (one-way ANOVA plus Tukey,  $P < 0.05$ ). The protection provided by 400- $\mu\text{g}/\text{mL}$  BSA was slightly higher than that provided by 400- $\mu\text{g}/\text{mL}$  AfrLEA2, but both provided significantly less protection than did trehalose alone (one-way ANOVA plus Tukey,  $P < 0.05$ ). Both 400- $\mu\text{g}/\text{mL}$  AfrLEA2 plus trehalose and 400- $\mu\text{g}/\text{mL}$  BSA plus trehalose provided a significantly higher level of protection than did trehalose alone (one-way ANOVA plus Tukey,  $P < 0.05$ ). Symbols to indicate significance have been omitted from the graph for clarity.

path length of 0.01 cm was used for conversion of dry data to mean residue ellipticity.

Secondary-structure analyses were performed with the DICHROWEB Web server (Whitmore and Wallace 2004, 2008), using the algorithms CONTINLL (Provencher and Glockner 1981; van Stokkum et al. 1990) and SELCON3 (Sreerama et al. 1999; Sreerama and Woody 2000). Reference data set 7 was used for all analyses because this data set is optimized for 190–240-nm wavelengths and contains denatured proteins (Sreerama and Woody 2000).

#### Desiccation and Activity Assays of Target Enzymes

Lactate dehydrogenase (LDH) from rabbit muscle was obtained from Sigma Aldrich (product code L2500). Before use, LDH was exchanged into LEA storage buffer using Amicon Ultra Centrifugal filters (Ultracel-10K; Millipore). Then, 10- $\mu\text{L}$  droplets of 50- $\mu\text{g}/\text{mL}$  LDH, with or without protectants, were dried at room temperature for 1 wk in 1.5-mL microcentrifuge tubes in a dry box containing Drierite. Samples were rehydrated on ice for 1 h with 20  $\mu\text{L}$  of LEA storage buffer (diluted twofold). Control assays of LDH activity were performed before desiccation by adding 5  $\mu\text{L}$  of LDH sample (50  $\mu\text{g}/\text{mL}$ ) to a final reaction volume of 1.0 mL, which contained 0.2 M Tris-HCl buffer (pH 7.3), 660  $\mu\text{M}$  NADH, and 3 mM sodium pyruvate. LDH activity after desiccation was measured as described for controls, except that 10  $\mu\text{L}$  of LDH sample was added to ac-

count for the twofold dilution of the enzyme during rehydration. Change in absorbance at 340 nm ( $A_{340}$ ) was recorded for 1.5 min, and LDH activity was reported as a percentage of the rate measured for nondried controls. Each sample was compared to control values that contained the same mixture of protectants in order to account for the increase in LDH activity observed in the presence of higher concentrations of protectant protein. It is appropriate to note that the use of LDH in droplets at up to 20  $\mu\text{g}/\text{mL}$  yields artifactual results because of non-specific binding of LDH protein to vial surfaces and time-dependent inactivation that can readily be detected with control samples. Reported values are the average of three separate drying trials, each with three nested replicates ( $n = 9$ ).

The phosphofructokinase (PFK) used in this study was a purified recombinant form of the rabbit muscle enzyme and was a generous gift from Dr. Simon Chang. Before use, PFK was exchanged into a 100 mM sodium phosphate buffer (pH 8.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM dithiothreitol (DTT) using Amicon Ultra Centrifugal filters. Then 10- $\mu\text{L}$  droplets of 150- $\mu\text{g}/\text{mL}$  PFK, with or without protectants, were dried at room temperature for 24 h in 1.5-mL microcentrifuge tubes in a dry box containing Drierite. Samples were then rehydrated on ice for 1 h with 20  $\mu\text{L}$  of 50 mM sodium phosphate buffer (pH 8.0) containing 0.5 mM EDTA and 2.5 mM DTT. Following Carpenter and Crowe (1988), who reported residual activity of air-dried PFK in phosphate buffer as a function of water content (their fig. 5), we

