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Biostabilization of Lipid Bilayers: Dealing with Water Stress in Embryos of Artemia franciscana

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BIOSTABILIZATION OF LIPID BILAYERS: DEALING WITH WATER STRESS IN EMBRYOS OF *ARTEMIA FRANCISCANA*

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in The Department of Biological Sciences

by

Daniel Moore
B.S., Louisiana College, 2006
December 2015
For Erin, Taylor, and Ashton
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ABSTRACT

The aim of this dissertation is to investigate stabilization of lipid bilayers during water stress by protectants found in embryos of *Artemia franciscana*. Two LEA proteins were used: AfrLEA2 and AfrLEA3m. AfrLEA3m was experimentally demonstrated to reside in the matrix. Two detergents were used to differentially solubilize the outer and inner membranes of mitochondria isolated from *A. franciscana*. Release of AfrLEA3m occurred simultaneously with the release of fumarase, a matrix-resident marker. As a second independent method to corroborate the above findings, I demonstrated that recombinant AfrLEA3m can be imported into mitochondria isolated from rat liver. Molecular modeling of AfrLEA2 and AfrLEA3m revealed structural features that are consistent with amphipathic proteins able to interact with and stabilize cell membranes. The abilities of trehalose and LEA proteins to protect liposomes of various compositions from desiccation-induced damage were evaluated by carboxyfluorescein leakage. AfrLEA2 (cytoplasmic) and AfrLEA3m (mitochondrial) were able to offset damage during drying of liposomes that mimicked the lipid compositions of the inner mitochondrial membrane, outer mitochondrial membrane, and the inner leaflet of the plasma membrane. LEA proteins were more effective than trehalose at preventing desiccation-induced damage when these protectants were confined to one side of the lipid bilayer. When LEA proteins were used in conjunction with trehalose, additive protection was measured in some cases. Little to no additional damage occurred to liposomes dried for one week compared to liposomes dried overnight. The capacity of trehalose and LEA proteins to protect liposomes from freeze-thaw damage was also assessed. Damage to liposomes was less severe after freezing than desiccation. Trehalose provided liposomes with greater protection than LEA proteins from freeze-thaw damage. The greatest stabilization during freezing occurred when trehalose was present on both
sides of liposome membranes. Only liposomes mimicking the outer mitochondrial membrane were significantly protected from freeze-thaw damage by LEA proteins. Based on bioenergetic properties assessed by respirometry, the outer membrane of isolated mitochondria (rat liver) remained intact after freezing in 300 mM trehalose solution. Respiratory control ratios were depressed by approximately 30% compared to non-frozen mitochondria, which indicated a limited retention of at least some inner-membrane-dependent properties.
A variety of small animals can tolerate extreme dehydration, where the percent tissue water can be reduced to 2% and even lower (Clegg, 1978; Crowe et al., 1992). The ability to survive the virtual absence of water is termed ‘anhydrobiosis’ (Crowe et al., 1997; Crowe et al., 1992). *Artemia franciscana* is one species of brine shrimp whose embryos are often used as models for desiccation tolerance in animals. Under favorable conditions, embryos of brine shrimp will develop to free-swimming nauplii prior to release from the mother (Clegg and Conte, 1980). However, when conditions are not favorable (i.e. shortened photoperiod, cold temperatures, limited food availability, etc.), embryos enter a state of developmental and metabolic arrest known as diapause and are released from the mother inside of cysts. Diapausing embryos of *Artemia franciscana* are able to survive extreme dehydration as well as a number of other environmental insults (Clegg, 2005). When conditions are favorable, embryonic development resumes and desiccation tolerance is lost upon reaching the emergence and larval stages.

During water stress, a profound metabolic downregulation occurs in desiccation-tolerant animals (Clegg et al., 1996; Hand and Hardewig, 1996; Hand et al., 2011a; Hochachka and Guppy, 1987). Water loss in anhydrobiotic organisms is also correlated with the accumulation of intracellular molecules that can stabilize biological structures -- organic solutes (Yancey, 2005; Yancey et al., 1982) and/or proteins, such as small heat shock proteins and late embryogenesis abundant (LEA) proteins (Clegg, 2011; Hand et al., 2011b). Embryos of brine shrimp, *Artemia franciscana*, accumulate both trehalose (Carpenter and Hand, 1986) and LEA proteins (Hand et al., 2007; Menze et al., 2009; Warner et al., 2012; Warner et al., 2010), a phenomenon that is at
least partly responsible for the ability to survive severe water stress (Clegg, 2005; Hand and Hagedorn, 2008). The primary aim of this dissertation is to investigate stabilization of lipid bilayers by trehalose and two LEA proteins, AfrLEA2 and AfrLEA3m, expressed in embryos of *A. franciscana*. I will provide evidence that AfrLEA3m localizes in the matrix of mitochondria. Using computer-generated models, I will demonstrate that both AfrLEA3m and AfrLEA2 structure is consistent with amphipathic proteins that interact with membranes. I will then investigate the ability of trehalose and LEA proteins, alone and in combination to stabilize liposomes against freezing- and desiccation-induced damage. Finally, I will investigate the ability of trehalose to protect mitochondria isolated from rat liver from freeze-induced damage.

1.1 Biostabilization by Trehalose and LEA Proteins

Trehalose is an organic solute accumulated by a wide variety of anhydrobiotic animals (Clegg, 1965; Crowe et al., 1984; Madin and Crowe, 1975; Tapia and Koshland, 2014). Trehalose is a non-reducing disaccharide of glucose joined by an α-1,1 glycosidic bond. Accumulation of trehalose as a protective solute by anhydrobiotic and freeze-tolerant organisms has garnered much attention over the last half century, both for its protective role in vivo as well as potential applications for in vitro stabilization of mammalian cells and cellular components (Crowe et al., 2005; Hand and Hagedorn, 2008). The efficacy of trehalose at stabilizing lipid bilayers is more than can be explained by a colligative effect, as Crowe et al. (1988) demonstrate by comparing trehalose stabilization to equal amounts of other sugars. Trehalose exerts its protective effect through two probable mechanisms: formation of sugar glasses (i.e. vitrification) and water replacement (Crowe et al., 1984; Crowe et al., 1992; Webb, 1965). Sugar glasses are amorphous solids that prevent deleterious interactions between cellular components during dehydration, as well as provide structural support to the cell (Crowe et al., 1998; Crowe et al.,
1984; Sun et al., 1996). Direct comparisons of trehalose to sucrose and other carbohydrates indicate that the ability of trehalose to stabilize macromolecules during dehydration is greater than that of other carbohydrates (Crowe and Crowe, 1992; Sun et al., 1996). The superior protection of macromolecules by trehalose during drying is primarily attributed to its higher glass transition temperature ($T_g$) (Crowe et al., 1998; Crowe et al., 1989). During severe drying, water bound to proteins and membranes is removed, leading to protein misfolding and phase transitions (Crowe et al., 1989; Webb, 1965). There is evidence that trehalose can replace water that was bound to macromolecules and prevent protein misfolding and lipid phase transitions (Crowe et al., 1997; Crowe et al., 1987; Webb, 1965). In order for trehalose to fully exert its protective effect on cellular membranes, the sugar must be present on both sides of the lipid bilayer (Chen et al., 2001; Crowe et al., 1985). Importantly, trehalose is preferentially excluded from the hydration shell of proteins (Timasheff, 2002), which favors the retention of secondary structures at moderate and low water contents.

The capacity of trehalose to stabilize dried or frozen mammalian cells has received considerable attention (Crowe et al., 2005; Hand and Hagedorn, 2008). Trehalose is an effective cryoprotectant as demonstrated by Buchanan et al (2004). Human TF-1 cells freeze-thawed with trehalose present on both sides of the membrane were able to retain 91% of their ability to form colonies prior to freeze-thawing (Figure 1.1) (Buchanan et al., 2004). Traditional freeze-thawing protocols using DMSO resulted in retention of only 66% of colony forming units (CFUs). Buchanan et al’s 2005 study reinforces the requirement of having trehalose present on both sides of a plasma membrane (Chen et al., 2001). With trehalose present only in the freezing medium, TF-1 cells retained only 11% of control (non-frozen) CFU; removing trehalose from the
Figure 1.1. Effects of intra- and extra- cellular trehalose versus dimethyl sulfoxide (DMSO) and no cryopreservant during freeze-thawing of TF-1 cells. TF-1 cells were porated via P2Z receptors in the presence of 200 mM trehalose for 60 min. One set of samples were immediately frozen with trehalose present both intra- and extra-cellularly. Extracellular trehalose was removed prior to freezing for another set of samples. A third set of samples was not porated and frozen in the presence of 200 mM extracellular trehalose. DMSO (10%) was used in a traditional freeze-drying protocol. All frozen samples were stored at -80°C, rapidly thawed, and assayed for colony-forming units (CFU). Results are expressed as percentage of control (non-frozen) CFU (from Buchanan et al., 2004).
extracellular medium prior to freezing had similar detrimental effects (17% of control CFU). Human platelets have also been successfully freeze-dried with trehalose (Wolkers et al., 2001b), and trehalose improves the plasma membrane stability of dried mammalian cells (Chen et al., 2001; Elliott et al., 2006; Li et al., 2012). However, trehalose alone is unable to fully stabilize nucleated mammalian cells in the desiccated state, likely because (a) subcellular compartments in these cells remain inaccessible to the trehalose (Hand and Hagedorn, 2008) and (b) other protective factors, like specialized proteins, are required (Crowe et al., 2005).

Damage to organelles during dehydration or freezing will likely result in cell death (Hand and Hagedorn, 2008). Therefore, it is pertinent to the study of cell preservation to determine what is required to stabilize an organelle like the mitochondrion. Yamaguchi et al. (2007) found that mice liver mitochondria freeze-thawed in a 300 mM trehalose solution retained their ultrastructure as well as a number of outer membrane functions. However, metabolic function was compromised in freeze-thawed mitochondria, as indicated by diminished oxidative phosphorylation capacity. These results are consistent with the requirement that trehalose needs to be present on both sides of a biological membrane in order to fully exert its stabilizing effects; thus, trehalose needs to be present in the matrix of mitochondria in order to maximally preserve inner membrane function (Hand and Hagedorn, 2008). It is possible to permeabilize isolated mammalian mitochondria to trehalose by temporarily opening the mitochondrial permeability transition pore, as demonstrated by Liu et al. (2005). When trehalose is present in the matrix as well as in the surrounding medium, mitochondria air dried to 0.1 g water/1 g solids retain substantial capacity to regenerate a membrane potential ($\Delta \psi$) (Figure 1.2). By comparison, mitochondria dried to 0.1 g water/1 g solids with trehalose present in the media but not in the matrix only regenerated 30% of their original $\Delta \psi$, and only 20% of $\Delta \psi$ was regenerated by
Figure 1.2. Normalized membrane potential of rehydrated mitochondria as a function of the final water content reached during desiccation. Isolated rat mitochondria were dried to different water contents in buffers that contained 125 mM KCl (without trehalose), 250 mM trehalose (extra-mitochondria trehalose only), and 250 mM trehalose after permeabilization (i.e. with both extra-mitochondrial and matrix trehalose present). After rehydration, ΔΨ was measured by the JC-1 fluorescence intensity ratio (590/538 nm), which was normalized to that of non-desiccated (control) mitochondria. Dash lines show 95% confidence interval for the data (From: Liu et al., 2005).
mitochondria dried without any trehalose at all. Thus, the stability of the inner membrane is increased during drying when trehalose is present in the matrix. However, mitochondrial respiratory function was compromised, as indicated by diminished respiratory control ratios (Liu et al., 2005). Diminished respiratory function in mitochondria dried with trehalose in the matrix was attributed to damage to the electron transport system or phosphorylation system. Yamaguchi et al (2007) point out that it is also possible the outer membrane was compromised during transient opening of the permeability transition pore, which would result in loss of cytochrome c (cyt c) and diminished electron flux through the electron transport system.

In addition to accumulating trehalose, embryos of A. franciscana express a number of late embryogenesis abundant (LEA) proteins (Hand et al., 2007; Menze et al., 2009; Warner et al., 2012; Warner et al., 2010). LEA proteins are a family of intrinsically disordered proteins (Uversky and Dunker, 2010) whose expression levels are tightly correlated to desiccation tolerance in numerous desiccation-tolerant plants (Tunnacliffe and Wise, 2007; Wise, 2003) and animal (Hand et al., 2011b) species. LEA proteins were initially discovered in germinating cotton seed nearly 30 years ago (Dure et al., 1981) and subsequently discovered in a number of other plant species (for review, see Tunnacliffe and Wise, 2007). More recently, LEA proteins have been reported in numerous organisms other than plants: prokaryotes (Battista et al., 2001), rotifers (Tunnacliffe et al., 2005), nematodes (Browne et al., 2002), larvae of an insect, Polypedilum vanderplanki, the African chironomid (Kikawada et al., 2006) and in the embryos of the brine shrimp, Artemia franciscana (Hand et al., 2007). While the precise mechanism(s) by which LEA proteins stabilize cells and cellular components is not yet fully resolved, several studies have shed light on potential roles of these unique proteins. LEA proteins prevent protein aggregation (Furuki et al., 2012; Goyal et al., 2005) and preserve function of target enzymes
(Boswell et al., 2014a; Goyal et al., 2005; Grelet et al., 2005). Interestingly, there is evidence of synergistic protection of enzyme function by LEA proteins and stabilizing disaccharides (i.e. trehalose, sucrose) (Boswell et al., 2014a; Goyal et al., 2005). It is unclear how LEA proteins and protective sugars interact, but there is evidence that LEA proteins can stabilize sugar glasses formed during drying (Shimizu et al., 2010; Wolkers et al., 2004). LEA proteins also protect lipid bilayers from freezing and desiccation-induced damage (Artus et al., 1996; Furuki and Sakurai, 2014; Steponkus et al., 1998; Thalhammer et al., 2014; Tolleter et al., 2010; Tolleter et al., 2007).

Two LEA proteins from embryos of *A. franciscana* will be used extensively in this dissertation: AfrLEA2 and AfrLEA3m. Expression of multiple LEA proteins in a single organism is not unusual, and is at least partially due to subcellular targeting of LEA proteins (Hand et al., 2011b). AfrLEA2 is located in the cytoplasm of *A. franciscana* (Boswell and Hand, 2014), while AfrLEA3m is targeted to the mitochondrial network (Boswell and Hand, 2014; Menze et al., 2009). Four isoforms of AfrLEA3m were discovered in mitochondrial extracts from embryos of *A. franciscana* (Boswell et al., 2014b). Both AfrLEA2 and AfrLEA3m share features common to many LEA proteins, such as high expression levels during the desiccation-tolerant life stages and the ability to form alpha-helical secondary structure upon drying (Boswell et al., 2014a; Boswell et al., 2014b). *In vitro* drying studies reveal that AfrLEA2 and AfrLEA3m protect target enzymes (Boswell et al., 2014a) (Figure 1.3 A). Furthermore, desiccation protection by both LEA proteins is enhanced in the presence of trehalose (ex. PFK, Figure 1.3 A). Expression of AfrLEA2 and AfrLEA3m in transfected HepG2 cells results in improved retention of membrane integrity after cells were rapidly dried to 0.12 g water/g dry weight and then immediately rehydrated (Figure 1.3 B) (Li et al., 2012). Expression of AfrLEA3m alone
Figure 1.3. Protection of PFK and cell membrane integrity by AfrLEA2 and AfrLEA3m. (A) Residual phosphofructokinase (PFK) activity after air drying for 24 h with the indicated amounts of AfrLEA2 (left panel) and AfrLEA3m (right panel) in the presence and absence of 100 mM trehalose. Enzymes were dried with equal amounts of BSA for comparison to a protein not associated with desiccation tolerance. Activity was expressed as percent of non-dried values (mean ± s.d., n=9). (from: Boswell et al., 2014) (B) Membrane integrity of HepG2 cells after drying and rehydration. HepG2 cells were transfected with genes encoding AfrLEA2, AfrLEA3m, and/or TRET1, a trehalose transporter. Membrane integrity is expressed as a percentage of non-dried values. Data represents means ± SD of 3-6 samples. ‘a’ indicates a significant improvement compared to cells dried without protectants (control). “b” indicates significant improvement compared to cells with intracellular trehalose alone (TRET1). (from: Li et al., 2012)
results in retention of 94% of original membrane integrity. While AfrLEA3m is targeted to the mitochondria, like most mitochondrial proteins it is synthesized in the cytoplasm. The results presented by Li et al. (2012) indicate that sufficient quantities of AfrLEA3m remain in the cytoplasm of transfected HepG2 cells to effectively stabilize the plasma membrane.

Stabilization of membranes by LEA proteins is thought to be linked to formation of class A α-helices similar to those found in apolipoproteins (Tolleter et al., 2010). Positively charged amino acids in amphipathic α-helices interact with negatively charged headgroups of phospholipids and the non-polar residues face the hydrophobic core of the lipid bilayer (Mishra et al., 1994; Segrest et al., 1992). LEA proteins positioned between the phospholipid headgroups of membranes promotes retention of spacing between lipids at low water contents (Tolleter et al., 2010). Preserving spacing between headgroups of phospholipids in a membrane during drying is important for maintaining membrane fluidity and preventing phase transitions (Crowe et al., 1989; Tolleter et al., 2010). Furthermore, non-bilayer forming lipids found in biological membranes promote formation of an inverted hexagonal II phase, especially at low water contents (Cullis et al., 1986; Tilcock, 1986). Positioning of LEA proteins between phospholipid headgroups relieves this inversion stress and promotes retention of the lamellar phase (Steponkus et al., 1998; Thalhammer et al., 2014). While several LEA proteins have been identified that form amphipathic alpha-helices capable of interacting with lipid bilayers (Popova et al., 2011; Pouchkina-Stantcheva et al., 2007; Thalhammer et al., 2010; Tolleter et al., 2010), stabilization of lipid bilayers during water stress has only been experimentally demonstrated with three LEA proteins from plants. COR15a and COR15b (LEA23 and LEA24) from Arabidopsis thaliana have been shown to protect chloroplast membranes from freeze-induced damage both in vitro and in vivo (Artus et al., 1996; Steponkus et al., 1998; Thalhammer et al., 2014) and
PsLEAm from the pea plant (*Pissum sativum*) was found to improve the stability of dried or frozen liposomes (Tolleter et al., 2010). To date, no LEA protein from an animal has been demonstrated to stabilize lipid bilayers. Additive protection of lipid bilayers by LEA proteins and protective sugars (e.g., trehalose) has also not been investigated.