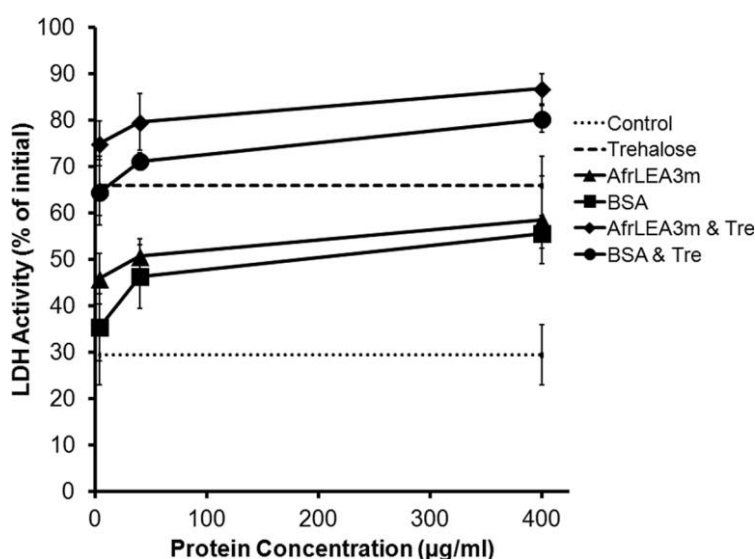


Figure 5. Residual lactate dehydrogenase (LDH) activity after desiccation for 1 wk without additives (control) or in the presence of different protectants. Late embryogenesis abundant (LEA) protein concentrations were 4, 40, and 400  $\mu\text{g}/\text{mL}$ . The protective ability of AfrLEA3m both with and without 100 mM trehalose (Tre) is compared to those of equivalent bovine serum albumin (BSA) solutions and 100 mM trehalose alone. Values are reported as percent of the initial LDH activity measured before desiccation (mean  $\pm$  SD,  $n = 9$ ). A significant level of protection was provided by 100 mM trehalose alone, compared to control (one-way ANOVA plus Tukey,  $P < 0.05$ ). Trehalose provided significantly higher protection than did 400- $\mu\text{g}/\text{mL}$  BSA. Both 400- $\mu\text{g}/\text{mL}$  AfrLEA3m plus trehalose and 400- $\mu\text{g}/\text{mL}$  BSA plus trehalose provided a significantly higher level of protection than did trehalose alone (one-way ANOVA plus Tukey,  $P < 0.05$ ). Symbols to indicate significance have been omitted from the graph for clarity.

estimate the water content of our dried PFK samples to be approximately 3%–5% of the initial.

Activity was assayed essentially as described by Bock and Frieden (1974). Briefly, reactions (1-mL assay volume) were performed at 25°C in a 42 mM Tris-acetate buffer (pH 8.0) containing 51 mM KCl and 5.1 mM  $\text{NH}_4\text{Cl}$ , with final concentrations of 0.16 mM NADH, 2 mM ATP, 2 mM fructose-6-phosphate, 2 mM magnesium acetate, 2.5 units of glycerol-3-phosphate dehydrogenase (Sigma Aldrich, product code G6715), 25 units of triosephosphate isomerase (Sigma Aldrich, product code T2391), and 2 units of aldolase (Sigma Aldrich, product code A8811). Before use, all accessory enzymes were exchanged into a 100 mM Tris-acetate buffer (pH 8.0, at 6°C) with 0.1 mM EDTA. PFK activity was measured for control samples before desiccation by adding 5  $\mu\text{L}$  of PFK sample (150  $\mu\text{g}/\text{mL}$ ) to a total reaction volume of 1 mL of the reaction mixture described above; for dried samples, 10  $\mu\text{L}$  of PFK was added to account for the twofold dilution of the enzyme during rehydration. Change in  $A_{340}$  was recorded for 2 min, and PFK activity was reported as a percentage of the rate measured for nondried controls. Each sample was compared to control values that contained the same mixture of protectants in order to account for the increase in PFK activity observed in the presence of higher concentrations of protectant protein. Reported values are the average of three separate drying trials, each with three nested replicates ( $n = 9$ ).

Citrate synthase (CS) from porcine heart was obtained from Sigma Aldrich (product code C3260). Before use, CS was ex-

changed into 1 M Tris-HCl (pH 8.1) using Amicon Ultra Centrifugal filters. Then, 10- $\mu\text{L}$  droplets of 50- $\mu\text{g}/\text{mL}$  CS, with or without protectants, were dried at room temperature for 24 h in a dry box containing Drierite. After one round of drying, each sample was resuspended with 10  $\mu\text{L}$   $\text{H}_2\text{O}$  and dried a second time for 24 h. “Double-dried” samples were rehydrated on ice for 1 h with 20  $\mu\text{L}$  of 0.5 M Tris-HCl (pH 8.1). Control CS activity assays were performed before desiccation by adding 5  $\mu\text{L}$  of CS sample (50  $\mu\text{g}/\text{mL}$ ) to a final reaction volume of 1 mL, which contained 0.1 M Tris-HCl buffer (pH 8.1), 0.1 mM 5,5'-dithiobis-(2-nitrobenzoic acid), and 0.2 mM acetyl-CoA. The reaction was initiated by the addition of 0.5 mM oxaloacetate. CS activity after desiccation was measured as described for controls, except that 10  $\mu\text{L}$  of CS sample was added to a final reaction volume of 1 mL in order to account for the twofold dilution of the enzyme during rehydration. Change in  $A_{340}$  was recorded for 2.0 min, and CS activity was reported as a percentage of the rate measured for nondried controls. Each sample was compared to control values that contained the same mixture of protectants in order to account for the increase in CS activity observed in the presence of higher concentrations of protectant protein. Reported values are the average of three separate drying trials, each with three nested replicates ( $n = 9$ ).

#### Statistical Analysis

A one-way ANOVA, paired with a Tukey post hoc test, was used in order to determine significance between treatment

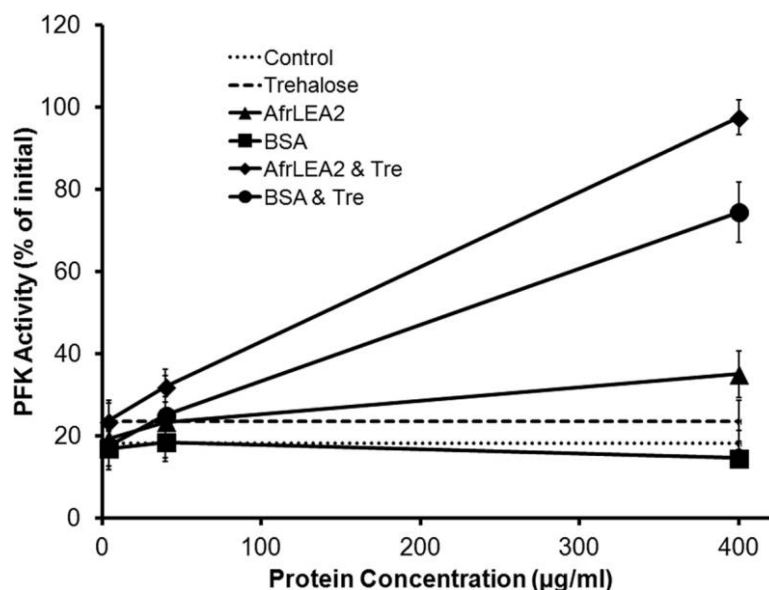


Figure 6. Residual phosphofructokinase (PFK) activity after desiccation for 24 h without additives (control) or in the presence of different protectants. Late embryogenesis abundant (LEA) protein concentrations were 4, 40, and 400  $\mu\text{g}/\text{mL}$ . The protective ability of AfrLEA2 both with and without 100 mM trehalose (Tre) is compared to those of equivalent bovine serum albumin (BSA) solutions and 100 mM trehalose alone. Values are reported as percent of the initial PFK activity measured before desiccation (mean  $\pm$  SD,  $n = 9$ ). A significant level of protection was provided by 400- $\mu\text{g}/\text{mL}$  AfrLEA2, compared to control, and 400- $\mu\text{g}/\text{mL}$  BSA plus trehalose preserved more activity than did AfrLEA2 alone (one-way ANOVA plus Tukey,  $P < 0.05$ ). Of all treatments tested, 400- $\mu\text{g}/\text{mL}$  AfrLEA2 plus trehalose preserved the highest residual PFK activity (one-way ANOVA plus Tukey,  $P < 0.05$ ). Symbols to indicate significance have been omitted from the graph for clarity.

groups for all enzyme drying studies (Prism; GraphPad Software, La Jolla, CA). Interaction between LEA proteins and trehalose was determined with a two-way ANOVA (SigmaPlot; Systat Software, San Jose, CA).