1.2 Research Aims of this Dissertation

The overall objective of this dissertation is to improve our understanding of the mechanisms responsible for water stress tolerance of anhydrobiotic species, particularly the roles of LEA proteins and trehalose found in embryos of the brine shrimp, *Artemia franciscana*. Chapter 2 describes the purification of recombinant AfrLEA3m and identifies the sub-mitochondrial compartment in which AfrLEA3m is located. Molecular models of AfrLEA2 and AfrLEA3m were generated in order to determine if either protein contained structural features consistent with amphipathic proteins able to interact with cell membranes. Determining the location and structure of AfrLEA3m provides clues as to the role this protein plays in tolerance to water stress by brine shrimp.

In Chapter 3, liposomes with lipid compositions mimicking the inner mitochondrial membrane (IMM), the outer mitochondrial membrane (OMM), and the inner leaflet of the plasma membrane (ILPM) were air dried overnight and for one week with various combinations of AfrLEA2, AfrLEA3m, and trehalose. Protection of lipid bilayers was assessed by measuring leakage of entrapped fluorescent dye, carboxyfluorescein (CF), after drying and rehydration. Protection by LEA proteins was compared to liposomes dried with lysozyme, a non-stabilizing (control) protein. By comparing stabilization of liposomes mimicking various cellular membranes, I was able to evaluate whether AfrLEA2 or AfrLEA3m are specialized to protect components within the compartments to which they are localized. Liposomes composed of
100% phosphatidylcholine (PC) were also prepared for comparison to a non-biological membrane. If the proposed mechanism by which AfrLEA2 and AfrLEA3m stabilize membranes is true, liposomes comprised entirely of PC should not be as effectively protected as those with compositions similar to biological membranes.

In Chapter 4, liposomes with lipid compositions identical to those in Chapter 3 were frozen with LEA proteins and trehalose. The effects of lipid composition on stabilization by AfrLEA2 and AfrLEA3m were again evaluated. Mitochondria isolated from rat liver were frozen in 300 mM trehalose, and respiratory measurements were used to evaluate mitochondrial function before and after freezing. Finally, tests were performed to determine whether loading trehalose into the matrix would improve freeze-tolerance of mitochondria compared to those frozen with only external trehalose only or with no trehalose at all.
CHAPTER 2.
SUBMITOCHONDRIAL LOCALIZATION OF AFRLEA3M AND MOLECULAR MODELING OF AFRLEA2 AND AFRLEA3M FROM ARTEMIA FRANCISCANA EMBRYOS

2.1 Introduction
Animal species capable of surviving at low water content accumulate specialized molecules that protect critical cellular components. Important among these protective molecules are disaccharides such as sucrose or trehalose and low molecular weight proteins such as LEA proteins (Crowe et al., 1997; Crowe et al., 1992; Hand et al., 2011b; Tapia and Koshland, 2014). Embryos of the brine shrimp Artemia franciscana are a classic model species for studying desiccation tolerance and are capable of surviving extended periods of time at less than 2% water content (Clegg, 1978; Clegg, 2005). A. franciscana embryos accumulate trehalose and express several Group 1 and Group 3 LEA proteins (Chen et al., 2009; Clegg, 2005; Hand et al., 2007; Menze et al., 2009; Sharon et al., 2009; Warner et al., 2012; Warner et al., 2010; Wu et al., 2011). Grouping of LEA proteins is based on possession of conserved sequence motifs, which are often tandemly repeated (Tunnacliffe and Wise, 2007; Wise, 2003). Many Group 3 LEA proteins, like AfrLEA3m used extensively in the studies reported in this dissertation, contain a repeated 11-mer motif (Dure, 1993). It has been suggested that these sequences may be conserved because they are critical to LEA protein function, a hypothesis supported by the ability of artificially synthesized 11-mer repeats to perform several functions associated with LEA proteins, including formation of structural networks, stabilization of sugar glasses, prevention of protein aggregation, and protection of liposomes (Furuki and Sakurai, 2014; Furuki et al., 2012; Shimizu et al., 2010). Like most LEA proteins, AfrLEA3m is unstructured in solution due to its high hydrophilicity (Menze et al., 2009). AfrLEA3m forms primarily α-helices at low water contents (Boswell et al., 2014). Models of α-helices formed by a Group 3 LEA protein from pea seeds contain axial bands of charged amino acids that allow the protein to...
interact with and stabilize biological membranes at low water contents (Tolleter et al., 2007). In this chapter, the sub-mitochondrial localization of AfrLEA3m will be determined along with its import kinetics into isolated mitochondria, and models of two LEA proteins from A. franciscana will be evaluated to determine if they share structural features consistent with proteins that stabilize lipid bilayers.

The mechanism(s) by which LEA proteins exert their protective effect has received considerable attention. In vitro, LEA proteins have been shown to stabilize sugar glasses (Hoekstra, 2005; Wolkers et al., 2001a), target enzymes (Chakrabortee et al., 2012; Goyal et al., 2005; Tompa and Kovacs, 2010) and liposomes (Tolleter et al., 2010; Tunnaccliffe and Wise, 2007). Furthermore, LEA proteins are capable of sequestering divalent cations (Grelet et al., 2005) and can form structural networks that may increase cellular resistance to physical stresses imposed by desiccation (Goyal et al., 2003; Tunnaciffe and Wise, 2007).

In order for cells to survive water stress, it is predicted that organelles responsible for critical cell processes must retain their function upon rehydration or thawing (Hand and Hagedorn, 2008). Supporting the supposition that a mechanism must be in place to stabilize subcellular components is the observation that LEA proteins are targeted to specific subcellular compartments within the cells of anhydrobiotic plants and animals (Hand et al., 2011b; Tunnaciffe and Wise, 2007). LEA proteins localize to the cytoplasm, nucleus, chloroplast, mitochondria, endoplasmic reticulum, vacuoles, peroxisomes, and the plasma membrane of plants (Tunnaciffe and Wise, 2007). In animals, LEA proteins have been shown to accumulate in the endoplasmic reticulum, Golgi, mitochondria, and extracellular space (Hand et al., 2011b).

Prior to the discovery of AfrLEA3m (Menze et al., 2009), the only LEA protein known to be targeted to mitochondria was PsLEAm found in seeds of the pea plant, Pisum sativum.
(Grelet et al., 2005; Menze et al., 2009). Many of the characteristics reported for LEAM are shared by AfrLEA3m (Boswell et al., 2014a). These include the formation of a predominantly α-helical structure upon water removal, accumulation in the matrix of mitochondria, and ability to stabilize certain enzymes and liposome models of mitochondrial membranes (Boswell et al., 2014a; Tolleter et al., 2010; Tolleter et al., 2007; Chapter 3). One intriguing difference between mitochondrial LEA proteins in pea plants versus *A. franciscana* is the number of different LEA proteins found in *A. franciscana* mitochondria. Whereas pea plants accumulate only one LEA protein, *A. franciscana* mitochondria contain 4 variants of AfrLEA3m as well as at least two Group 1 LEA proteins (Boswell et al., 2014b; Toxopeus et al., 2014; Warner et al., 2012; Warner et al., 2010). The concentration of AfrLEA3m and its variants is estimated to be 1.2-2.2 mg/ml matrix volume (Boswell et al., 2014b), which is in good agreement with the hypothesized values Tolleter et al. (2010) calculated as sufficient to stabilize the non-protein portion of the IMM. Models of LEAM indicate that bands of positive and negative charges form along the axial spine of the dried protein which interact with charged headgroups of phospholipid membranes (Tolleter et al., 2007). Settling of amphipathic proteins into a curved membrane has the effect of stabilizing membrane ultrastructure at low water levels (Hincha and Thalhammer, 2012; Thalhammer et al., 2014; Thalhammer et al., 2010; Tolleter et al., 2010). In this chapter, molecular modeling demonstrated that AfrLEA3m contain bands of charged amino acids characteristic of membrane-stabilizing proteins. A second LEA protein (AfrLEA2), which by comparison is cytoplasmically-localized in *A. franciscana* embryos, was also found to contain similar bands of charged amino acids.

Like most proteins found in mitochondria, AfrLEA3m proteins are encoded by DNA in the nucleus, synthesized in the cytoplasm, and transferred into or across the mitochondrial
membranes. Proteins destined for the mitochondrial matrix contain an N-terminal leader sequence referred to as a ‘presequence’ or ‘matrix-targeting signal.’ Matrix-targeting signals are usually composed of 20-60 amino acids, most of which have a positive charge. Presequences are largely unstructured in solution but form amphipathic alpha-helices in membranous environments, which is thought to aid in their specific recognition by the translocase of the outer membrane, TOM (von Heijne, 1986; Wiedemann et al., 2004). After passing through the outer membrane, the majority of proteins that contain a presequence are guided to the TIM$_{23}$ complex for passage into the matrix (Bolender et al., 2008; Marom et al., 2011b). Insertion into the TIM$_{23}$ complex is dependent on the presence of a membrane potential (Marom et al., 2011b). Transfer of proteins into the matrix space is completed by the ATP-driven Presequence Translocase-Associated Motor (PAM), of which mt-HSP-70 located on the matrix side of the inner membrane is the major component (Wiedemann et al., 2004). The precise mechanism by which PAM interacts with proteins during import into the matrix is still unknown (Gebert et al., 2011; Wiedemann et al., 2004). After import into the matrix, the majority of presequences are cleaved by the mitochondrial matrix processing peptidase (MPP) and the mature protein forms, often aided by a specialized chaperone system (For a review of protein import, see Marom et al., 2011a; Wiedemann et al., 2004). In this chapter, I show that recombinant AfrLEA3m is imported and processed in the matrix of mitochondria isolated from rat liver. Cleavage of the N-terminal leader sequence indicates that AfrLEA3m is targeted to the matrix of mitochondria in *A. franciscana* embryos.
2.2 Materials and Methods

Expression of Recombinant AfrLEA3m

The gene encoding AfrLEA3m from *A. franciscana* was cloned, inserted into a pET-30a expression vector (Novagen, Rockland, MA, USA) and incorporated into Rosetta 2 *E. coli* cells (Merck, Darmstadt, Germany) (Boswell et al., 2014). The protein sequence for recombinant AfrLEA3m contained a hexa-his tag at the C-terminus used to purify the recombinant protein by affinity chromatography with immobilized nickel. The C-terminus location of the his-tag does not interfere with the matrix targeting sequence at the N-terminus. The plasmid used to transfect Rosetta 2 bacteria also contained genes that confer resistance to the antibiotics kanamycin (Kan) and chloramphenicol (Cam), which were used to select for successfully-transfected bacteria. For protein expression, 20 ml of LB broth (1% Tryptone, 1% NaCl, and 0.5% yeast extract) containing 30 µg/ml Cam and 35 µg/ml Kan were inoculated from glycerol stocks of transfected bacteria and incubated overnight at 37°C with shaking. The overnight starter culture was diluted into 1 L of LB broth with Kan and Cam and shaken at 37°C for 3 h. Protein expression was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After 2 h of expression, bacteria were pelleted by centrifugation at 5,000 x g for 15 min (4°C).

Purification of AfrLEA3m and Antibody Production.

Protein extraction was performed using a detergent to solubilize bacterial membranes or by sonication. For detergent lysis, bacterial pellets were frozen overnight, thawed, and resuspended in Bugbuster® extraction reagent (Merck) at a ratio of 0.5 g of pellet to 5 ml of reagent. Protease inhibitor (p8849, Sigma) was added to the suspension, which was then incubated with gentle shaking for 20 min at room temperature. Unwanted cell debris was removed by centrifugation at 20,000 x g for 30 min, and the supernatant, which contained the
recombinant protein, was retained. Alternatively, bacterial pellets collected after expression were immediately resuspended in the initial mobile phase used for affinity chromatography, composed of 20 mM imidazole, 500 mM NaCl, 20 mM sodium phosphate, pH 7.5. Protease inhibitor was added and the suspension frozen overnight. After thawing, the bacterial suspension was sonicated with a probe tip sonicator (VWR Scientific) and then heated to 95°C for 30 min. Due to their highly hydrophilic nature, LEA proteins remain soluble at elevated temperatures, while most other proteins denature and precipitate. After cooling on ice for ~ 15 min, the bacterial lysate was centrifuged at 20,000 x g for 30 min at 4°C to remove cell debris and aggregated protein, and the supernatant was collected. This sonication-based procedure was better suited for large-scale protein purification.

An ACTA Prime Plus FPLC (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used to purify recombinant AfrLEA3m. Initial purification was performed by Ni²⁺ affinity chromatography with a HisTrap FF crude column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) equilibrated with the initial buffer for the mobile phase specified above. Prior to loading onto the column, samples prepared by detergent lysis were diluted 10-fold in the initial buffer for the mobile phase. Bound protein was eluted with 500 mM imidazole prepared in the mobile phase buffer. For preparations made by detergent lysis, the elution fractions containing recombinant LEA protein were pooled and immediately heated as described above. The sample was then dialyzed overnight against 20 mM triethanolamine, 10 mM NaCl, pH 7. Samples were applied to a HiTrap Q FF column (GE Healthcare Bio-Sciences AB) and bound protein eluted with a NaCl gradient from 10 mM to 1 M. This purified LEA protein was used for antibody production and subsequent experiments. Polyclonal antibodies against purified AfrLEA3m were produced by Aves Labs Inc. (Tigard, OR).
Mitochondrial Isolation from Embryos of A. franciscana and from Rat Liver

Dehydrated post-diapause embryos from the Great Salt Lake, UT, were obtained from *Artemia* LLC (Ogden, UT) and stored at -20 °C. Embryos were hydrated overnight at 4 °C in 0.25 M NaCl solution, after which embryos were transferred to room temperature 0.25 M NaCl and allowed to develop under normoxic conditions for the desired amount of time (0-8 h). Embryos were dechorionated as previously described (Kwast and Hand, 1993; Reynolds and Hand, 2004). Ten grams of dechorionated embryos were transferred to a 55 ml Potter-Elvehjem tissue homogenizer (Thomas Scientific, Swedesboro, NJ) that contained 40 ml of homogenization medium composed of 0.5 M sucrose, 150 mM KCl, 1 mM EGTA, 0.5% (w/v) BSA, and 20 mM HEPES (pH 7.5) and homogenized on ice with six passes of a motor-driven Teflon pestle (clearance 0.15 mm) at 1000-1100 rpm. Mitochondria were isolated from this homogenate by differential centrifugation following published procedures (Kwast and Hand, 1993; Reynolds and Hand, 2004). Isolated mitochondria were resuspended at a final concentration of 40 mg/ml mitochondria proteins as determined by Coomassie Plus (Bradford) protein assay (Thermo Fisher Scientific Inc., Rockford, IL).

Mitochondrial isolation from rat liver was performed as outlined by Gnaiger et al. (2000). Briefly, male Spraque-Dawley rats were euthanized by CO₂ asphyxiation in accordance with IACUC protocol 12-061. The liver (approx. 10-15 g) was excised and immediately placed in ice-cold homogenization medium A (HMA), which was composed of 250 mM trehalose, 10 mM Tris, 1 mM EGTA (pH 7.4). The large lobes of the liver were finely diced using small surgical scissors in order to facilitate easier homogenization and any connective tissue observed was removed. The minced liver was rinsed up to 4 times in approximately 35 ml of HMA to remove as much blood as possible, then transferred to a 40 ml Dounce homogenizer (Kontes Glass Co.,
Vineland, NJ). The liver was suspended in 30 ml of homogenization medium B (HMB), which was HMA fortified with 0.2% BSA. Optimal homogenization of the liver required 4 to 6 passages of the loose-fitting pestle, which had a total clearance between the pestle tube of 0.15 mm. The homogenate was then transferred to two 50 ml centrifuge tubes and centrifuged at 700 x g for 10 min at 4°C. The supernatant was centrifuged at 9,000 x g for 10 min at 4°C. The lipid layer that formed on top of the supernatant was carefully removed. After decanting the supernatant, the pellet was gently resuspended in 30 ml of fresh HMB and centrifuged once more at 9,000 x g for 10 min at 4°C. Any remaining lipid was once more carefully removed and the supernatant decanted. The resulting mitochondrial pellet was resuspended in HMA for trehalose loading experiments or HMB for all other experiments. Generally 40-60 mg mitochondrial protein was obtained per liver and suspended at 40-60 mg protein/ml or higher as determined by the protein assay above.

Electrophoresis and Western Blotting

Samples were prepared with 4X Laemmli sample buffer by adding one part sample buffer to 3 parts sample (final concentration: 62.8 mM Tris-HCl (pH 6.8), 25% glycerol (v/v), 2% SDS (w/v), 5% 2-mercaptoethanol (v/v), 0.025% bromophenol blue (w/v)). The SDS-treated samples were then analyzed by SDS polyacrylamide gel electrophoresis. Typically, 50 µg mitochondrial or bacterial protein or less than 10 µg purified protein was loaded per lane. Prior to loading, all samples were boiled for 5 min and vortexed. Proteins were separated on a 4% stacking gel and 11% resolving gel.

Silver staining was carried out following the manufacturer’s directions with a Silver Stain Plus kit (Bio Rad, Cat. # 161-0449). After electrophoresis, gels were placed in fixative solution (50% methanol, 10% acetic acid, 10% Fixative Enhancer Concentrate v/v) and gently shaken for
20 min at room temperature. Gels were rinsed twice for 10 min with distilled water then placed in the staining and developing solution (5% Silver Complex Solution, 5% Reduction Moderator Solution, 5% Image Development Reagent, 50% Development Accelerator Solution v/v). After staining had reached the desired end point, gels were placed in a 5% acetic acid (v/v) solution to stop development.