## Results

### Secondary Structure of LEA Proteins

In solution, the CD spectrum of AfrLEA2 exhibits features typical of a predominantly disordered, random-coiled protein with a minimal ellipticity at around 200 nm (fig. 1A). The presence of either 70% TFE or 2% SDS promotes AfrLEA2 to adopt substantial  $\alpha$ -helical structure, as indicated by spectra containing a double minimum near 208 and 222 nm and a strong positive band at 191 nm (fig. 1A). Secondary-structure estimates confirm the apparent gain of  $\alpha$ -helix content by AfrLEA2 in the presence of 2% SDS and 70% TFE, with an increase from 4%  $\alpha$ -helix content in solution to 24% and 41%, respectively (fig. 1B). AfrLEA2 in the dry state also exhibits spectral properties that are characteristic of  $\alpha$ -helix, and the spectrum is quite similar to that induced by the presence of TFE (fig. 1A). Desiccation of AfrLEA2 causes an increase in  $\alpha$ -helix content from 4% in solution to 46% dry, as determined from the CD spectra (fig. 1B).

Similar to what was observed for AfrLEA2, AfrLEA3m was found to be predominantly disordered in solution, with a propensity to adopt a more  $\alpha$ -helical structure in 2% SDS or 70% TFE solutions (fig. 2A);  $\alpha$ -helix content increases from 2% for

the hydrated protein to 41% and 36%, respectively (fig. 2B). Drying of AfrLEA3m does not seem to induce a structural shift in the CD spectra as large as that seen for AfrLEA2 (fig. 2A), but the secondary-structure estimation indicates that  $\alpha$ -helix content does increase notably, from 2% in solution to 18% dry (fig. 2B). AfrLEA3m possesses a greater percentage of  $\beta$ -sheet in the dry state than does AfrLEA2, which could explain the lower  $\alpha$ -helix content of AfrLEA3m. Finally, it is appropriate to note that both recombinant proteins contain approximately 10% sequence that is atypical of mature LEA protein. AfrLEA2 contains a 4.2-kDa segment that represents the 6X-His tag and associated vector sequence, while the AfrLEA3m protein includes a hydrophobic targeting sequence of 3.2 kDa.

CD spectroscopy measurements were also gathered for the globular protein BSA. BSA spectra served two purposes: first, as a control protein to check the accuracy of both CD measurements and deconvolution software and second, as a control protein against which we could compare the structural changes of AfrLEA2 and AfrLEA3m. The spectrum of BSA in solution was that of a predominantly  $\alpha$ -helical protein (fig. 3A). Takeda et al. (1987) estimated the secondary structure of BSA to contain 66%  $\alpha$ -helix, 3%  $\beta$ -sheet, and 31% random coil. Secondary-structure estimates from our CD data are similar: 56%  $\alpha$ -helix, 6%  $\beta$ -sheet, and 26% random coil. The presence of either 2% SDS or 70% TFE did not cause a substantial shift in the BSA spectrum (fig. 3A). However, drying did shift the CD spectra of BSA (fig. 3A), but toward a pattern that indicates a greater percentage of random coil (26% in solution vs. 52%



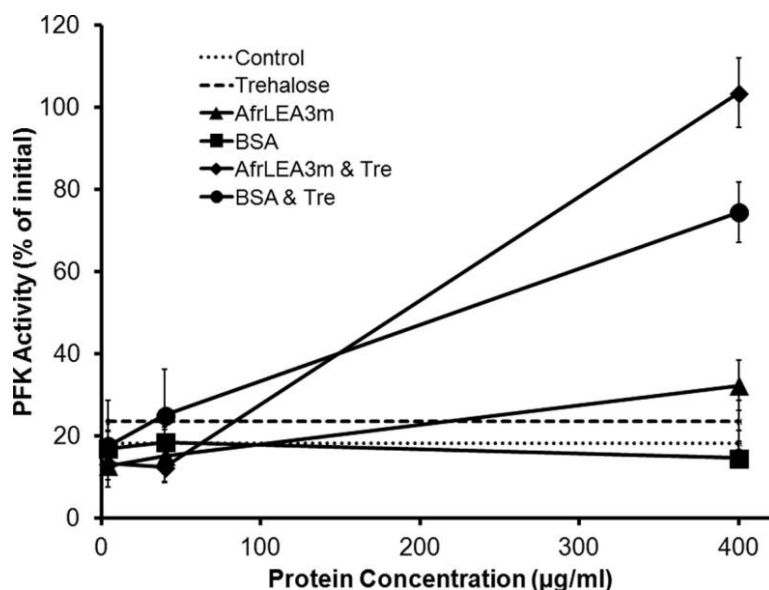


Figure 7. Residual phosphofructokinase (PFK) activity after desiccation for 24 h without additives (control) or in the presence of different protectants. Late embryogenesis abundant (LEA) protein concentrations were 4, 40, and 400  $\mu\text{g}/\text{mL}$ . The protective ability of AfrLEA3m both with and without 100 mM trehalose (Tre) is compared to those of equivalent bovine serum albumin (BSA) solutions and 100 mM trehalose alone. Values are reported as percent of the initial PFK activity measured before desiccation (mean  $\pm$  SD,  $n = 9$ ). A significant level of protection was afforded by 400- $\mu\text{g}/\text{mL}$  BSA plus trehalose, compared to control, and 400- $\mu\text{g}/\text{mL}$  AfrLEA3m plus trehalose provided a significantly higher level of protection than did any other treatment (one-way ANOVA plus Tukey,  $P < 0.05$ ). Symbols to indicate significance have been omitted from the graph for clarity.

when dry; fig. 3B). This spectrum change is consistent with denaturation of the globular protein in the dried state.

#### Protection of Enzyme Activity by LEA Proteins during Desiccation

Drying studies with combinations of target enzyme and LEA protein were performed to test the protective abilities of the two recombinant AfrLEA proteins against dehydration-induced damage. To evaluate whether a cytoplasmic LEA protein preferentially protects cytoplasmic enzymes while a mitochondrial LEA protein preferentially protects mitochondria-localized enzymes, we used a mix-and-match design by choosing target enzymes that reside in each of these compartments.

After desiccation and storage in the dry state for 1 wk, LDH, when rehydrated, exhibits a residual activity of  $31\% \pm 8\%$  (mean  $\pm$  SD), compared to nondried control activity (figs. 4, 5). The residual activity of LDH increases to  $66\% \pm 6\%$  (mean  $\pm$  SD) when dried in the presence of 100 mM trehalose. The protection afforded LDH by the two LEA proteins is significant and similar to but not statistically different from the protection seen with BSA (one-way-ANOVA plus Tukey,  $P > 0.05$ ), both in the presence and in the absence of 100 mM trehalose (figs. 4, 5).

PFK is considered one of the most dehydration-sensitive enzymes known (Crowe et al. 1987). It is completely and irreversibly inactivated during freeze-drying (Carpenter et al. 1987; Carpenter and Crowe 1989) and during air-drying to no

more than 3% initial sample water (superfused with  $\text{CaSO}_4$ -dried nitrogen at  $33^\circ\text{--}35^\circ\text{C}$ ; Carpenter and Crowe 1988), perhaps because of the formation of inactive dimers (Crowe et al. 1992). After drying at room temperature for 24 h, PFK displays a residual activity of  $18\% \pm 3\%$  (mean  $\pm$  SD), which is increased only to  $24\% \pm 5\%$  in the presence of 100 mM trehalose (figs. 6, 7). AfrLEA2 and AfrLEA3m again perform similarly, but both LEA proteins protect PFK far better than does BSA with or without trehalose. In fact, BSA alone does not afford any protection to PFK, while a remarkable  $98\% \pm 4\%$  of control (nondried) activity is preserved when the enzyme is dried in the presence of 400- $\mu\text{g}/\text{mL}$  AfrLEA2 plus 100 mM trehalose (fig. 6), and  $103\% \pm 8\%$  of control activity is preserved when PFK is dried in the presence of 400- $\mu\text{g}/\text{mL}$  AfrLEA3m plus 100 mM trehalose (fig. 7). To our knowledge, this is the first time the protective ability of LEA proteins has been tested with a target protein possessing such high sensitivity to desiccation.

Finally, we tested the mitochondrial enzyme CS, which was double-dried (see "Desiccation and Activity Assays of Target Enzymes") before each assay for residual activity. CS retains  $20\% \pm 3\%$  of control activity after the final rehydration (figs. 8, 9). Trehalose provides a high level of protection to CS, as evidenced by a residual activity of  $76\% \pm 5\%$ . As seen with the target enzyme LDH, the protective abilities of the two LEA proteins plus 100 mM trehalose are similar to but are not statistically different from the protection observed with BSA plus trehalose (one-way ANOVA plus Tukey,  $P > 0.05$ ). However, the presence