For immunoblotting, proteins were transferred to nitrocellulose membrane at 4°C for 1 h at 80 V in a buffer composed of 25 mM Tris, 192 mM glycine, 20% methanol, 0.025% SDS. Ponceau S stain was used to confirm transfer, and membranes were then blocked in 5% fat free dry milk solution for at least 1 h. Membranes were incubated with properly diluted primary antibody for 15 h at 4°C with shaking, after which they were rinsed for at least 1 h with 0.1% Tween-20, 500 mM NaCl, 20 mM Tris-HCl, pH 7.6 (TBST). Membranes were then incubated with HRP-linked secondary antibody on a shaker for 1 h at 25°C and subsequently washed with TBST for at least 2 h prior to visualization. To detect protein bands, membranes were incubated with LumiGLO chemiluminescent substrate (Cell Signaling Technology) for 10 min, wrapped in cellophane, exposed to ECD Film (GE Healthcare), and the film developed.

Submitochondrial Localization of AfrLEA3m.

In order to determine the submitochondrial location of the four AfrLEA3m isoforms, mitochondria isolated from 4 h post-diapause embryos were subjected to detergent treatments designed to differentially solubilize the outer and inner membranes (cf. Grelet et al. 2005). Approximately 2 mg mitochondrial protein were suspended in 200 µl of homogenization buffer (500 mM Sucrose, 150 mM KCl, 1 mM EGTA, 20 mM HEPES, 0.5% BSA, pH 7.5) that included various concentrations of digitonin or Triton X-100. After 15 min of incubation on ice, mitochondria were centrifuged at 10,000 x g for 10 min, and the supernatant was analyzed for
the release of AfrLEA3m proteins and marker enzymes for the inner membrane space and matrix. Specific solubilization of the outer membrane was achieved at low concentrations (0.5-2 mg/ml) of digitonin, while higher concentrations (3-4 mg/ml) resulted in at least partial rupture of the inner membrane as well. Triton X-100 (1-2% v/v) was used to completely solubilize both the outer and inner membranes. Release of components from the inner membrane space after solubilization of the outer membrane was indicated by the presence of adenylate kinase activity. Adenylate kinase activity was detected by monitoring NADPH production at 340 nm in a SpectraMaxPlus microplate reader (Molecular Devices, Sunnyvale, CA). The reaction medium contained 50 mM Tris, pH 8.0, 15 mM Glucose, 5 mM MgCl$_2$, 5 mM KCN, 3 mM ADP, 0.75 mM NADP$^+$, 0.5 units/mL Glucose-6-Phosphate dehydrogenase, and 4 units/mL hexokinase. Fumarase activity was used as a marker for solubilization of the inner membrane and release of matrix contents. The reaction medium contained 50 mM Tricine, pH 7.5, 0.1% Triton X-100 and 50 mM Malate. Fumarate production by fumarase was detected by monitoring the change in optical density at 250 nm. Equal volumes of supernatant were assayed for all treatments. After measuring enzyme activity, the remaining supernatant was heated to 95°C for 20 min and centrifuged at 20,000 x g for 30 min in order to enrich the LEA protein fraction. The presence of LEA proteins in the heat-soluble fraction was detected by Western blotting performed with AfrLEA3m-specific antibodies.

Purification of Su9-DHFR.

Su9-DHFR is a chimeric protein comprised of the 69-amino acid presequence of subunit 9 (subunit c) of the F$_{1}$F$_{0}$ ATP synthase and dihydrofolate reductase (DHFR; 30 kDa) with a C-terminal His-tag. DHFR is not naturally localized to mitochondria and therefore it can be used as a positive control for mitochondrial import by immunoblotting with DHFR antibody (see
below). Dr. Dejana Mokranjac from the laboratory of Professor Walter Neupert graciously provided us with the expression vector for Su9-DHFR (Stan et al., 2000), which also conferred Amp resistance. BL21(DE3) cells (Invitrogen, Carlsbad, CA) were transfected with the plasmid and grown on LB Agar that contained 100 µg/ml Amp. Expression of Su9-DHFR was performed as described above, and the protein was purified with a Histrap FF crude column using an imidazole gradient from 2-500 mM.

Import of Recombinant Proteins into Isolated Mitochondria.

Import of purified Su9-DHFR and AfrLEA3m into isolated rat mitochondria was performed as described by Ryan et al. (2001). Mitochondria (1-5 mg/ml) were suspended in homogenization buffer that contained 3% BSA. The high BSA concentration stabilizes the outer membrane during prolonged incubation times that are often required for protein import. Prior to the addition of Su9-DHFR or AfrLEA3m, mitochondria were incubated for 5 min in the presence of 10 mM glutamate and 1 mM malate in order to establish a membrane potential (ΔΨm), which is necessary for import of proteins into the matrix. ATP (2 mM) was added because certain cytosolic chaperones that aid in protein recognition by the TOM complex require ATP to release their bound protein destined for import.

To measure the time course for protein import, aliquots of mitochondria were removed from the reaction mixture at various points and import arrested in these aliquots by addition of 0.5 µM valinomycin to dissipate ΔΨm. Each aliquot was then divided equally, and Proteinase K was added to one half to degrade any protein not fully imported through the outer membrane. The other half of the sample was retained and used to evaluate whether Proteinase K degraded any matrix-processed protein. After 10 min incubation on ice with Proteinase K, phenylmethylsulfonyl fluoride (PMSF) was added to inhibit the protease. The mitochondrial
suspensions were centrifuged at 9,000 x g for 10 min at 4°C. The mitochondrial pellets were resuspended in SDS sample buffer and immediately heated to 95°C for 5 min to denature Proteinase K, which is still active in SDS sample buffer. To serve as a negative control for protein import, a parallel reaction mixture was prepared in which generation of $\Delta \Psi_m$ was blocked by addition of antimycin A, valinomycin, and oligomycin (prevents reversal of the ATP synthase). Import of precursor protein was assayed by Western blotting with either AfrLEA3m antibody or a commercially-available antibody for DHFR (Abcam, Cambridge, MA) that recognized Su9-DHFR.

Modeling of AfrLEA3m and AfrLEA2

Primary amino acid sequences of AfrLEA3m and AfrLEA2 were determined from our existing cDNA library for *A. franciscana* as published previously (Hand et al., 2007; Menze and Hand, 2010; GenBank accession no. ACA47268.1 for AfrLEA2, ACM16586.1 for AfrLEA3m). Sequences were modeled as $\alpha$-helices using UCSF Chimera version 1.10, which was obtained at cgl.ucsf.edu/chimera. Images of the resulting protein model were generated with DeepView version 4.1 (formerly Swiss-PDB Viewer), which was downloaded from spdbv.vital-it.ch.

2.3 Results

AfrLEA3m Expression and Purification

Recombinant AfrLEA3m protein was successfully expressed and purified from transfected *E. coli* (Fig 2.1). During purification, AfrLEA3m is highly susceptible to proteolytic attack, a characteristic of both intrinsically disordered proteins and proteins containing matrix-targeting sequences (Receiveur-Brechot et al., 2006; Uversky and Dunker, 2010). However, the fragments did not bind to the anion exchange column at pH 7 (Fig 2.2A). Thus it was possible to obtain highly purified AfrLEA3m, but only in reduced quantities (0.25-0.5 mg per liter of cell culture).
Low molecular weight break down products can be detected by His6 antibody (Fig 2.1 B) and AfrLEA3m antibodies (data not shown) in SDS samples of bacterial lysates after expression of AfrLEA3m (Fig. 2.1 B, lane 2). Because these fragments still contain a His6-tag and are heat soluble, they cannot be separated from the full length recombinant protein by affinity chromatography or incubation at elevated temperatures (Fig. 2.1B, lanes 3 and 4).

Sub-mitochondrial Localization of Endogenous AfrLEA3m

Mitochondria isolated from *A. franciscana* embryos were incubated with a range of digitonin and Triton X-100 concentrations in order to differentially solubilize the outer and inner membrane. All four AfrLEA3m isoforms are localized in the mitochondrial matrix based on the observation that their release with detergent coincides with that of fumarase, a matrix marker enzyme (Fig. 2.3). When mitochondria were incubated with 2 mg/ml digitonin, a large increase in adenylate kinase activity (an intermembrane space marker) was measured, but AfrLEA3m release was not detected, which indicates that AfrLEA3m is not present at appreciable levels in the IMS (Fig. 2.1, lanes 2-4). When membranes were solubilized by 3 mg/ml digitonin, fumarase activity (a matrix marker) doubles and AfrLEA3m was detected, which indicates that AfrLEA3m is primarily located in the matrix of mitochondria. After solubilization of mitochondria by Triton X-100, the mitochondrial fragments and membranes collected by centrifugation were also analyzed by immunoblotting, and LEA protein was not detected (data not shown).

Recombinant AfrLEA3m Import in to Isolated Rat Mitochondria

In order to confirm the existence of a functional matrix-targeting sequence for the AfrLEA3m precursor protein, we evaluated the ability of the pre-protein to be imported into rat
Figure 2.1. Expression and purification of recombinant AfrLEA3m expressed in transfected bacteria. (A) Silver stained polyacrylamide gel of recombinant AfrLEA3m purified by affinity chromatography before (lane 2) and after (lanes 3-6) anion exchange. (B) A Western blot performed with anti-His\(_6\) antibody detected both full-length AfrLEA3m and proteolytic breakdown product in an SDS extract of bacteria after 2 h of recombinant AfrLEA3m expression (lane 2). Both full-length protein and breakdown product remain after affinity chromatography (lane 3) and heat treatment (lane 4). Full-length AfrLEA3m (34 kDa) is labeled to the right of each panel.
liver mitochondria. The AfrLEA3m precursor was successfully imported (Fig. 2.3A). A ~29 kDa protein product could be detected after just 2 min of incubation with isolated mitochondria at room temperature. This protein size would be approximately that predicted for mature AfrLEA3m after cleavage of the N-terminal leader sequence (estimated by bioinformatic analysis to be 3.2 kDa; Menze et al., 2009) by the mitochondrial processing peptidase in the matrix. The import is clearly dependent on the presence of an electric potential across the inner membrane ($\Delta \psi_m$), which is required for movement through the TIM$_{23}$ complex (Weidemann et al, 2004). In the absence of a membrane potential (Fig 2.3A, ‘-\(\Delta \psi\)’), mature AfrLEA3m is not detected and no protein remains after addition of external protease. The fusion protein Su9-DHFR was used to confirm that the isolated mitochondria were competent for import of matrix-targeted proteins for extended periods of time (up to 60 m) and that the incubations conditions used were sufficient (Fig 2.3 B and C). Import of Su9-DHFR was greatly improved when rabbit reticulocyte lysate was included in the incubation media (Fig 2.3C). Su9-DHFR continued to accumulate in the matrix for up to 1 h under the conditions employed. By comparison, AfrLEA3m appears to accumulate more quickly than Su9-DHFR but did not continue to increase in amount beyond several minutes. Some of the mature AfrLEA3m product is susceptible to externally added Proteinase K, which may be indicative of incomplete import for a fraction of the AfrLEA3m (Fig 2.3A, ‘+ PK’). Mature Su9-DHFR was also susceptible to externally added protease when rabbit reticulocyte lysate was absent from the import media (Fig 2.3B, ‘+ PK’). However, the addition of rabbit reticulocyte lysate had no effect on the import of AfrLEA3m (data not shown).
Figure 2.2 Sub-mitochondrial localization of AfrLEA3m isoforms in mitochondrial extracts from *A. franciscana*. Mitochondria were incubated with different concentrations of the detergents Digitonin or Triton X-100. Control mitochondria (lane ‘C’) were not exposed to detergent. After treatment, organelles were pelleted by centrifugation and the supernatants assayed for marker enzyme activity. Adenylate kinase (ADK) was chosen as the marker enzyme for the inner membrane space and fumarase for the matrix. Enzyme activity is expressed as change in optical density (OD) per minute per milliliter of supernatant. Immunoblotting with AfrLEA3m-specific antibody was used to detect release of AfrLEA3m proteins from the mitochondria (lower panel). Lane ‘M’ is an SDS extract of intact mitochondria in which the four AfrLEA3m isoforms (A-D) can be observed for comparison.
Figure 2.3 Protein import into isolated rat mitochondria. Recombinant AfrLEA3m (A) was imported for the indicated time periods. As a positive control, the fusion protein Su9-DHFR was also imported into mitochondria in the presence (B) and absence (C) of rabbit reticulocyte lysate (RRL). The full-length precursor protein (p) and the imported and processed protein (i) are indicated. Mitochondria were incubated for indicated periods of time prior to dissipation of the membrane potential by the addition of valinomycin. For comparison, some mitochondria lacked a membrane potential (−Δψ) during incubation with pre-protein, which prevents translocation across the inner mitochondrial membrane. In panel A, some mitochondria were incubated in the absence of any added AfrLEA3m (‘Mitochondria’) to ensure no cross-reactivity occurred with the AfrLEA3m antibody. Proteinase K (PK) was added to half of each sample in order to confirm complete transport across the outer membrane. Equal volumes of sample with equal amounts of mitochondria were loaded in each well. In panels B and C, the alpha subunit of the F₁-domain of the ATP Synthase (α F(1)) was used as a loading control.
Molecular Modeling of AfrLEA3m and AfrLEA2

Drying of AfrLEA3m and AfrLEA2 promotes formation of substantial $\alpha$-helical structure (Boswell et al., 2014a). Therefore, models of AfrLEA3m and AfrLEA2 were generated as helical rods (Figs. 2.4 and 2.5). The distribution of acidic and basic residues revealed a distinctive organization associated with amphipathic proteins that interact with lipid bilayers (Hristova et al., 1999; Mishra et al., 1994; Segrest et al., 1992). For both AfrLEA2 and AfrLEA3m, stripes of acidic residues were flanked by bands of basic residues along the length of the protein. In AfrLEA3m, the arrangement of charged residues formed parallel bands that extended linearly along the axis of the helix with a single sharp twist around residues 120-130 (Fig. 2.4A). To more clearly view the distinctive pattern made by charged amino acids in AfrLEA3m, the acidic and basic residues between amino acids 48-219 are shown with the side chains removed (Fig. 2.4B). Figure 2.4C shows a front view of residues 149-240 with only the positive and negative residues displayed. Both panels B and C highlight the distinctive organization of acidic and basic amino acids found in AfrLEA3m, which are characteristic of class A $\alpha$-helices originally described for apolipoproteins (Hincha and Thalhammer, 2012).

Somewhat differently compared to AfrLEA3m, where charged residues were arranged in a straight line for the majority of the protein’s length, bands of charged amino acids in AfrLEA2 wrap around the axis of the helix for the entire length of the protein (Fig. 2.5A). As with AfrLEA3m, a smaller region of AfrLEA2 (amino acids 131-232) is displayed with the side chains removed (Fig. 2.5B) in order to emphasize the arrangement of charged residues. A front view of residues 29-98 also revealed an amphipathic motif similar to apolipoproteins (Fig. 2.5C).
Figure 2.4 Helical model of AfrLEA3m. (A) Two views of AfrLEA3m modeled as an α-helix with Chimera Software; images were generated with Swiss-PDB Viewer (DeepView, version 4.1). Charged amino acids are depicted in red (acidic: D or E) or blue (basic: H, K, or R). Hydrophobic (non-polar: A, G, I, L, M, V, or W) residues are colored gray and hydrophilic (polar: N, Q, S, T, or Y) residues are depicted as yellow. (B) The α-helical backbone (white) is depicted with the charged residues (colored as above) between positions 48-219. The three dimensional rendering of the side chains was removed to highlight the linear banding pattern formed by the residues. (C) End-on view of residues 149-240 with only the charged amino acids visible.
Figure 2.5 Helical model of AfrLEA2. (A) Two views of AfrLEA2 modeled as a single helical rod. The amino acid sequence for AfrLEA2 was modeled as an alpha helix with Chimera Software and images were generated with Swiss-PDB Viewer (DeepView, version 4.1). Charged amino acids are depicted in red (acidic: D or E) or blue (basic: H,K, or R). Hydrophobic (non-polar: A, G, I, L, M, V, or W) residues are colored gray and hydrophilic (polar: N, Q, S, T, or Y) residues are depicted as yellow. (B) The α-helical backbone (white) is depicted with the charged residues (colored as above) between positions 131-232. The three dimensional rendering of the side chains was removed to highlight the linear banding pattern formed by the residues. (C) End-on view of residues 29-98 with only the charged amino acids visible.
2.4 Discussion

In order to predict the role of LEA proteins in desiccation tolerance, it is important to determine their location within the cell. In the present study I experimentally demonstrated that AfrLEA3m is located in the matrix of mitochondria isolated from embryos of *A. franciscana*. In response to detergent treatment, four previously identified AfrLEA3m isoforms (Boswell et al., 2014b) were released from mitochondria in concert with fumarse, an enzyme marker for the matrix (Figure 2.2). Furthermore, when recombinant AfrLEA3m was incubated with isolated rat mitochondria, import of the protein and cleavage of the N-terminal leader sequence occurred, the latter event presumably accomplished by a mitochondrial processing peptidase found in the matrix (Figure 2.3). The processed protein did not accumulate in mitochondria that lacked a membrane potential, which is necessary for import across the inner mitochondrial membrane. Therefore, it is clear that recombinant AfrLEA3m is imported into the matrix compartment of isolated rat mitochondria. Localization of AfrLEA3m in the matrix of mitochondria is consistent with the presence of a N-terminal leader sequence as predicted by bioinformatics (Menze et al., 2009). Structural features also hold important clues for predicting the function of proteins. Molecular modeling of AfrLEA3m and AfrLEA2 revealed structural features consistent with amphipathic proteins that associate with lipid bilayers (Figure 2.4 and 2.5). Similar structural features have been described for LEA proteins able to effectively protect liposomes that were air-dried or frozen (Thalhammer et al., 2014; Tolleter et al., 2010). The proposed mechanism by which LEA proteins with amphipathic character stabilize lipid bilayers is described briefly below.
Matrix Localization of AfrLEA3m in *Artemia franciscana* Embryos

All four AfrLEA3m variants were experimentally demonstrated to reside in the matrix of mitochondria from *A. franciscana* embryos. Demonstrating the matrix localization is important for understanding the role these proteins play in desiccation tolerance of *A. franciscana* embryos. The location of the AfrLEA3m isoforms suggests that these proteins are involved in protecting macromolecules found within the matrix compartment and/or associated with the inner mitochondria membrane, which includes much of the electron transport system and phosphorylation machinery that are critical for mitochondrial metabolic function. In this regard, an intrinsically disordered protein (COR15) related to LEA proteins that is targeted to the soluble stroma fraction of chloroplasts has recently been shown to possess remarkable specificity of function; it stabilizes chloroplast membranes but not plastidic proteins during freezing (Hincha and Thalhammer, 2012; Thalhammer et al., 2014; Thalhammer and Hincha, 2014; Thalhammer et al., 2010). It is important to point out that while travelling from the cytoplasm, where AfrLEA3m is synthesized, to the mitochondria, the protein could provide protection to other cellular components as well. Indeed, when recombinant AfrLEA3m is expressed in mammalian cells, plasma membrane integrity is improved after acute drying and rehydration compared to cells without the protein (Li et al., 2012). Thus, AfrLEA3m is capable of stabilizing non-mitochondrial cellular components and effectively reducing damage to lipid bilayers *in vivo*.