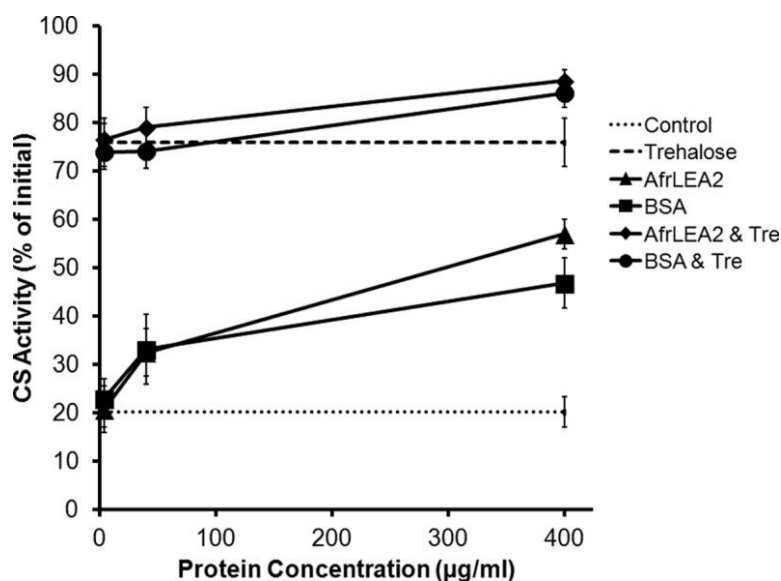


Figure 8. Residual citrate synthase (CS) activity after “double drying” without additives (control) or in the presence of different protectants. Late embryogenesis abundant (LEA) protein concentrations were 4, 40, and 400  $\mu\text{g}/\text{mL}$ . The protective ability of AfrLEA2 both with and without 100 mM trehalose (Tre) is compared to those of equivalent bovine serum albumin (BSA) solutions and 100 mM trehalose alone. Values are reported as percent of the initial CS activity measured before desiccation (mean  $\pm$  SD,  $n = 9$ ). A significant level of protection was provided by 100 mM trehalose alone, compared to 400- $\mu\text{g}/\text{mL}$  AfrLEA2, which in turn provided a higher level of protection than did 400- $\mu\text{g}/\text{mL}$  BSA (one-way ANOVA plus Tukey,  $P < 0.05$ ). The protection provided by both 400- $\mu\text{g}/\text{mL}$  AfrLEA2 plus trehalose and 400- $\mu\text{g}/\text{mL}$  BSA plus trehalose was significantly higher than that provided by trehalose alone (one-way ANOVA plus Tukey,  $P < 0.05$ ). Symbols to indicate significance have been omitted from the graph for clarity.

of 400- $\mu\text{g}/\text{mL}$  AfrLEA3m alone protects  $69\% \pm 4\%$  of the control CS activity (fig. 9), which is significantly more protection than is provided by the same concentration of either AfrLEA2 or BSA (one-way ANOVA plus Tukey,  $P < 0.05$ ; fig. 8).

## Discussion

Despite the high content of  $\alpha$ -helix predicted by bioinformatics software (Hand et al. 2007; Menze et al. 2009), both recombinant AfrLEA2 and AfrLEA3m are predominantly disordered in solution. This intrinsic disorder in the hydrated state is a common theme for LEA proteins and is attributed to their highly hydrophilic nature (Tunnacliffe and Wise 2007). Both AfrLEA2 and AfrLEA3m adopt an increased percentage of  $\alpha$ -helical structure in the presence of SDS and TFE and also are found to gain structure in the dried state. The ability of LEA proteins to gain structure after drying was first documented by Wolkers et al. (2001). We also show that both AfrLEA2 and AfrLEA3m protect sensitive enzymes from the damage imposed by desiccation, thereby preserving enzyme activity that is typically lost after drying.

The ability of AfrLEA2 and AfrLEA3m to gain structure during drying is in agreement with recent studies on LEA proteins (for review, see Tunnacliffe and Wise 2007; Tunnacliffe et al. 2010; Hand et al. 2011; Hinch and Thalhammer 2012). A predominant lack of secondary structure in solution places LEA proteins within a large class of proteins most commonly called intrinsically disordered proteins, or IDPs (for review, see Uver-

sky and Dunker 2010). Buffer solutions containing 70% TFE were used in order to probe the conformational propensities of recombinant AfrLEA2 and AfrLEA3m. Both recombinant LEA proteins were found to gain  $\alpha$ -helical structure in the presence of 70% TFE, while the secondary structure of the control protein BSA remained effectively unchanged. Although the exact mechanism through which this desolvating agent promotes secondary structure is debated,  $\alpha$ -helix induction is thought to be due largely to desolvation of the polypeptide backbone (Kentsis and Sosnick 1998). Therefore, it can be argued that the promotion of  $\alpha$ -helical structure in AfrLEA2 and AfrLEA3m by TFE is pertinent to the secondary structure gained during desiccation. SDS was also found to induce  $\alpha$ -helical structure in both AfrLEA2 and AfrLEA3m, although not as effectively as TFE. The induction of  $\alpha$ -helical structure in LEA proteins by SDS has been previously documented (Ismail et al. 1999; Shih et al. 2004, 2012; Tolleter et al. 2007).

The spectra for AfrLEA2 and AfrLEA3m in the dried state indicate that both proteins gain structure during desiccation. AfrLEA2 adopts substantial  $\alpha$ -helical structure, while AfrLEA3m gains both  $\alpha$ -helix and turns. In contrast, BSA becomes more disordered during drying. This observed loss of structure suggests denaturation, which would not be unusual for a globular protein such as BSA. Of the three conditions evaluated by CD in the present study (TFE, SDS, and dried), the  $\alpha$ -helix content of 59% predicted for AfrLEA2 by bioinformatics software best matches the content observed for the

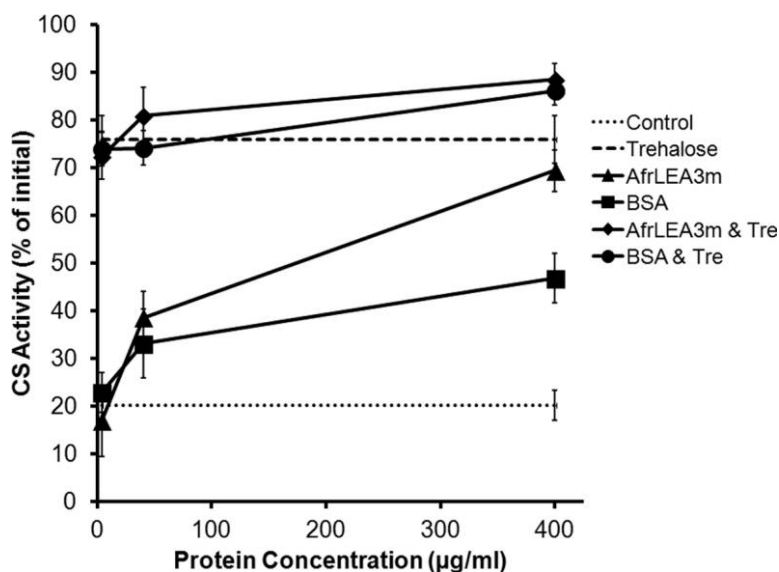


Figure 9. Residual citrate synthase (CS) activity after “double drying” without additives (control) or in the presence of different protectants. Late embryogenesis abundant (LEA) protein concentrations were 4, 40, and 400  $\mu\text{g}/\text{mL}$ . The protective ability of AfrLEA3m both with and without 100 mM trehalose (Tre) is compared to those of equivalent bovine serum albumin (BSA) solutions and 100 mM trehalose alone. Values are reported as percent of the initial CS activity measured before desiccation (mean  $\pm$  SD,  $n = 9$ ). A significant level of protection was provided by 100 mM trehalose alone, compared to 400- $\mu\text{g}/\text{mL}$  AfrLEA3m, which in turn provided a higher level of protection than did 400- $\mu\text{g}/\text{mL}$  BSA (one-way ANOVA plus Tukey,  $P < 0.05$ ). The protection provided by both 400- $\mu\text{g}/\text{mL}$  AfrLEA3m plus trehalose and 400- $\mu\text{g}/\text{mL}$  BSA plus trehalose was significantly higher than that provided by trehalose alone (one-way ANOVA plus Tukey,  $P < 0.05$ ). Symbols to indicate significance have been omitted from the graph for clarity.

dried state (45.6%). This outcome is consistent with the general case that predictive algorithms, when applied to LEA protein, often predict the structure of the dried molecule (Tolletier et al. 2007; Thalhhammer et al. 2010; Popova et al. 2011). In contrast to AfrLEA2, AfrLEA3m is predicted to contain 73%  $\alpha$ -helix but, on the basis of CD spectroscopy, adopts only 18%  $\alpha$ -helix when desiccated. Secondary structure of a group 3 LEA protein from pollen was shown to be dependent on both the speed of drying and the presence of sucrose (Wolkers et al. 2001). Rapid drying, as opposed to the slow drying performed for AfrLEA2 and AfrLEA3m, promoted a higher proportion of  $\alpha$ -helical structure (Wolkers et al. 2001). Changes in ionic composition can also affect secondary structure of LEA proteins (Furuki et al. 2011). How such factors might alter the final dried structures of AfrLEA2 and AfrLEA3m should be tested in the future.