Import of Recombinant AfrLEA3m into Isolated Rat Mitochondria

Recombinant AfrLEA3m was successfully imported into mitochondria isolated from rat liver. Formation of a lower molecular weight product suggests processing by a mitochondrial processing peptidase (MPP) in the matrix. The absence of any mature product in mitochondria lacking a membrane potential verifies that this lower molecular weight peptide is not a result of
non-specific degradation. Formation of mature AfrLEA3m in the matrix is detectable after just 2 min of incubation, which is rapid compared to the positive control protein (Su9-DHFR) that requires 5 min. Partial degradation of the matrix-processed AfrLEA3m by externally added protease is consistent with formation of a translocation intermediate spanning the outer and inner membrane (Cyr et al., 1993). Thus, while the N-termini of some AfrLEA3m molecules were exposed to the matrix and processed, some the C-termini could still remain outside of the mitochondria. Translocation intermediates spanning both mitochondrial membranes have been described for mitochondria with low levels of ATP in the matrix (Cyr et al., 1993). When matrix ATP is depleted, the protein is allowed to slip back out of the import machinery where it can be degraded by externally added protease. If degradation of mature AfrLEA3m were due to low levels of ATP in the matrix, it would imply that more ATP is required to import AfrLEA3m than Su9-DHFR, which was successfully imported and fully protected from externally-added protease.

Molecular Modeling of AfrLEA2 and AfrLEA3m

Models of AfrLEA2 and AfrLEA3m reveal regions where amino acids of positive and negative charge align in parallel bands, with acidic (negative) residues flanked to either side by basic (positive) residues. Such a unique arrangement of amino acids closely resembles class A α-helices found in apolipoproteins. Amphipathic α-helices align perpendicular to lipid bilayers with their hydrophobic domains facing the interior of the membrane and the positive charges interacting with phosphate head groups (Hristova et al., 1999; Mishra et al., 1994; Segrest et al., 1992). Positioning of LEA proteins between phospholipid headgroups preserves spacing between lipids during water stress, which is thought to prevent phase transitions and membrane inversion (Steponkus et al., 1998; Thalhammer et al., 2014; Tolleter et al., 2010). Consistent
with formation of amphipathic $\alpha$-helices, several LEA proteins are capable of interacting with lipid bilayers (Popova et al., 2011; Pouchkina-Stantcheva et al., 2007; Thalhammer et al., 2010; Tolleter et al., 2010), although membrane stabilization during water stress has only been experimentally demonstrated for three LEA proteins from plants: PsLEAm (drying), COR15a and COR15b (freezing; also known as LEA23 and LEA24) (Artus et al., 1996; Steponkus et al., 1998; Thalhammer et al., 2014; Tolleter et al., 2010). It is appropriate to note that formation of bands of charged amino acids does not necessarily mean that a LEA protein will stabilize lipid bilayers. LEA7 from *Arabidopsis thaliana* forms amphipathic $\alpha$-helices and interacts with lipid bilayers but does not improve the stability of air-dried liposomes (Popova et al., 2011; Popova et al., 2015). Therefore, while molecular models of LEA proteins are valuable for predicting the function of the protein, it is necessary to experimentally demonstrate liposome stabilization to confirm that a specific LEA protein indeed protects lipid bilayers.
CHAPTER 3.
LIPOSOMES WITH DIVERSE COMPOSITIONS ARE PROTECTED DURING DESICCATION BY LEA PROTEINS FROM ARTEMIA FRANCISCANA AND TREHALOSE

3.1 Introduction
Organisms adapted to water stress often possess low molecular weight solutes inside their cells that counteract osmotic fluctuation (Yancey, 2005; Yancey et al., 1982). Some of these solutes, such as trehalose, can stabilize biological structures (Auton et al., 2011; Bolen and Rose, 2008; Crowe et al., 1997; Crowe et al., 2005; Street et al., 2006; Sun et al., 1996; Tapia and Koshland, 2014; Timasheff, 2002). A suite of specialized proteins are also expressed by animals adapted to such conditions. Prominent among these are small stress proteins (Clegg, 2011; Feder and Hofmann, 1999; Hand et al., 2015; King and MacRae, 2015; Ma et al., 2005) and intrinsically disordered proteins (Tompa and Kovacs, 2010; Uversky and Dunker, 2010), the latter group including Late Embryogenesis Abundant (LEA) proteins. In this study, I will evaluate the protection of liposomes in the dried state by two LEA proteins from Artemia franciscana (AfrLEA3m and AfrLEA2), by the sugar trehalose, and by LEA protein and trehalose in combination.

LEA proteins are a family of intrinsically disordered proteins that were first discovered in germinating cottonseeds in 1981 (Dure et al., 1981). Since that time they have been found in seeds and desiccation tolerant tissues of many plant species (Tunnacliffe and Wise, 2007). In recent years, LEA proteins have been found to accumulate during dehydration tolerant stages of development in a number of non-plant organisms. These include prokaryotes (Battista et al., 2001), rotifers (Tunnacliffe et al., 2005), nematodes (Browne et al., 2002), larvae of an insect (Polypedilum vanderplanki, the African chironomid (Kikawada et al., 2006)), and embryos of the brine shrimp (Artemia franciscana (Hand et al., 2007)). Many LEA proteins are unstructured in solution and adopt a secondary structure as water is removed. Due to a biased amino acid
composition, LEA proteins are extremely hydrophilic. LEA proteins are divided into 6 groups
based on possession of repeated sequence motifs. The majority of LEA proteins found in
animals belong to Group 3 (Hand and Menze, 2015; Hand et al., 2011b; Tunnaciffe and Wise,
2007). In vitro experiments indicate that LEA proteins may perform several functions in
desiccation-tolerant cells. During drying, LEA proteins protect target enzymes (Boswell et al.,
2014a; Grelet et al., 2005) and prevent protein aggregation (Goyal et al., 2005; Pouchkina-
Stantcheva et al., 2007). Several LEA proteins form amphipathic alpha-helices capable of
interacting with lipid bilayers (Popova et al., 2011; Pouchkina-Stantcheva et al., 2007;
Thalhammer et al., 2010; Tolletter et al., 2010), although membrane stabilization during water
stress has only been experimentally demonstrated for three LEA proteins from plants: PsLEAm
(drying), COR15a and COR15b (freezing; also known as LEA23 and LEA24) (Artus et al.,
1996; Steponkus et al., 1998; Thalhammer et al., 2014; Tolletter et al., 2010). Additionally, LEA
proteins may form structural networks (Shimizu et al., 2010) and strengthen glasses formed by
protective sugars (Wolkers et al., 2001a).

Targeting of LEA proteins to different compartments within the cell explains some of the
multiplicity of LEA proteins within a given species (Hand et al., 2011b). Subcellular
localization emphasizes the necessity of protecting organelles from water stress-induced damage.
To date, mitochondria-targeted LEA proteins have been identified definitively in two species:
*Pisum sativum*, the pea plant (Grelet et al., 2005), and the brine shrimp *Artemia franciscana.*
The identification and predicted mitochondrial localization of AfrLEA3m from *A. franciscana*
was first reported by Menze et al. (Menze et al., 2009). Experimental evidence now supports a
mitochondrial location for AfrLEA3m, based on Western blots of isolated mitochondria and
immunohistochemistry of embryos (Boswell and Hand, 2014; Boswell et al., 2014b).
Interestingly, *A. franciscana* mitochondria contain four isoforms of AfrLEA3m and at least two Group 1 LEA proteins (Boswell et al., 2014b; Marunde et al., 2013; Toxopeus et al., 2014; Warner et al., 2012; Warner et al., 2010). Functionally, AfrLEA3m protects target enzymes against desiccation-induced damage (Boswell et al., 2014a), and when transfected into human HepG2 cells, preserves membrane integrity and viability after acute desiccation and rehydration (Li et al., 2012).

To date, no cytoplasmically-localized LEA protein has been reported to stabilize lipid bilayers *in vitro*. AfrLEA2 is located in the cytoplasm of *A. franciscana* embryos (Boswell and Hand, 2014; Hand et al., 2007), and shares similar abilities with AfrLEA3m to protect target enzymes and HepG2 cells during drying (Boswell et al., 2014a; Li et al., 2012). It has been suggested that a given LEA protein may preferentially stabilize membranes of a particular lipid composition based on the protein’s subcellular location (Hincha and Thalhammer, 2012; Thalhammer et al., 2010; Tolleter et al., 2010). Using both cytoplasmic-localized and mitochondrial-targeted LEA proteins to study protection of liposomes during drying should allow a direct test of this concept. For example, one prediction would be that AfrLEA3m will stabilize liposomes mimicking the inner mitochondrial membrane to a greater degree than AfrLEA2.

Numerous studies have demonstrated the ability of trehalose to stabilize biological structures subjected to desiccation and freezing (Auton et al., 2011; Crowe et al., 2005; Sun et al., 1996; Tapia and Koshland, 2014). The efficacy of trehalose during severe dehydration is partially attributed to its ability to form sugar glasses (Crowe et al., 1998; Sun et al., 1996) and to replace the hydration water normally associated with phospholipid head groups of lipid bilayers, i.e., the ‘water replacement hypothesis’ (Crowe et al., 1987; Webb, 1965). Damage to lipid
bilayers during drying is primarily attributed to two stresses: fusion and phase transitions (Crowe et al., 1984). Trehalose has been documented to protect both liposomes and native membranes (Crowe et al., 1984; Crowe et al., 2005; Li et al., 2012; Liu et al., 2005; Sun et al., 1996; Wolkers et al., 2001b). Trehalose prevents membrane fusion of dried liposomes, presumably due to its ability to foster glass formation (Crowe et al., 1998; Hincha and Hagemann, 2004; Sun et al., 1996). The phase transition temperature \( T_m \) of lipid bilayers increases dramatically during desiccation, such that the liquid-crystalline state can convert to a gel state at room temperature. Bilayers become leaky during phase transitions (Crowe et al., 1989). In order to depress the \( T_m \) and prevent damaging phase transitions during drying and rehydration, and the sugar must be present on both sides of the bilayer for optimal protection (Crowe et al., 1992). One proposed mechanism for the strong depression of \( T_m \) by trehalose is the replacement of water bound to phospholipid head groups as discussed above.

In the present study, liposomes with compositions simulating the inner mitochondrial membrane, the outer mitochondrial membrane, and inner leaflet of the plasma membrane were air dried with and without AfrLEA2, AfrLEA3m, and trehalose. Additionally, each LEA protein was tested in combination with trehalose to determine whether liposome protection (as assessed with a carboxyfluorescein leakage assay) would be enhanced beyond that afforded by LEA protein alone; additivity and in some cases synergistic effects have demonstrated in drying studies with target enzymes (Boswell et al., 2014a; Goyal et al., 2005). Consistent with the formation of amphipathic \( \alpha \)-helices depicted in molecular models of AfrLEA3m and AfrLEA2 (Chapter 2), both LEA proteins clearly stabilized all three types of liposomes significantly better than a control protein (lysozyme). However, there were no clear differences in the degree of protection afforded by either LEA protein that were correlated with specific liposome
compositions. Additive protection by LEA proteins in combination with trehalose was observed in some but not all cases, which indicates that additivity is dependent on liposome composition.

3.2 Materials and Methods

Biochemicals

Lipids and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). All lipids were naturally derived in order to mimic the fatty acid distribution found in eukaryotic cells. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) were derived from bovine liver (Avanti Polar Lipids; product numbers 840055, 840026, 840042). Phosphatidylserine (PS) was purified from bovine brain tissue (Avanti Polar Lipids; product number 840032) and cardiolipin (CL) from bovine heart (Avanti Polar Lipids; product number 840012). Cholesterol was isolated from ovine wool (Avanti Polar Lipids; product number 700000P). 5(6)-Carboxyfluorescein (CF) (product number 21877) was obtained from Sigma-Aldrich (St. Louis, MO). Trehalose (product number T-104-4) was purchased from Pfanstiehl (Waukegan, IL).

Liposome Preparation

Lipids were combined with mass ratios simulating the inner mitochondrial membrane, outer mitochondrial membranes, and inner leaflet of the plasma membrane of mammalian cells. The lipid compositions of mammalian membranes are well-defined (Horvath and Daum, 2013; van Meer et al., 2008), and these membranes are known to be sensitive to water stress-induced damage (Crowe et al., 1989; Crowe et al., 2005). Consequently the lipid contents of these membranes were used as a first approximation to prepare representative liposomes (Table 3.1). For comparison to a non-physiological bilayer, liposomes composed entirely of phosphatidylcholine were also prepared. Lipid mixtures were dried under a stream of nitrogen,
and then stored under vacuum overnight to remove residual solvent. For a typical batch, 20 mg of total lipids were used. Lipids were rehydrated in 1 ml of 100 mM CF, 10 mM TES (2-[[1,3-dihydroxy-2-(hydroxymethyl)-2-propanyl]amino]ethanesulfonic acid), 0.1 mM EDTA (pH 7.4) for at least 1 h with intermittent vortexing at 70°C, a temperature sufficiently high to ensure all phospholipids were in the liquid crystalline phase. Then small unilamellar liposomes (SULs) were prepared with a hand held mini extruder (Avanti Polar Lipids, Alabaster, AL). Lipid preparations were passed through two stacked polycarbonate membranes with 1 µm pore size prior to passage through two stacked membranes with 0.1 µm pore size at 70°C. Liposomes were extruded through the 0.1 µm membranes at least 10 times to ensure uniform size distribution. After extrusion, liposomes were eluted through a Sephadex G-25 column (NAP-5, GE Healthcare, Buckinghamshire, UK) that was equilibrated in TEN buffer (50 mM NaCl, 10 mM TES, 0.1 mM EDTA, pH 7.4) to remove CF not entrapped by the liposomes. Liposomes were mixed with 250 mM trehalose and/or LEA proteins to yield various protein:lipid mass ratios between 0.1 and 0.4. For some experiments (i.e., Fig. 3.2 D), trehalose was included during the preparation of SULs, so that the sugar also was present inside of the liposomes. The final concentration of liposomes was approximately 5 mg total lipid/ml.

In order to compare the impact of LEA proteins to a negative control (i.e., a protein predicted to be non-stabilizing), liposomes were also dried with lysozyme at identical protein:lipid mass ratios. Lysozyme was chosen because it retains its native structure when dried and does not interact with membranes (Nagendra et al., 1998; Pap et al., 1996). Finally, it is appropriate to note that desiccation-tolerant embryos of brine shrimp contain large concentrations of trehalose (Carpenter and Hand, 1986; Clegg, 1965; Clegg, 2005), and
consequently it is relevant to include this solute in the present studies of liposome protection by LEA proteins that originate from the same species.

CF Leakage Measurements

For CF leakage assays, 2 µl droplets of liposome mixtures were placed in the wells of opaque 96-well plates (OptiPlate 96-F, PerkinElmer, Waltham, MA). Droplets were air dried in the dark overnight or for one week at room temperature in an airtight desiccation cabinet (Fisher Scientific, Hampton, NH). Droplets were rehydrated by the addition of 300 µl of TEN buffer at room temperature and aspirated by pipet to achieve a uniform suspension. Fluorescence of CF is strongly quenched at the high concentration of 100 mM contained inside of the liposomes, but increases when CF is released into the medium (Weinstein et al., 1986). After measuring CF fluorescence of rehydrated liposomes (F₀), Triton X-100 was added to a final concentration 1 % in order to fully lyse the liposomes (Fₜ). The percent CF leakage was calculated as (F₀/Fₜ) x 100. Measurements were made with a Victor 3 Multilabel Counter (PerkinElmer, Waltham, MA) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. In the presence of 1 % Triton X-100, significant quenching of CF fluorescence was observed. Therefore, Fₜ measurements were corrected using standard curves of CF fluorescence versus known concentrations of the dye in the presence and absence of detergent. While this correction was essential, it sometimes led to control values (no protectant) slightly above 100% CF leakage.

LEA Protein Purification

AfrLEA3m and AfrLEA2 were expressed and purified as described in detail in Chapter 2.

Statistics

Two-way ANOVA paired with a Tukey post-hoc test were used to compare protection of each type of liposome by AfrLEA3m, AfrLEA2 or lysozyme across the range of protein:lipid
Table 3.1: Composition of liposomes used to mimic mammalian membranes. Mixtures are expressed as percent (w/w).