As discussed above, gain of structure upon desiccation has led to the prediction that LEA proteins may function preferentially in the dry state. However, the ability of LEA proteins to prevent protein aggregation also has been shown in solution (e.g., Chakrabortee et al. 2007). Thus, it appears more likely that LEA proteins are functional across a broad range of hydration states, although the nature of the protection conferred could differ. For example, an individual LEA protein could function as a molecular shield in solution, and the same LEA protein could gain structure as water is removed to further protect the cell in the dry state by interacting with membranes, stabilizing sugar glasses, and forming filamentous networks (for

extended reviews, see Tunnacliffe and Wise 2007; Hand et al. 2011).

We have shown that the capacity of AfrLEA2 and AfrLEA3m to protect desiccation-sensitive target enzymes from damage during drying depends on the target protein tested. For LDH, neither AfrLEA2 nor AfrLEA3m was able to afford protection better than that provided by BSA, which is in apparent contrast with reports for certain LEA proteins in the literature (Goyal et al. 2005). However, closer inspection reveals that AfrLEA2 and AfrLEA3m did afford a high degree of protection to LDH, similar to that reported by Goyal et al. (2005), but the difference is that we found BSA to stabilize LDH far more than previously reported. Small differences in the final water content of dried samples could explain this inconsistency. Reyes et al. (2005) reported that, in the presence of BSA, LDH exhibited 75% residual activity after being dried to 2% water content but activity dropped below 40% at a water content of less than 2%. Another aspect that has differed substantially among studies is the concentration of LDH in the test mixture. In our study, LDH was dried at an initial concentration of 50  $\mu\text{g}/\text{mL}$  because preliminary observations showed that at lower concentrations the enzyme lost activity in a time-dependent fashion when simply stored on ice for 1 h during rehydration (data not shown). In comparison, multiple groups have dried or frozen LDH at concentrations lower than 10  $\mu\text{g}/\text{mL}$  (Miller et al. 1998; Honjoh et al. 2000; Sanchez-Ballesta et al. 2004; Goyal et al. 2005; Reyes et al. 2005; Nakayama et al. 2007). The use of such

low concentrations of LDH could result in nonspecific adsorption of LDH to vial surfaces.

In our study, AfrLEA2 and AfrLEA3m, when combined with trehalose, are able to protect nearly 100% of control PFK activity when rehydrated 24 h after desiccation, and this protection is far greater than that seen with BSA plus trehalose. Furthermore, the combined effect of LEA protein and trehalose is synergistic, compared to that of either of the agents alone; significant interaction between LEA protein and trehalose was confirmed (two-way ANOVA,  $P < 0.001$ ). The stabilization of PFK with trehalose alone is virtually identical to that reported by Carpenter et al. (1987) under their “slow-drying” regime, which is very similar to the drying conditions we used here. Results for the two cytosolic enzymes tested (LDH and PFK) do not indicate that a cytosolic LEA protein is able to provide better protection to cytosolic enzymes than is a mitochondrial LEA protein.

Compared to PFK, CS shows significant resistance to desiccation damage and therefore was “double-dried” in order to cause more perturbation to the enzyme. This double-drying technique has been utilized previously with CS (e.g., see Pouchkina-Stantcheva et al. 2007). The protective abilities of the two LEA proteins when combined with trehalose are similar to each other and also to that of BSA plus trehalose. However, AfrLEA3m alone, at the highest concentration tested, provided significantly more protection to CS than did AfrLEA2 or BSA. A somewhat similar situation of preferential protection has been reported for the interaction of a LEA protein with lipid membranes, where a mitochondria-targeted LEA protein preferentially protected liposomes with a lipid composition that mimicked the endogenous composition of the inner mitochondrial membrane, as compared to liposomes with a more generic composition (Tolleter et al. 2010).

In conclusion, AfrLEA2 and AfrLEA3m are intrinsically disordered proteins that gain secondary-structural motifs upon desiccation. In addition, both proteins are able to protect desiccation-sensitive enzymes from the deleterious effects of desiccation and subsequent rehydration. These findings serve not only to further define the molecular characteristics and possible functions of AfrLEA2 and AfrLEA3m but also to add to the pool of evidence that supports a role for LEA proteins in desiccation tolerance.

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