<table>
<thead>
<tr>
<th>Mammalian Membrane</th>
<th>Composition of liposomes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inner Leaflet of the Plasma Membrane</td>
<td>50 % PC</td>
<td>(van Meer et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>30 % PE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 % PS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 % Cho</td>
<td></td>
</tr>
<tr>
<td>Inner Mitochondrial Membrane</td>
<td>50 % PC</td>
<td>(Horvath and Daum, 2013)</td>
</tr>
<tr>
<td></td>
<td>30 % PE</td>
<td>(Tolleter et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>20 % CL</td>
<td></td>
</tr>
<tr>
<td>Outer Mitochondrial Membrane</td>
<td>55 % PC</td>
<td>(Horvath and Daum, 2013)</td>
</tr>
<tr>
<td></td>
<td>30 % PE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 % PI</td>
<td></td>
</tr>
</tbody>
</table>

*Cho- Cholesterol; CL- Cardiolipin; PC-phosphatidylcholine; PE- Phosphatidylethanolamine; PI-phosphatidylinositol; PS- Phosphatidylserine
mass ratios tested (Prism 6; GraphPad Software, La Jolla, CA). A Bonferroni multiple comparisons test of sample means was used to identify statistical differences between trehalose and protein combinations compared to each component alone (Prism 6). Multiple t-tests combined with a Holm-Sidak post hoc test were used to compare CF leakage from liposomes after short-term versus long-term drying (Prism 6). Significance level was set at $p \leq 0.05$.

3.3 Results

Short-term Drying of Liposomes with LEA Proteins

Stability of liposomes during drying and rehydration was assessed by measuring leakage of entrapped carboxyfluorescein (CF) upon rehydration. Leakage from liposomes simulating the inner mitochondrial membrane (IMM), the outer mitochondrial membrane (OMM) or inner leaflet of the plasma membrane (ILPM) was reduced in a concentration dependent manner by both AfrLEA2 and AfrLEA3m (Fig. 3.1). In order to compare multiple liposome mixtures, leakage was normalized to that of control liposomes, which were dried without any protectant. When liposomes with realistic biological compositions were dried overnight with AfrLEA2 or AfrLEA3m at a 0.4 (protein:lipid) mass ratio, liposomes retained 38-48 % more of entrapped CF than liposomes dried without any protectant (Fig. 3.1A and B). No additional protection was measured for PC and IMM-like liposomes dried with LEA proteins at a 0.8 (protein:lipid) mass ratio (data not shown). By comparison, lysozyme (a control protein known for its lack of interaction with lipids, see Materials and Methods), did not exhibit concentration-dependent protection of any liposome tested; liposomes retained only ~13 % of entrapped dye at all protein:lipid ratios tested. When dried with LEA proteins, retention of entrapped dye by liposomes mimicking biological membranes was significantly greater at all protein:lipid mass ratios compared to liposomes dried with lysozyme (2-way ANOVA, $p \leq 0.05$, n = 6). Non-
Figure 3.1  Dye leakage from liposomes dried overnight and rehydrated in the presence of LEA proteins and lysozyme. The relative stability of the membranes was determined by measuring the loss of entrapped fluorescent dye carboxyfluorescein (CF), expressed as a percentage of complete dye release quantified by detergent solubilization. Experimental treatments were then normalized to control treatments (no protectant), which was necessary due to inherent differences in control leakage across compositional types. Lipid compositions simulated those of the inner mitochondrial membrane, the outer mitochondrial membrane, the inner leaflet of the plasma membrane, or a non-biological composition of 100 % phosphatidylcholine. Liposomes were dried with the indicated protein:lipid mass ratios of (A) AfrLEA2, (B) AfrLEA3m, (C) lysozyme. Data represent the mean ± SD of n = 6 samples; where error bars are absent, the SD was less than the size of the symbol. Statistical analysis was omitted from figures for clarity. At all protein:lipid mass ratios, stabilization by LEA proteins was significantly greater than that afforded by lysozyme for all membranes except 100 % PC liposomes (2-way ANOVA, p ≤ 0.05, n= 6).
biological membranes composed of 100% phosphatidylcholine (PC) leaked 76-85% of entrapped CF when dried with LEA proteins at the highest protein concentrations tested. CF leakage in the presence of LEA proteins was not significantly less than leakage from PC liposomes dried with the same amount of lysozyme except at the highest concentration of AfrLEA2.

Short-term Drying of IMM-like Liposomes with LEA Proteins and Trehalose

When trehalose was present only outside of liposomes that simulated the IMM, the sugar provided little protection (CF leakage, 95.7 ± 1.6%; mean ± SD, n = 3-6) (Fig. 3.2). These results are similar to other liposome drying studies where trehalose was confined to the outside of liposomes (Crowe and Crowe, 1992; Hincha and Hagemann, 2004). At the highest concentration tested with AfrLEA2 alone, CF leakage was depressed to 64.2 ± 0.4% (mean ± SD, n = 3-6) for IMM liposomes dried and rehydrated (Fig. 3.2A). At the same concentration of AfrLEA2 but with the addition of 250 mM trehalose outside, CF leakage decreased to 56.1 ± 0.8%. The amount of protection afforded by the combination of AfrLEA2 and trehalose was significantly greater than that with either component alone at the highest and lowest protein:lipid mass ratios tested (2-way ANOVA, Bonferroni multiple comparisons test, p ≤ 0.05, n=6). AfrLEA3m alone decreased CF leakage from IMM liposomes to 65.2 ± 2.1% (mean ± SD, n = 3-6) at the highest protein:lipid mass ratios tested (Fig. 3.2B). When both trehalose and AfrLEA3m were present, CF leakage was further decreased to 56.6±6.1% (mean ± SD, n = 3-6) at the same protein:lipid mass ratio (Fig. 2B). CF leakage was significantly lower at all protein:lipid mass ratios tested (2-way ANOVA, p ≤ 0.05, n = 6) when trehalose and AfrLEA3m were both present compared to either component alone; thus protection by these factors was additive. The presence of lysozyme also enhanced the ability of external trehalose to stabilize
IMM-like liposomes (Fig. 3.2 C), but the overall impact was small; CF leakage was only depressed to 76.9 ± 0.6 % (mean ± SD, n = 3-6) at the highest lysozyme:lipid ratio tested in the presence of 250 mM trehalose outside, which was far less protection than that afforded by LEA proteins alone or in combination with trehalose.

In order to quantify maximal stabilization by trehalose, IMM-like liposomes were also prepared with 250 mM trehalose present inside of the liposomes as well as outside (Fig. 3.2D). Not surprisingly, when dried with both internal and external trehalose, CF leakage was diminished to 38.9±0.6 %, which was a 56 % decrease in leakage compared to liposomes dried with external trehalose only (Fig. 3.2D). Furthermore, overall CF leakage was significantly lower than that seen with any combination of LEA protein and trehalose when these constituents are restricted to the outside of liposomes. No further improvement in CF retention was observed when liposomes containing 250 mM trehalose inside were dried with both trehalose and LEA proteins present outside in the medium (data not shown).

Short-term Drying of ILPM-like Liposomes with LEA Proteins and Trehalose

When dried in the presence of external trehalose, ILPM-like liposomes leaked 93.3 ± 7.9% (mean ± SD, n = 6) of entrapped CF (Fig. 3.3). The greatest protection by AfrLEA2 alone reduced CF leakage to 65.2 ± 4.2 % (mean ± SD, n = 6) (Fig. 3.3A). When AfrLEA2 and external trehalose were used in conjunction, protection was significantly greater than with either component alone at the lower concentrations of AfrLEA2 tested but not at the highest (2-way ANOVA, Bonferroni multiple comparisons test, p ≤ 0.05, n=6). AfrLEA3m depressed CF leakage from ILPM-like liposomes to 58.6 ± 4.3 % (mean ± SD, n = 6) (Fig. 3.3B). Unexpectedly, the addition of external trehalose did not significantly increase protection by AfrLEA3m at any of the protein:lipid mass ratios tested. Once again, protection by lysozyme
Figure 3.2. Dye leakage from liposomes simulating the inner mitochondrial membrane dried overnight and rehydrated in the presence of trehalose, LEA proteins and lysozyme. The relative stability of the membranes was determined by measuring the loss of entrapped fluorescent dye carboxyfluorescein (CF). CF leakage was expressed as a percentage of complete dye release, which was measured by detergent solubilization. (A) AfrLEA2 and (B) AfrLEA3m were added to drying media at the indicated protein:lipid mass ratios in the presence and absence of 250 mM trehalose. (C) Liposomes were also dried with equal concentrations of lysozyme as a control protein. Data represent mean ± SD of n = 3-6 samples; where error bars are absent, the SD was less than the size of the symbol. Asterisks indicate significantly greater protection by trehalose and protein combined than either component alone (2-way ANOVA, Bonferroni multiple comparisons test, p ≤ 0.05, n =3-6). (D) IMM-like liposomes were dried with 250 mM trehalose present on both sides of the lipid bilayer for comparison to liposomes dried without protectant and with trehalose present only outside of the liposomes. Data represents the mean ± SD of n= 6 samples.
Figure 3.3 Dye leakage from liposomes simulating the inner leaflet of the plasma membrane dried overnight and rehydrated in the presence of trehalose, LEA proteins, and lysozyme. The relative stability of liposomes was determined by measuring the loss of entrapped carboxyfluorescein (CF). CF leakage was expressed as a percent of total CF loss, which was measured by detergent solubilization. (A) AfrLEA2 and (B) AfrLEA3m were added to drying media at the indicated protein:lipid mass ratios in the presence and absence of 250 mM trehalose. (C) Liposomes were also dried with equal concentrations of lysozyme, a non-stabilizing protein, for comparison. Data represent mean ± SD of n = 3-6 samples; where error bars are absent, the SD was less than the size of the symbol. Asterisks indicate significantly greater protection by trehalose and protein combined than either component alone (2-way ANOVA, Bonferroni multiple comparisons test, p ≤ 0.05, n = 6).
and external trehalose was found to be additive. However, maximal protection by lysozyme and trehalose (CF leakage, 70.2 ± 4.9 %; mean ± SD, n = 6) was statistically less than that of liposomes dried with AfrLEA2 in conjunction with external trehalose (2-way ANOVA, Bonferroni multiple comparisons test, p ≤ 0.05, n=6).

Short-term Drying of OMM-like Liposomes with LEA Proteins and Trehalose

Liposomes that simulated the OMM lost 81.0 ± 6.3 % (mean ± SD, n = 6) of entrapped CF when dried in the presence of 250 mM trehalose outside (Fig. 3.4). CF leakage was depressed to 53.2 ± 7.8 % (mean ± SD, n = 6) when OMM-like liposomes were dried with AfrLEA2 (Fig. 3.4A). The combination of trehalose and AfrLEA2 did not significantly improve stability of OMM-like liposomes. Stabilization of OMM model liposomes by AfrLEA3m was similar to that of AfrLEA2; maximal protection resulted in 59.8±3.4 % CF leakage (mean ± SD, n = 6) (Fig. 3.4B). Once again, no additivity occurred during drying when 250 mM trehalose was included in the medium with AfrLEA3m. When OMM-like liposomes were dried with lysozyme and trehalose, CF leakage was not statistically different than with 250 mM trehalose alone, but leakage was significantly reduced compared to lysozyme alone (2-way ANOVA, Bonferroni multiple comparisons test, p<0.05, n= 6) (Fig. 3.4C). Stability of OMM-like liposomes dried with a combination of lysozyme and 250 mM trehalose was less than that for AfrLEA3, except at the highest lysozyme concentration (2-way ANOVA, Bonferroni multiple comparisons test, p ≤ 0.05, n= 6). Maximal protection by AfrLEA2 at all protein concentrations tested was greater than that of lysozyme and external trehalose (2-way ANOVA, Bonferroni multiple comparisons test, p<0.05, n= 6).
Figure 3.4 Dye leakage from liposomes simulating the outer mitochondrial membrane dried overnight and rehydrated in the presence of trehalose, LEA proteins, and lysozyme. The relative stability of liposomes was determined by measuring the loss of entrapped carboxyfluorescein (CF). CF leakage was expressed as a percent of total CF loss, which was measured by detergent solubilization. (A) AfrLEA2 and (B) AfrLEA3m were present at the indicated protein:lipid mass ratios in the presence and absence of 250 mM trehalose. (C) Liposomes were also dried with equal concentrations of lysozyme for comparison to a non-stabilizing protein. Data represent mean ± SD of n = 6 samples; where error bars are absent, the SD was less than the size of the symbol. Asterisk indicates significantly greater protection by trehalose and protein combined than either component alone (2-way ANOVA, Bonferroni multiple comparisons test, p ≤ 0.05, n =6).
Figure 3.5  Dye leakage from liposomes composed of phosphatidylcholine dried overnight and rehydrated in the presence of trehalose, LEA proteins, and lysozyme. The relative stability of liposomes was determined by measuring the loss of entrapped carboxyfluorescein (CF). CF leakage was expressed as a percent of total CF loss, which was measured by detergent solubilization. (A) AfrLEA2 and (B) AfrLEA3m were added to drying media at physiologically relevant protein:lipid mass ratios in the presence and absence of 250 mM trehalose. (C) Liposomes were also dried with equal concentrations of lysozyme as a control protein. Data represent mean ± SD of n = 6 samples; where error bars are absent, the SD was less than the size of the symbol.
Short-term Drying of 100 % PC Liposomes with LEA Proteins and Trehalose

Liposomes composed of 100 % PC were prepared in order to assess whether LEA proteins would protect a bilayer with a non-biological composition. Loss of CF from PC liposomes dried with LEA proteins was not less than leakage from PC liposomes dried without any additive except at the highest protein:lipid mass ratio tested (2-way ANOVA, Bonferroni multiple comparisons test, $p \leq 0.05$, n=6). PC liposomes dried overnight with AfrLEA2 lost 69.0 ± 2.3 % (mean ± SD, n = 6) of entrapped CF and 76.6 ± 2.4 % with AfrLEA3m (Fig. 3.5A and B). Leakage from PC liposomes dried with lysozyme was equivalent to that for LEA proteins except at the highest protein:lipid mass ratio tested, where leakage with lysozyme was greater than that with AfrLEA2 (2-way ANOVA, Bonferroni multiple comparisons test, $p \leq 0.05$, n=6). There was no significant difference in CF leakage from PC liposomes when trehalose was used in conjunction with either LEA protein compared to protection by the proteins alone.

Long-term Drying of Liposomes with LEA Proteins and Trehalose

The stability of dried liposomes after 7 days of storage at room temperature and 0 % relative humidity was also examined (Fig. 3.6). Protection by combinations of trehalose, AfrLEA2, and AfrLEA3m was evaluated for all four liposome compositions; CF leakage from liposomes dried with lysozyme or without any protectants was also measured. With only four exceptions, significant increases in CF leakage were not observed after 7 days of drying compared to liposomes dried overnight (multiple t-test, Holm-Sidak post hoc test, $p \leq 0.05$). When there were significant increases with extended drying, the magnitude of the effects was very modest. Most cases for which CF leakage increased were observed for OMM-like liposomes, i.e., those dried with lysozyme, AfrLEA2, or AfrLEA3m at a 0.4 protein:lipid mass ratio (multiple t-test, Holm-Sidak post hoc test, $p \leq 0.05$). These increases in CF leakage from
Figure 3.6. Comparison of CF leakage from liposomes air-dried overnight (dark grey bars) or for 7 days (light grey bars) at room temperature with LEA proteins (0.4 protein:lipid mass ratio) and 250 mM external trehalose. Lipid compositions simulated that of the inner (IMM) and outer (OMM) mitochondrial membranes and the inner leaflet of the plasma membrane (ILPM), or a non-biological composition of 100% phosphatidylcholine (100% PC). Control values without additives (no protectant) are also presented. The relative stability of liposomes was assessed by measuring leakage of entrapped dye carboxyfluorescein (CF). CF leakage was expressed at a percentage of total CF loss, which was measured by detergent solubilization. Data represents mean ± SD of n = 3-6 samples. Asterisks indicate a significant difference between samples dried overnight or 7 days (Multiple t-tests, Holm-Sidak post hoc test, p ≤ 0.05).
OMM-like liposomes did not occur when 250 mM trehalose was also present in the external medium (multiple t-test, Holm-Sidak post hoc test, \( p \leq 0.05 \)). The fourth exception to the general lack of increased CF leakage after extended drying occurred with 100% PC liposomes dried with AfrLEA2 and external trehalose (multiple t-test, Holm-Sidak post hoc test, \( p \leq 0.05 \)).

3.4 Discussion

In this study I have shown that two LEA proteins from *A. franciscana* embryos, AfrLEA2 and AfrLEA3m, are effective at protecting liposomes with lipid compositions similar to biological membranes against damage incurred during desiccation. Liposomes with a non-biological composition of 100% phosphatidylcholine are not as effectively protected by either LEA protein, which is consistent with the mechanism of action for amphipathic proteins. The ability of AfrLEA3m or AfrLEA2 to stabilize liposomes does not appear to be dependent on lipid composition, provided there are physiological amounts of bilayer and non-bilayer-forming lipids present. Protection of IMM- and ILPM-like liposomes by AfrLEA3m and AfrLEA2 is significantly enhanced in the presence of the sugar trehalose, but this finding does not hold for OMM-like membranes. Thus additive protection by LEA proteins and trehalose is dependent on the lipid composition of the target membrane. In most cases, additional damage does not occur to liposomes dried for 7 days compared to overnight drying, which suggests that most damage occurs during the initial drying phase or during rehydration.

Liposome Stabilization by AfrLEA2 and AfrLEA3m.

Stabilization of IMM-like liposomes has been demonstrated with one other LEA protein, PsLEAm (Tolleter et al., 2010). PsLEAm is a LEA protein found in the matrix of mitochondria from the pea plant, *Pisum sativum* (Grelet et al., 2005). There is evidence that PsLEAm stabilizes liposomes through direct interaction with the lipid bilayer (Tolleter et al., 2010). As shown for
AfrLEA2 and AfrLEA3m (Chapter 2), models of PsLEAm reveal regions where amino acids form parallel bands of charged residues (Tolleter et al., 2007). These features allow PsLEAm to interact with membranes in a manner similar to that of amphipathic proteins (Mishra et al., 1994; Segrest et al., 1992). The a-helix of amphipathic proteins aligns perpendicular to the phospholipid molecules (i.e. lies flat against the membrane surface), such that the protein settles in between the phospholipid head groups. The positively charged amino acids interact with the negatively charged phospholipids headgroups, and the nonpolar residues face the hydrophobic core of the membrane. (Mishra et al., 1994; Segrest et al., 1992). Positioning of LEA proteins between polar headgroups is thought to maintain spacing between lipids and increase acyl-chain mobility at low water contents, which results in depression of the Tm (Tolleter et al., 2010).

Furthermore, non-bilayer forming lipids such as PE and CL promote inverted hexagonal II (HexII) phase transitions in lamellar membranes, a problem exacerbated by low water content (Cullis et al., 1986; Tilcock, 1986). Arranging of amphipathic molecules between phospholipid headgroups of membranes is thought to alleviate membrane inversion stress and promote retention of the lamellar phase (Steponkus et al., 1998; Thalhammer et al., 2014; Timasheff, 2002). Consistent with this, freezing studies show COR15 proteins (LEA23 and LEA24) are able to retard the transition from lamellar to HexII phase in chloroplast (Steponkus et al., 1998; Thalhammer et al., 2014). Interestingly, protective sugars are less effective at stabilizing membranes that contain certain non-bilayer forming lipids, and in some cases the sugars exacerbate water stress-induced damage (Hincha and Crowe, 1998; Hincha et al., 1998; Oliver et al., 2002). The ability to stabilize membranes with a high representation of non-bilayer forming lipids may be one advantage for the accumulation of LEA proteins in cells despite the presence of protective sugars. In future experiments it could be revealing to dry liposomes with LEA
protein present on both sides of the lipid bilayer, which to our knowledge has not been attempted thus far.

The Effect of Lipid Composition on Protection by LEA Proteins.

AfrLEA3m is predicted to be located in the matrix of *A. franciscana* mitochondria (Menze et al., 2009). Therefore, the membrane with which AfrLEA3m would be expected to primarily interact is the IMM. However, because AfrLEA3m is translated in the cytoplasm, it could potentially interact with the ILPM and OMM prior to import into mitochondria. To date, only models of the IMM and 100% PC membranes have been dried with a matrix-targeted LEA protein, specifically PsLEAm from the pea plant (Tolletter et al., 2010). Logically, it was suggested by these investigators that the presence of cardiolipin, which is found predominantly in the IMM, might be important for facilitating interaction of the matrix-localized PsLEAm with this membrane. A similar specificity has been documented for a chloroplast-targeted stress protein (COR15) and its interactions with the chloroplast lipid monogalactosyldiacylglycerol for membrane stabilization during freezing (Hincha and Thalhammer, 2012; Thalhammer and Hincha, 2014). Therefore, we hypothesized that AfrLEA3m, which is also matrix-targeted, would provide greater protection to IMM-like liposomes than to the OMM or ILPM liposomes. Instead, the data in our study indicate that AfrLEA3m provides similar protection to all three liposome types. Stabilization of ILPM liposomes is consistent with the observation that AfrLEA3m, when transfected into HepG2 cells, improves plasma membrane integrity during acute spin-drying and rehydration (Li et al., 2012). Liposome protection by AfrLEA2 was similar to that of AfrLEA3m for all types of liposomes tested, including the IMM. AfrLEA2, which is located in the cytoplasm (Boswell and Hand, 2014), would not be expected to contact the IMM of intact mitochondria *in vivo*. Thus, it appears that AfrLEA2 and AfrLEA3m stabilize
liposomes without bias for lipid composition, provided the liposomes mimic biological membranes. Note that our findings demonstrate LEA proteins are largely ineffective at stabilizing 100% PC liposomes (non-biological); this result is similar to that reported for arbutin (a glycosylated hydroquinone), apparently due to the tight packing of PC in the hydrated state at high, non-physiological concentrations (Hincha et al., 1999).

Additive Protection of Liposomes with Combinations of Trehalose and LEA Proteins.

The present study demonstrates for the first time the additive protection of lipid bilayers by trehalose plus LEA proteins. Protection of liposomes simulating the IMM and ILPM by the combination of 250 mM trehalose and AfrLEA2 or AfrLEA3m was greater than that afforded by either protectant alone. The lack of additional protection of OMM-like liposomes by combinations of trehalose and LEA protein indicates that cooperative stabilization by these components is dependent on the lipid composition of the lipid bilayer. At this point, the molecular explanation for this result is unclear. Nevertheless, previous results with stabilization of target enzymes during drying also show that additive protection by trehalose and LEA proteins is affected by the target enzyme chosen (Boswell et al., 2014a). Additive protection of cellular components could partially explain why many, but not all (Hengherr et al., 2008; Tunnaciffe and Lapinski, 2003; Tunnaciffe et al., 2005), anhydrobiotic animals accumulate both trehalose and LEA proteins. Precisely how these protective molecules interact with each other is not known, although it has been shown that LEA proteins and model peptides of LEA proteins can stabilize sugar glasses (Shimizu et al., 2010; Wolkers et al., 2001a). The stability of sugar glasses corresponds to an increased glass transition temperature ($T_g$) as well as vitrification at higher water content (Crowe et al., 1998). Formation of sugar glasses is known to be
important for preventing fusion, which results in passive leakiness of components entrapped within compartments formed by lipid bilayers (Crowe et al., 1984; Sun et al., 1996).

Long term drying of liposomes with trehalose and LEA proteins.

Under the majority of conditions tested herein, liposomes do not appear to undergo additional damage after seven days of dry storage at room temperature compared to liposomes dried overnight. OMM-like liposomes were an exception; an increase in CF leakage occurred after extended drying in the presence of LEA proteins alone. While the addition of trehalose did not improve short-term protection of OMM liposomes dried with LEA proteins, it improved long-term stability of these liposomes in the dry state. The data indicate that during extended periods of desiccation, certain compositional types of membranes may be most effectively stabilized by a combination of trehalose and LEA protein, as opposed to either component alone.
CHAPTER 4
CRYOPRESERVATION OF LIPOSOMES AND MEMBRANES BY LEA PROTEINS FROM ARTEMIA FRANCISCANA AND TREHALOSE

4.1 Introduction
Some organisms, including embryos of brine shrimp (Artemia franciscana), are able to survive the virtual absence of water (Clegg, 2005; Crowe et al., 1997; Crowe et al., 1992). Many organisms that tolerate extreme water stress utilize common protective mechanisms. These features include depressed cellular metabolism and accumulation of specialized proteins and osmolytes (Clegg, 1978, 2011; Crowe et al., 1997; Hand and Hardewig, 1996; Yancey et al., 1982). Prominent among the protective molecules found to accumulate in embryos of brine shrimp are late embryogenesis abundant (LEA) proteins (Boswell et al., 2014b; Hand et al., 2007; Menze et al., 2009; Warner et al., 2010) and the sugar trehalose (Clegg, 1965; Patil et al., 2013). LEA proteins are a family of specialized proteins often expressed in desiccation-tolerant plants and animals (Hand et al., 2011b; Tunnacliffe and Wise, 2007). First identified over 30 years ago in germinating cotton seeds, LEA proteins are expressed during desiccation-tolerant life stages and water stress-tolerant tissues of several plant species (Tunnacliffe and Wise, 2007). More recently, interest in LEA proteins has expanded since their discovery in a number of anhydrobiotic animal species (Hand et al., 2011b; Tunnacliffe and Wise, 2007). Trehalose is a disaccharide composed of two glucose molecules joined by an α-1-1-glycosidic bond that accumulates in desiccation tolerant animal species in response to water deficit (Clegg, 1965; Madin and Crowe, 1975; Tapia and Koshland, 2014). In the study presented below, I evaluate the ability of two LEA proteins from A. franciscana and trehalose to protect liposomes with lipid compositions similar to membranes found in mammalian cells from freeze-induced damage. Furthermore, I will investigate the ability of trehalose to stabilize isolated mitochondria during freezing and thawing.
Several LEA proteins form amphipathic alpha-helices capable of interacting with lipid bilayers (Popova et al., 2011; Pouchkina-Stantcheva et al., 2007; Thalhammer et al., 2010; Tolleter et al., 2010). However, stabilization of lipid bilayers during water stress has only been experimentally demonstrated with three LEA proteins from plants. COR15a and COR15b (LEA23 and LEA24) from *Arabidopsis thaliana* have been shown to protect chloroplast membranes from freeze-induced damage both *in vitro* and *in vivo* (Artus et al., 1996; Steponkus et al., 1998; Thalhammer et al., 2014). PsLEAm from the pea plant (*Pissum savitum*) was found to improve the stability of both dried or frozen liposomes (Tolleter et al., 2010). Liposomes frozen in the study by Tolleter et al. (2010) were comprised entirely of phosphatidylcholine. In drying studies LEA proteins are far more effective at stabilizing liposomes if the lipid compositions are similar to biological membranes as opposed to 100% phosphatidylcholine (Chapter 3; Tolleter et al., 2010). Therefore, I will investigate the ability of two LEA proteins from *A. franciscana* (AfrLEA2 and AfrLEA3m) to protect liposomes mimicking the inner mitochondrial membrane, outer mitochondrial membrane, and inner leaflet of the plasma membrane from freeze-induced damage and compare the results to protection of liposomes composed of phosphatidylcholine.

Numerous studies have demonstrated the ability of trehalose to protect liposomes and membranes from freezing and desiccation-induced damage (Buchanan et al., 2004; Crowe et al., 1984; Crowe et al., 2005; Liu et al., 2005; Sun et al., 1996; Wolkers et al., 2001b; Yamaguchi et al., 2007). Like many sugars, trehalose forms a glass at low water contents and/or temperatures (Crowe et al., 1998). Trehalose is often superior to other sugars at protecting macromolecules due to its relatively high glass transition temperature (Crowe et al., 2001; Crowe et al., 2005). Sugar glasses provide structural support and immobilize cellular components, which is important
for preventing deleterious interactions. In addition to sugar glass formation, there is evidence that in the dry state trehalose has the ability to bind to membranes in the place of water (Crowe et al., 1987; Webb, 1965). This “water replacement” favors the retention of the liquid crystalline phase of lipid bilayers. For maximal protection of a membrane trehalose must be present on both sides of a lipid bilayer (Crowe et al., 2005; Crowe et al., 1987). There is some evidence that trehalose protection during drying is dependent on the lipid composition of the target membrane (Hincha and Crowe, 1998; Hincha et al., 1998). Below we investigate the ability of trehalose, both in the presence and absence of LEA proteins, to protect liposomes with various lipid compositions mimicking membranes found in mammalian cells from freeze-thaw damage.

For cells to survive water stress, it is critical that cellular components such as the mitochondria be preserved (Hand and Hagedorn, 2008). To preserve mitochondria, trehalose must permeate both the outer and inner membranes (Crowe and Crowe, 1992; Liu et al., 2005). The outer membrane of mitochondria is permeable to solutes with a molecular weight less than 5 kDa due to non-specific protein channels like porin (Mannella, 1992; Walther and Rapaport, 2009). It is possible to load disaccharides into the matrix of isolated mammalian mitochondria via transient opening of the mitochondrial permeability transition pore (MPTP) (Al-Nasser and Crompton, 1986; Liu et al., 2005). The MPTP is a mega-pore of the inner membrane that is comprised of dimers of the ATP synthase (Bernardi, 2013; Giorgio et al., 2013). The formation of the pore in the inner mitochondrial membrane allows passage of solutes up to 1500 Da (Al-Nasser and Crompton, 1986; Zoratti and Szabo, 1995). Transition to the open state is physiologically regulated by calcium, phosphate, pH, cellular adenylates, reactive oxygen species, and redox state (Bernardi et al., 2006; Rasola and Bernardi, 2007). The MPTP plays a
pivotal role in calcium homeostasis and mitochondria-mediated apoptosis (Rasola and Bernardi, 2011).

Transient opening of the MPTP was utilized by Liu et al (2005) to load trehalose into the matrix of isolated rat mitochondria. As a result, these mitochondria retained a larger capacity to generate a membrane potential after drying and rehydration than mitochondria dried in the absence of trehalose or with 250 mM trehalose present only outside of the matrix. However, respiratory function was compromised. Trehalose was also used by Yamaguchi et al (2007) to protect isolated mouse mitochondria from damaged incurred during freeze-thawing. These authors report the OMM remained fully intact when mitochondria were frozen in 300 mM trehalose. Trehalose was the only sugar tested that was capable of full protection of the OMM. Because the primary focus was stabilization of the outer membrane, trehalose was not loaded into the matrix of mitochondria. Again, respiratory function was compromised. I propose to load trehalose in the matrix of isolated mitochondria prior to freezing to evaluate whether mitochondria will retain greater respiratory function.

4.2 Materials and Methods

Liposome Preparation

Liposomes were prepared as described in Chapter 3. In this study, liposomes were frozen with combinations of LEA proteins derived from *A. franciscana* embryos in the presence and absence of trehalose. Bovine serum albumin (fraction V; Sigma Aldrich, St. Louis MO) was used as a control protein. Lysozyme, which was used successfully as a control protein in studies of liposome drying with LEA proteins (Tolleter et al., 2010; Chapter 3), was not selected for freezing experiments because it destabilizes liposomes during freezing (Tolleter et al., 2010; Moore and Hand, data not shown). As in Chapter 3, lipids were combined with mass ratios
simulating the inner mitochondrial membrane, outer mitochondrial membrane, and inner leaflet of the plasma membrane of mammalian cells. For comparison to a non-physiological bilayer, liposomes composed entirely of phosphatidylcholine were also prepared.

Transient Opening of the MPTP

Mitochondrial isolation from rat liver was performed as described in Chapter 2. Loading the matrix of isolated mitochondria with trehalose via transient opening of the mitochondrial permeability transition pore (MPTP) was performed as described by Al-Nassar and Crompton (1986). Mitochondria (20-40 mg/ml) suspended in homogenization medium A HMA (250 mM trehalose, 10 mM Tris, 1 mM EGTA pH 7.4) were diluted to 0.5-1 mg/ml in HMA that contained no EGTA, which resulted in a buffer composed of 250 mM trehalose, 10 mM Tris, and 25 µM EGTA (pH 7.4). Respiratory substrates glutamate (10 mM) and malate (2 mM) were added to the suspension in order to establish a membrane potential, which promotes calcium accumulation. Potassium phosphate (1 mM) was also added because the sensitivity of the MPTP to calcium-induced opening is much higher in the presence of P$_i$ (Bernardi, 1996; Bernardi and Forte, 2007; Zoratti and Szabo, 1995). During initial optimization of the conditions for pore opening, a range of 25-150 µM total Ca$^{2+}$ was tested at exposure times of 1, 5, and 15 min. For freezing studies, MPTP opening was induced by the addition of 35 µM Ca$^{2+}$. After 1 min the pore was closed by the addition of 2 mM EGTA, 1 mM MgCl$_2$, 1 mM NAD$^+$, and 2 mg/ml BSA. EGTA chelates free Ca$^{2+}$ and Mg$^{2+}$ is a competitive inhibitor of Ca$^{2+}$ (Bernardi et al., 2006; Zoratti and Szabo, 1995). NAD$^+$ was added to help replace any lost from the matrix while the pore was open. Addition of BSA (2 mg/ml) during closing was found to increase coupling of mitochondria and outer membrane integrity. Mitochondria were incubated for ~8 min after the addition of EGTA to allow complete closure of the pore (Al-Nasser and Crompton, 1986; Zoratti
and Szabo, 1995). Control (i.e. nonpermeabilized) mitochondria were incubated under identical conditions with the exception that calcium was not added to the medium. For comparison of trehalose-incubated mitochondria to ones that were not exposed to trehalose, mitochondria were also isolated and frozen in a buffer composed of 150 mM KCl, 10 mM Tris, 2 mM EGTA, and 2 mg/ml BSA (pH 7.4).

Quantification of Trehalose by HPLC

After opening and reclosing of the MPTP, mitochondria were sedimented by centrifugation (10,000 x g for 10 min at 4°C). To remove extra-mitochondrial trehalose, mitochondrial pellets were resuspended in buffer composed of 250 mM sucrose, 10 mM Tris, and 1 mM EGTA (pH 7.4) and centrifuged once more at 10,000 x g for 10 min at 4°C, after which the supernatant was discarded and the mitochondrial pellet resuspended in water. The suspension was frozen at -80°C and thawed to insure complete release of trehalose from the matrix. The sample was heated at 95°C for 20 min then centrifuged at 20,000 x g for 30 min at 4°C to remove precipitated protein. Samples were then filtered with a syringe filter (0.2 µm pore size; Nalgene Nunc; Penfield, New York). HPLC analysis was performed with a Dionex HPLC system (Dionex, Sunnydale, CA). Samples were applied to a CarboPac MA1 column (Dionex), and carbohydrate peaks were separated by isocratic elution at 0.1 ml/min flow rate with 600 mM NaOH as the mobile phase (Patil et al., 2013). Carbohydrate elution was detected with an ED50 electrochemical detector (Dionex). Trehalose was quantified by comparison of peak areas to a standard curve of known trehalose concentrations. Values were expressed as µmol trehalose per mg mitochondrial protein.
Freezing of Liposomes and Mitochondria

Liposomes were frozen by placing 2 µl droplets in the wells of opaque 96-well plates (OptiPlate 96-F, PerkinElmer, Waltham, MA). Plates were covered with a plastic wrap and frozen at -80°C for at least 1 week and thawed at room temperature.

Mitochondria were frozen as described by Yamaguchi et al. (2007). Mitochondria were suspended at 40 mg mitochondrial protein/ml in HMB (HMA plus 2 mg/ml BSA). Small (0.6 ml) Eppendorf tubes were placed on a bed of dry ice pellets, and 5 µl droplets of mitochondrial suspension were pipetted into the tubes. Care was taken to fully dispense the mitochondrial suspension without allowing the pipette tip to contact the tube, which would result in the suspension freezing inside of the pipette tip. The tubes were transferred in to liquid nitrogen and then placed in a -80°C freezer overnight. Droplets were thawed quickly by warming between fingers.

CF Leakage Measurements

CF leakage was measured with a Victor 3 Multilabel Counter (PerkinElmer, Waltham, MA) as described in Chapter 3. Percent CF leakage was calculated as defined in Chapter 3. When comparing liposomes with different lipid compositions, experimental treatments were normalized to control treatments (no protectant), which was necessary due to inherent differences in control leakage across compositional types.

Measurements of Respiration Rate

Oxygen consumption of mitochondria was measured at 37°C with an Oxygraph 2K (OROBOROS Instruments, Innsbruck, Austria), and the data were analyzed with DatLab software (OROBOROS Instruments). The rate of oxygen consumption was calculated as the time derivative of oxygen concentration and normalized per mg mitochondrial protein (pmol O$_2$ s$^{-1}$)
mg mitochondrial protein\(^{-1}\)). Complex II-stimulated respiration was measured in the presence of succinate (10 mM) and rotenone (0.5 µM; inhibitor of Complex I). Proton leak respiration (LEAK state, or state 4) was measured in the presence of substrate without ADP. The addition of ADP (5 mM) stimulated maximum oxidative phosphorylation (OXPHOS state, or state 3). The respiratory control ratio (RCR) was calculated by dividing OXPHOS by LEAK respiration. Cytochrome c (cyt c) was added to the respiration medium during the OXPHOS state in order to assess the integrity of the outer membrane; an increase in oxygen consumption was indicative of compromised membrane integrity. Finally, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) was titrated step-wise to estimate the maximum respiratory capacity of the electron transport system in the uncoupled state (ETS state).

Statistics

Two-way ANOVA paired with a Tukey post-hoc test were used to compare protection of each type of liposome by AfrLEA3m, AfrLEA2, or BSA across the range of protein:lipid mass ratios tested (Prism 6; GraphPad Software, La Jolla, CA). A Bonferroni multiple comparisons test of sample means was used to identify statistical differences between trehalose and protein combinations compared to each component alone (Prism 6). 1-way ANOVA paired with a Tukey multiple comparisons test was used to compare protection of each type of liposome by trehalose alone. Paired and unpaired t-tests were used to compare trehalose content and mitochondrial respiration rates and RCRs before and after freezing or permeabilization (Prism 6). Significance level was set at \( p \leq 0.05 \).
4.3 Results

Stability of Liposomes Frozen with LEA Proteins

Protection of liposomes by LEA proteins varied depending on the lipid composition of the liposomes (Fig. 4.1). The only statistically significant (2-way ANOVA, Bonferroni multiple comparisons test, p>0.05, n=3-6) improvement in stability during freezing and thawing occurred with liposomes that simulated the composition of the outer mitochondrial membrane (OMM). When quantified at the highest protein:lipid mass ratio tested, leakage of CF from OMM-like liposomes was 56.3±2.8% (mean ± SD, n=6) of control leakage in the presence of AfrLEA3m and 29.3±2.5% (mean ± SD, n=6) in the presence of AfrLEA2 (2-way ANOVA, Bonferroni multiple comparisons test, p>0.05, n=3-6) (Fig 4.1 A and B). After freeze-thawing in the presence of AfrLEA3m, there were no statistical differences compared to controls in CF loss from liposomes that mimicked the inner leaflet of the plasma membrane (ILPM), the inner mitochondrial membrane (IMM), or in those composed of 100% phosphatidylcholine (PC; 2-way ANOVA, Bonferroni multiple comparisons test, p>0.05, n=3-6) (Fig 4.1 A). Likewise, AfrLEA2 did not significantly diminish CF leakage after freeze-thawing from IMM, ILPM, or PC liposomes (2-way ANOVA, Bonferroni multiple comparisons test, p>0.05, n=3-6) (Fig. 4.1 B). The control protein, BSA, did not depress CF leakage from any liposomes tested except at the highest protein:lipid mass ratio tested with OMM-like liposomes (2-way ANOVA, Bonferroni multiple comparisons test, p>0.05, n=3-6) (Fig. 4.1 C).

Stability of Liposomes Frozen with Trehalose

Relative to controls (no sugar added), 250 mM external trehalose significantly depressed CF leakage from liposomes for all compositional types tested (Fig 4.2 A, 2-way ANOVA, Bonferroni multiple comparisons test, p<0.05). Trehalose protected non-biological liposomes
Figure 4.1. Dye leakage from liposomes frozen and thawed in the presence of LEA proteins and BSA. The relative stability of the membranes was determined by measuring the leakage of entrapped fluorescent dye carboxyfluorescein, expressed as a percentage of complete dye release quantified by detergent solubilization. Experimental treatments were then normalized to control treatments (no protectant), which was necessary due to inherent differences in control leakage across compositional types. Lipid compositions simulated those of the inner mitochondrial membrane, the outer mitochondrial membrane, the inner leaflet of the plasma membrane, or a non-biological composition of 100 % phosphatidylcholine. Liposomes were frozen with the indicated protein:lipid mass ratios of (A) AfrLEA3m, (B) AfrLEA2, or (C) BSA. Data represent mean ± SD of n = 3-6 samples; where error bars are absent, the SD was less than the size of the symbol. Asterisks indicate significantly less CF leakage than that of liposomes frozen without protectants (2-way ANOVA, Bonferroni multiple comparisons test, p ≤ 0.05, n =3-6).
composed of 100% PC and liposomes with lipid compositions similar to the OMM and ILPM with equal efficacy. However, CF loss from IMM-like liposomes in the presence of trehalose was significantly greater than for the other liposomes (1-way ANOVA, Tukey multiple comparisons test, p<0.05, n=3-6). Therefore, lipid composition did affect the degree to which trehalose improved liposome stability. In order to evaluate maximal protection by trehalose, IMM-like liposomes were prepared that contained 250 mM trehalose inside and outside. Loss of CF from IMM-like liposomes significantly decreased (1-way ANOVA, Tukey multiple comparisons test, p<0.05, n=3) from 22.9±1.3% (mean ± SD, n=3) in the presence of external trehalose alone to 7.1±0.2% (mean ± SD, n=3) when liposomes were equipped with both internal and external trehalose (Fig. 4.2 B).

Finally, stabilization of liposomes by LEA proteins in combination with trehalose was also examined. No significant increase in protection was observed with any of the liposomes tested when LEA proteins were used in conjunction with trehalose compared to CF leakage in the presence of trehalose alone (data not shown).

Optimization of Transient Opening of the MPTP

Because the presence of trehalose on both sides of the lipid bilayer improved the integrity of IMM model liposomes during freezing, I wished to compare stabilization of isolated mitochondria frozen with and without trehalose in the matrix. Transient opening of the MPTP has been used to load sugars into the matrix of mitochondria isolated from rat liver (Al-Nasser and Crompton, 1986; Liu et al., 2005). However, the opening and closing of the pore needed to be optimized to preserve mitochondrial function. Mitochondria that were permeabilized by the addition of 35 µM Ca^{2+} for 1 min prior to resealing accumulated substantial trehalose and retained respiratory function and outer membrane integrity (Fig. 4.3). After inducing MPTP
Figure 4.2. Dye leakage from liposomes frozen and thawed in the presence of trehalose. (A) Cryoprotection by external trehalose of liposomes with variable lipid composition. The relative stability of the membranes was determined by measuring the leakage of entrapped fluorescent dye carboxyfluorescein (CF), expressed as a percentage of complete dye release quantified by detergent solubilization. Experimental treatments were then normalized to control treatments (no protectant), which was necessary due to inherent differences in control leakage across compositional types. Lipid compositions simulated those of the inner mitochondrial membrane (IMM), the outer mitochondrial membrane (OMM), the inner leaflet of the plasma membrane (ILPM), or a non-biological composition of 100 % phosphatidylcholine (PC). Data represents mean ± SD of n = 3-6 samples. * indicates significant decrease of CF loss compared to controls (2-way ANOVA, Bonferroni multiple comparisons test, p<0.05). (B) IMM-like liposomes were prepared with and without trehalose inside of the liposomes and frozen in a 250 mM trehalose solution. Control liposomes were frozen without any trehalose. The relative stability of the membranes was determined by measuring the loss of (CF), expressed as a percentage of complete dye release quantified by detergent solubilization. Data represents mean ± SD of 6 samples. * indicates significant decrease of CF loss compared to controls. ** indicates significant decrease of CF leakage compared to liposomes frozen with external trehalose only (1-way ANOVA, Tukey multiple comparisons test, p<0.05, n=3).
opening by the addition of 35 µM Ca^{2+} for 1 min, mitochondria contained 0.24±0.03 µmol trehalose/mg mitochondria in the matrix (Fig 4.3 A). Increase in OXPHOS respiration after the addition of exogenous cytochrome c was not significantly greater than that measured for nonpermeabilized mitochondria (Student’s paired t-test, p<0.05, n=6), which indicated that the outer membrane remained intact after permeabilization with 35 µM Ca^{2+} (Fig 4.3 B). The respiratory control ratio (RCR) was unchanged as well after permeabilization with 35 µM Ca^{2+} (Student’s paired t-test, p<0.05, n=6). In contrast when 100 µM Ca^{2+} is used to induce pore formation, cytochrome c stimulation was greatly increased and RCR values were significantly decreased (Student’s paired t-test, p<0.05, n=6). The amount of trehalose loaded into the matrix of mitochondria after permeabilizing with of 100 µM Ca^{2+} (0.31±0.03 µmol trehalose/mg mitochondrial protein) was significantly greater than achieved with 35 µM Ca^{2+} (Student’s paired t-test, p<0.05, n=3-5). Nevertheless, to test whether trehalose could improve mitochondrial function after freeze-thawing, 35 µM Ca^{2+} was chosen for MPTP opening due the lack of impact on OMM integrity and RCR.

Cryoprotection of Isolated Mitochondria by Trehalose

Respiratory function of mitochondria before and after freezing was measured to assess protection of mitochondria by trehalose (Fig 4.4). Prior to freezing, all RCR values were > 4 when succinate was used as a substrate. In the absence of trehalose (replaced by 150 mM KCl), the respiratory control ratio was diminished to 0.98±0.36 (mean ± SD, n = 4) after freezing and thawing (Fig 4.4 A). The RCR value of unity indicated a lack of oxidative phosphorylation upon ADP addition. The decreased RCR was due to both an increase in LEAK respiration and a decrease in OXPHOS respiration. The addition of cyt c resulted in an increase in respiration above OXPHOS after freeze-thawing, which indicated compromised integrity of the outer
Figure 4.3. Matrix trehalose content, cytochrome c stimulation, and respiratory control ratios after 1 min of transient MPTP formation. Addition of 35 or 100 µM Ca$^{2+}$ was used to induce MPTP formation for 1 min prior to closing the pore by chelation of Ca$^{2+}$ with 2 mM EGTA. Control mitochondria were incubated under identical conditions as permeabilized mitochondria except that calcium was not added. (A) Matrix trehalose content was measured by HPLC and normalized to mitochondrial protein. (B) OXPHOS respiration was measured before and after the addition of cytochrome c (4 mM) and expressed as a percent increase after cytochrome c addition. OXPHOS respiration was stimulated by succinate (10 mM) and ADP (5 mM) in the presence of complex 1 inhibitor rotenone (1 µM). (C) Respiratory control ratios (RCR) were calculated by dividing OXPHOS respiration by LEAK respiration. * indicates significant difference from control values and ** indicates significant difference from 35 µM Ca$^{2+}$ values (Student’s unpaired t-test, p<0.05, n=3-6).
Figure 4.4. Respiratory measurements of mitochondria isolated from rat liver before and after freezing. Mitochondria were frozen in media that contained 150 mM KCl (A) or 300 mM trehalose (B and C). Trehalose was loaded into the matrix of mitochondria (C) via transient opening of the permeability transition pore. LEAK respiration was stimulated by the addition of the substrate succinate in the presence of rotenone. OXPHOS respiration was stimulated by the addition of ADP. Outer membrane integrity was assessed by the addition of exogenous cytochrome c (+ cyt c). Maximal capacity of the electron transport system respiration (ETS) was determined by titration with the chemical uncoupler FCCP. Respiratory control ratios were calculated as OXPHOS divided by LEAK. Data represent the mean ± SD of 3-6 samples. * Indicates a significant change from non-frozen values (Student’s paired t-test, p ≤ 0.05, n=3-6)
membrane. In contrast, mitochondria frozen with 300 mM external trehalose showed improved RCR values (3.8 ± 0.4) (Fig. 4.4 B) compared to those with no external trehalose (Student’s unpaired t-test, p≤ 0.05, n = 4-6; Fig. 4.4 A). Additionally, the external trehalose eliminated any cyt c effect after post-freezing; thus, the outer membrane was protected. Providing trehalose to the matrix compartment along with external trehalose did not improve freeze tolerance compared to mitochondria frozen with external trehalose alone (Fig 4.4 C). Indeed, the RCR value post freezing (2.6 ± 0.2, mean ± SD; n = 4) was actually less than that measured with external trehalose alone (Student’s unpaired t-test, p≤ 0.05, n = 4-6). It appears that the rather low amount of trehalose loaded into the matrix with 35 µM Ca^{2+} was insufficient to improve the RCR (see Discussion).

4.4 Discussion

In this study, I found that cryopreservation of liposomes by LEA proteins was dependent on the lipid composition of the bilayer. Only liposomes mimicking the outer mitochondrial membrane were significantly stabilized by LEA proteins when frozen and thawed. Additive protection of liposomes was not observed when trehalose was used in conjunction with LEA proteins. External trehalose alone significantly decreased damage to all liposomes tested. However, when trehalose was present on both sides of the lipid bilayer during freezing, as tested with IMM-like liposomes, the stabilization was much greater. Based on this result, I investigated whether isolated mitochondria provisioned with trehalose in the matrix would retain more respiratory function than mitochondria frozen without trehalose or with trehalose present only outside of the matrix. I showed that isolated rat mitochondria retained outer membrane integrity and improved respiratory function when frozen in a medium that contained 300 mM trehalose, compared to mitochondria without trehalose. However, loading matrix trehalose – to levels
obtainable via the MPTP without compromising OMM integrity - did not improve respiratory function after freeze-thawing compared to external trehalose alone.

Effect of LEA Proteins on the Stability of Frozen Liposomes

Freezing damage to liposomes with a lipid composition mimicking the OMM was significantly reduced by AfrLEA2 and AfrLEA3m. However, AfrLEA2 and AfrLEA3m did not have any effect on freeze-induced damage to liposomes with the other three lipid compositions tested, which indicated that protection is dependent on the lipid composition of the bilayer. This lack of stabilization of IMM-like, ILPM-like, and PC liposomes was unexpected because Tolleter et al. (2010) report a ~15-25% decrease in CF leakage from PC liposomes frozen with PsLEAm at similar protein:lipid mass ratios. Additionally, COR15a and COR15b have been shown to offset freeze-induced damage to chloroplast-like liposomes (Thalhammer et al., 2014). Liposomes in the study by Tolleler et al. (2010) were frozen for 1 h at -18°C and liposomes were frozen at -20°C by Thalhammer et al. (2014). In our study, liposomes were frozen at -80°C for at least 1 week. It could be that differences in the freezing method partially explain the differences in our results versus others. Indeed, when expressed as % CF leakage, freeze-thaw damage only resulted in 20-35% CF leakage from all liposomes tested in our study, compared to ~50% leakage from 100% PC liposomes in the study by Tolleler et al. (2010). Our values for control leakage (without LEA protein) are similar to the smallest amount of leakage reported by Tolleter et al. (2010) for liposomes protected with PsLEAm. Thus, in order to detect protection of liposomes by AfrLEA2 or AfrLEA3m across all compositional types, perhaps freezing conditions that promote more damage are needed. Apparently, freezing of liposomes at higher temperature causes more damage. Alternatively, AfrLEA2 and AfrLEA3m may simply be less effective in protecting liposomes during freezing than certain other LEA proteins.
Protection of Liposomes by Trehalose

In the present study, liposomes frozen with 250 mM trehalose retain significantly more CF than liposomes frozen without trehalose, regardless of the lipid composition of the liposomes. However, liposomes with an IMM-like distribution of lipids were not protected to the same degree as OMM-like, ILPM-like and 100% PC liposomes. Therefore, it does appear that lipid composition can affect the ability of trehalose to stabilize lipid bilayers, but it must be emphasized that most experiments herein were performed with trehalose only on the outside of liposomes. Many studies with trehalose have demonstrated that virtually no damage occurs to liposomes during freezing if at least 0.2 M trehalose is present on both sides of the lipid bilayer (e.g., (Anchordoguy et al., 1987; Crowe et al., 1990; Rudolph and Crowe, 1985). However, liposomes with lipid compositions similar to the IMM of mammalian mitochondria were not previously investigated. Consequently, IMM-like liposomes were frozen with internal and external trehalose (Fig 4.2 B), and CF leakage was significantly reduced, which extends the established paradigm to this compositional type as well.

Trehalose Loading Via Transient Opening of the MPTP

The impact of transient pore opening on the respiratory function of mitochondria has not been the primary focus of studies involving the MPTP. Often respiratory measurements before and after pore opening in isolated mitochondria are not reported at all. Al-Nasser and Crompton (1986) found that mitochondria isolated from rat liver retained comparable RCR values after loading sucrose via transient opening of the MPTP to those of non-permeablized mitochondria. However, respiration was measured in the presence of exogenous cyt c, and therefore any damage to the outer membrane would not have been detected. Liu et al (2005) demonstrated that mitochondria loaded with trehalose in the matrix via transient MPTP formation retained a greater
capacity to generate membrane potential after drying compared to mitochondria dried without trehalose or with trehalose present only outside of the matrix. However, these authors reported that respiratory function was compromised. RCR values were diminished from >5 before drying to less than 3 after rehydration, but respiratory measurements were not taken after transient MPTP opening prior to drying. Because Liu et al. (2010) permeabilized mitochondria by the addition of 150 \( \mu \text{M} \) Ca\(^{2+}\) for 5 min, it is possible the OMM was compromised prior to desiccation. Nevertheless, matrix trehalose does appear able to stabilize the inner mitochondrial membrane during water stress based on the results of Liu et al., but my results indicate there may be a trade-off between loading sufficient matrix sugar for stabilization and avoiding degraded respiratory function the longer the pore is left open.

Stability of Isolated Mitochondria Frozen with Trehalose

Mitochondria isolated from rat liver retain significant respiratory function when frozen in 300 mM trehalose, similar to that reported for mouse mitochondria by Yamaguchi et al. (2007). The fact that mammalian mitochondria exhibit some degree of coupling after freeze-thawing is noteworthy. Mitochondria isolated from mouse liver also retain outer membrane integrity when frozen in 300 mM trehalose medium (Yamaguchi et al., 2007), as shown here for rat liver mitochondria by the cyt c test (Fig. 4.4 B).

Loading trehalose into the matrix of mitochondria did not improve the respiratory performance of the organelle after freeze-thawing. This result is possibly due to insufficient trehalose in the matrix. Transient opening of the MPTP was optimized to preserve outer membrane integrity and respiratory function. As Figure 4.3 shows, there is a trade-off between retention of these characteristics and the amount of trehalose loaded into the matrix.

Stabilization of mammalian cells and mitochondria during freezing has been shown to be
dependent on the concentration of trehalose (Buchanan et al., 2004; Yamaguchi et al., 2007). Yamaguchi et al (2007) reported that mitochondria isolated from mouse liver retained outer membrane integrity when frozen in 300 mM trehalose but not in 250 mM trehalose. Thus small differences in effective trehalose concentration in the matrix could explain why stabilization is observed in some cases and not others, coupled with differences in the type and severity of water stress imposed.
CHAPTER 5.
CONCLUSIONS AND FUTURE DIRECTIONS

I found that both AfrLEA2 and AfrLEA3m protect liposomes with biological lipid compositions from desiccation-induced damage. Liposomes of 100% PC were not stabilized by either LEA protein. In some cases, but not all, stabilization of dried liposomes by LEA proteins and trehalose was additive. Liposomes were also frozen with mixtures of trehalose and LEA proteins. Only liposomes with lipid compositions similar to the OMM were significantly protected from freeze-thaw damage by AfrLEA2 and AfrLEA3m. However, trehalose improved CF retention of frozen liposomes for all lipid compositions tested. Mitochondria isolated from rat liver retained greater respiratory function when cryopreserved with external trehalose, compared to a medium in which the sugar was substituted with KCl.

Matrix Localization of AfrLEA3m

The matrix localization of AfrLEA3m should allow the protein to stabilize the inner mitochondrial membrane (IMM) as well as many enzymes responsible for cellular respiration. Specialization of LEA proteins to membranes located in specific compartments to which the proteins are targeted has been suggested based on freezing and drying studies with lipid bilayers (Hincha and Thalhammer, 2012; Thalhammer et al., 2014; Tolleter et al., 2010). However, neither Thalhammer et al. (2014) nor Tolleter et al. (2010) tested other liposomes that mimicked compositions of alternative biological membranes so that direct comparisons could be made to the bilayers that enclosed the subcellular compartments in which their LEA proteins resided (chloroplasts and mitochondria, respectively). Such comparisons in Chapters 3 and 4 show that AfrLEA3m has potential to play a wider role cell stabilization outside of specific protection of components in the matrix of mitochondria, and in vivo evidence supports this premise (Li et al., 2012). That is not to say that targeting of the protein to the matrix is insignificant; preventing
damage to mitochondria and other organelles is critical for cell stabilization (Hand and Hagedorn, 2008). Nevertheless, my results indicate that AfrLEA2 and AfrLEA3m have broad protective abilities.

Four isoforms of AfrLEA3m are located in the matrix of *A. franciscana* mitochondria (Boswell et al., 2014b), and at least two other LEA proteins are targeted to the mitochondria as well (Warner et al., 2010). One possible reason for accumulating several LEA proteins in a single organelle may be functional divergence among the proteins (Pouchkina-Stantcheva et al., 2007). Analysis of cDNA sequences corresponding to the four AfrLEA3m proteins reveal that there are four sequences that distinguish these proteins from each other, but each of these sequences is shared by at least one other isoform of the protein. Future stabilization studies with all four isoforms would clarify whether these differences in protein sequence translate into functional divergence. All four isoforms would first have to be cloned in to expression vectors and purified, a daunting proposition due to the susceptibility of LEA proteins to proteolytic attack. Yet owing to their high sequence similarity, such fine-resolution, functional comparisons would begin to help us understand why, for example, the genome of *Polypedilum vanderplanki* (anhydrobiotic insect) encodes 27 distinct LEA proteins (Gusev et al., 2014).

Mitochondria isolated from *A. franciscana* embryos show remarkable tolerance for water stress (Menze et al., 2009). Mammalian mitochondria, which do not contain any endogenous LEA proteins, are highly susceptible to such damage (Liu et al., 2005; Yamaguchi et al., 2007). Mammalian mitochondria equipped with LEA protein would likely have increased tolerance for water stress. The concentration of AfrLEA3m in the matrix of *A. franciscana* mitochondria is 1.8-2.2 mg/ml matrix volume (Boswell et al., 2014b). This concentration represents ~0.55% of protein in the matrix, which is estimated to be 400 mg/ml (Srere, 1980). Tolleter et al. (2010)
calculated that PsLEAm would need to comprise 0.6% (~2.4 mg/ml matrix volume) of protein in the matrix to stabilize the entire surface of the IMM. In future experiments it would be informative to import AfrLEA3m into mitochondria isolated from rat liver and evaluate whether water stress tolerance is improved. But in order to import the high physiological concentrations of AfrLEA3m mentioned above, it will be necessary to resolve why accumulation of the protein stops after two minutes (Chapter 2).

**Molecular Modeling of AfrLEA2 and AfrLEA3m**

The prevailing mechanism by which LEA proteins stabilize membranes is through formation of amphipathic α-helices that interact with phospholipids at low water contents (Hincha and Thalhammer, 2012; Thalhammer and Hincha, 2014; Tolleter et al., 2010). An alternative mechanism has been suggested based on drying liposomes with model peptides (Furuki and Sakurai, 2014) comprised of tandemly repeating 11-mer sequences found in many group 3 LEA proteins (Shimizu et al., 2010). Like true LEA proteins, the model peptides were unstructured in solution and formed α-helices when dehydrated in the absence of lipid bilayers. Yet when dried in the presence of liposomes, the secondary structure of the peptides was primarily β-sheet (Furuki and Sakurai, 2014), which led the authors to suggest that stabilization involved coating liposomes in a web of β-sheets. However, their model peptides stabilized dried liposomes composed of 100% PC, which contrasts strongly with the inability of true LEA proteins to do so (Tolleter et al., 2010; Chapter 3). Furthermore, α-helical content of true LEA proteins increases in the presence of membranes at low water content (Popova et al., 2011; Thalhammer et al., 2014; Thalhammer et al., 2010). As a result it does not appear that their model peptides were behaving as bona fide, full-length LEA proteins.
Studies of Liposome Drying

Protection during drying by trehalose and LEA proteins combined was greater than protection by either component alone for IMM- and ILPM-like liposomes but not for OMM-like or PC liposomes. Consistent with this finding, Boswell et al. (2014a) reported that additive or synergistic stabilization by trehalose and AfrLEA2 or AfrLEA3m depends on the target enzyme. Differences in additivity could involve the ability of one or both components to interact with specific lipids. Fourier transform infrared (FTIR) spectroscopy has been used to investigate interactions between liposomes and trehalose (Luzardo et al., 2000) or LEA proteins (Popova et al., 2011; Pouchkina-Stantcheva et al., 2007; Thalhammer et al., 2010; Tolleter et al., 2010). To my knowledge, FTIR analysis of interactions between liposomes and combinations of trehalose and LEA proteins has not been performed. Comparing the phase behavior of liposomes with diverse lipid compositions in the presence of trehalose and LEA proteins may help to explain differences in additivity.

In all studies to date, liposomes have not been dried with LEA proteins present on both sides of the lipid bilayer. It seems likely that protection by LEA proteins would increase if the protein had access to both sides of the lipid bilayer, as is the case with sugars like trehalose and sucrose (Crowe et al., 1988; Crowe et al., 1989). To test this prediction with liposomes, the only practical limitation is the availability of purified LEA protein. To that end, procedures to expand the production of AfrLEA3m and AfrLEA2 in our laboratory and improve the efficiency of the protocol are currently underway. If liposomes representing cell membranes are more effectively stabilized with LEA proteins on both sides of the lipid bilayer, this could partially explain why LEA proteins are distributed among different compartments inside of the cell, and even secreted into the extracellular space of some organisms (Hand et al., 2011b).
The model peptides described above for LEA proteins have been shown to form glasses with high melting points (Shimizu et al., 2010). Consistent with this observation, almost no fusion occurs between liposomes when frozen or dried with LEA proteins from plants (Thalhammer et al., 2014; Tolleter et al., 2007; Uemura et al., 1996). Therefore by extrapolation, it is likely that little fusion occurred between liposomes dried or frozen with AfrLEA2 or AfrLEA3m. Dynamic light scattering (DLC) measurements indicate that less fusion occurred in liposomes dried with AfrLEA2 or AfrLEA3m compared to liposomes dried without any protectant (D. Moore, data not shown). However, DLC is not suitable to quantify fusion between liposomes. Fluorescence resonance energy transfer (FRET) can be used to quantify fusion of liposomes (Struck et al., 1981). FRET analysis of fusion between liposomes is currently underway in our laboratory. However, preliminary results were obfuscated by artifactual fluorescence in samples that contained LEA protein. I am currently trying to determine the reason for this fluorescent signal.

Cooperative protection by LEA proteins and trehalose could partly explain why many animals acquire both protectants. However, some anhydrobiotic organisms that express LEA proteins do not accumulate trehalose or other sugars (Hengherr et al., 2008; Tunnclifffe and Lapinski, 2003; Tunnclifffe et al., 2005). The amount of LEA proteins that an organism accumulates could determine the need for trehalose, as suggested by Hand et al. (2011). It would be informative to quantify cellular titers of LEA proteins in anhydrobiotes that lack trehalose (Hengherr et al., 2008; Tunnclifffe et al., 2005) for comparison to organisms that contain both (e.g., A. franciscana, Boswell et al., 2014b). Furthermore, knowing the cellular titer of LEA proteins is also valuable when interpreting results of in vitro studies. For instance, both Tolleter et al. (2010) and I (Chapters 3 and 4) used protein:lipid ratios that were consistent with cellular
levels of LEA proteins reported by Boswell et al. (2014b) and thus can be considered physiologically relevant.

Liposomes used in Chapters 3 and 4 are representative of lipid compositions found in mammalian cells (Horvath and Daum, 2013; van Meer et al., 2008). Tolleter et al. (2010) showed that PsLEAm protected liposomes mimicking the IMM of pea seed mitochondria significantly better than liposomes imitating the IMM of mammalian cells. It would be informative to dry liposomes mimicking precisely the compositions of *A. franciscana* membranes. However, the lipid compositions of *A. franciscana* IMM, OMM, and ILPM membranes are not known. Determining the lipid composition of *A. franciscana* membranes could shed light on fundamental mechanisms behind this organism’s remarkable desiccation tolerance. For instance, Abusharkh et al. (2014) reported that a reduced PC content of membranes was correlated with acquisition of desiccation tolerance in dauer larva of the nematode *Caenorhabditis elegans*. Phospholipid extracts from desiccation-tolerant larva had a higher affinity for trehalose and greater acyl chain mobility than lipids extracted from developmental stages of *C. elegans* that were desiccation-intolerant (Abusharkh et al., 2014). It is possible to isolate plasma membrane (Cioffi and Wolfersberger, 1983; Schimmel et al., 1973; van Meer et al., 2008), OMM and IMM fractions (Horvath and Daum, 2013; Pallotti and Lenaz, 2007) from cells. It could be revealing to determine the lipid compositions of these fractions for embryos of *A. franciscana*, as well as for later stages of development that lack desiccation tolerance. Temperature is also known to affect the lipid composition of membranes (Hazel, 1979). However, at this point I am primarily interested in how LEA proteins interact with lipid bilayers from different parts of the cell. I intend to determine lipid composition at a common temperature, because differences across membrane types (for example, high cardiolipin content
of the IMM) are more pronounced than changes in membrane composition due to temperature variation. However, alternations during temperature acclimation, or a combination membrane type and temperature change, may be of interest in future studies.

Studies of Liposome Freezing

Lack of protection of PC, IMM, and ILPM liposomes by AfrLEA2 and AfrLEA3m during freezing was unexpected because LEA proteins from plants effectively stabilize frozen liposomes with diverse compositions (Thalhammer et al., 2014; Tolleter et al., 2010). As discussed earlier, differences in freezing temperature could be partially responsible for the dissimilar results. Also, small differences among LEA proteins are known to affect the functions of the proteins, as demonstrated with ArLEA1A and ArLEA1b from Adineta riciiae (Pouchkina-Stantcheva et al., 2007). These LEA proteins differ by only 12 amino acids, yet ArLEA1A was found to interact strongly with lipid bilayers while ArLEA1B did not. Thus, small sequence changes in AfrLEA2 and AfrLEA3m compared to LEA proteins from plants could explain the differences observed in my results with frozen liposomes.

Stabilization of liposomes by LEA proteins during freezing differs from results with drying in several ways (Chapters 3 versus 4). Because LEA protein secondary structure is dependent on hydration state, perhaps there are small differences in the structure of LEA proteins during the water stresses imposed by desiccation versus freezing. Newly developed applications of Raman spectroscopy could provide a method for analyzing the frozen structure of LEA proteins (Roach et al., 2012; Roessl et al., 2014). Also, lower concentrations of LEA proteins are required to improve freeze tolerance of lipid bilayers compared to desiccation tolerance. COR15a and COR15b effectively stabilized liposomes simulating chloroplast membranes during freezing at a 1:25 (0.04) protein:lipid mass ratio (Thalhammer et al., 2014), which is much lower
than the effective mass ratios used by either Tolleter et al. (2010) or me (Chapter 3) for
desiccation studies. Future experiments should evaluate protection of liposomes by AfrLEA2
and AfrLEA3m during freezing at lower protein:l lipid mass ratios than were used in Chapter 4.

Studies of Mitochondria Freezing

It would be important to determine more precisely the reasons for the compromised
function observed when mitochondria were frozen in 300 mM trehalose (outside the matrix
only). It is possible to analyze individual components of the electron transport system by
measuring oxygen consumption in the presence of specific substrates, inhibitors, and cofactors.
For example, the capacity of cytochrome c oxidase (Complex IV) can be analyzed in the
presence of TMPD and ascorbate (which together serve as an electron donor system) and
antimycin A that inhibits electron flux upstream at Complex III. After identifying the site(s) of
damage to frozen mitochondria, it may be possible to add protectants that specifically stabilize
these site(s). Finally, it is clear from the results in Chapter 4 that improved methods are required
to load higher concentrations of trehalose into the matrix without compromising the respiratory
capacity of mitochondria. It should be noted that the ability to stabilize mitochondria during
freezing would be of great practical value to researchers in mitochondria-related fields. Frozen
mitochondria that remain fully functional after storage could be used to quantitatively calibrate
bioenergetic assays for tissue biopsies in clinical studies of mitochondrial-based diseases. Such
mitochondrial preparations would eliminate the need to isolate fresh mitochondria from rat liver
each day in these clinical settings.

Broader Biological Implications

In addition to obvious implications for the applied area of stabilization of mammalian
cells for biomedical or clinical purposes (Chakrabortee et al., 2007; Huang and Tunnacliffe,
2007; Iturriaga, 2008; Li et al., 2012), the present work may be considered in the broader perspective of the evolution of desiccation tolerance. It is virtually impossible to explain the presence of LEA proteins in the various phyla and species in which they occur by traditional vertical gene transfer and sexual or asexual reproduction. For example, how is it possible to explain the occurrence of LEA proteins in *Polypedilum vanderplanki* and their complete absence in a congeneric midge *P. nubifer* (cf. Gusev et al, 2014)? In fact, *P. vanderplanki* is the only insect species reported to express these proteins. It is becoming increasingly clear that acquisition of LEA proteins in animals is more logically explained by horizontal (lateral) gene transfer from bacteria and fungi. Horizontally acquired genes account for 9.7% of the genome of the anhydrobiotic rotifer, *Adineta ricciae* (Boschetti et al., 2012; Boschetti et al., 2011; Szydlowski et al., 2015). At least 3 horizontally acquired genes are expressed as part of the desiccation response of *A. ricciae* (Boschetti et al., 2011). The same is likely true for the genus *Artemia* and perhaps all anhydrobiotic animals. A careful analysis of the *Artemia* genome, which will soon be available (scientific team at the University of Ghent, Belgium), would shed light on the function(s) of horizontally acquired genes in *A. franciscana*. It may be possible to determine the origin of LEA proteins in *A. franciscana* through bioinformatic analysis, as described by Boschetti et al. (2011), or by genome comparison to closely related species that lack some or all LEA proteins, as demonstrated by Gusev et al. (2014).


VITA

Daniel Steven Moore was born in Fayetteville, AK, where his father held a research position at the University of Arkansas. At the age of 5, Daniel’s family moved to Alexandria, LA, where his father worked at Dean Lee Research Station, a branch of the Louisiana Agriculture Center. Daniel grew up living in university housing at LSU of Alexandria surrounded by researchers, and therefore developed an interest in science from a very young age. Daniel earned his B.S. (Biology) from Louisiana College in 2006. After college, Daniel taught high school science for two years while his wife earned her nursing degree. In 2009, Daniel applied to the LSU Graduate School and was accepted into the lab of Dr. Steven Hand. For his doctoral research, Daniel studied the ability of trehalose and LEA proteins to prevent freezing and desiccation-induced damage to lipid bilayers and isolated mitochondria from rats. Daniel currently teaches high school science full time at the Dunham School. Daniel plans to pursue a career in academia that will allow him to exercise his love for teaching at the college level and pursue studies in the field of comparative physiology and biomedical research.