The K2 Dehydrin: an Intrinsically Disordered Membrane Protector

By
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Dehydrins are disordered plant proteins which are expressed when the plant is exposed to stresses such as drought, high salinity, and low temperatures. Cell membranes appear to be the primary site of injury, and dehydrins interact with membranes in vitro and in vivo, often exerting a protective effect. K₂ dehydrin from Vitis riparia provides an opportunity to study the protective effect of K-segment binding on membrane surfaces. Liposome fusion assays indicate that K₂ attenuates freeze-thaw-induced damage in PC/PA membranes. This effect is not a result of significant coverage of the membrane surface, since merocyanine 540 accessibility is unaffected. Protection is also not a result of microviscosity change in the hydrophobic interior or the water-phospholipid interface of PC/PA membranes. Differential scanning calorimetry experiments indicate that K₂ reduces the thermotropic phase transition temperature of 1,2-dimyristoyl-sn-glycero-3-phosphate/1,2-dimyristoyl-sn-glycero-3-phosphocholine membranes by 3°C. This effect might be due to increased hydration of phosphate head groups by K₂.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ABA</td>
<td>Abscisic Acid</td>
</tr>
<tr>
<td>ANS</td>
<td>1-Anilinonaphthalene-8-sulfonic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CBF</td>
<td>Cold Responsive Element Binding Factor</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>CF</td>
<td>Carboxyfluorescein</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical micelle concentration</td>
</tr>
<tr>
<td>CRT/DRE</td>
<td>Cold Responsive or Dehydration Response Element</td>
</tr>
<tr>
<td>DHN</td>
<td>Dehydrin</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>DMPA</td>
<td>1,2-dimyristoyl-sn-glycero-3-phosphate</td>
</tr>
<tr>
<td>DMPC</td>
<td>1,2-dimyristoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DPH</td>
<td>1,6-diphenyl-1,3,5-hexatriene</td>
</tr>
<tr>
<td>DPPG</td>
<td>1,2-dipalmitoyl-sn-glycero-3-[phospho-rac-(3-lysyl(1-glycerol))]</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential Scanning Calorimetry</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EPE</td>
<td>L-α-phosphatidylethanolamine (Egg)</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast Protein Liquid Chromatography</td>
</tr>
<tr>
<td>HII</td>
<td>Hexagonal-II phase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear Single Quantum Coherence</td>
</tr>
<tr>
<td>IDP</td>
<td>Intrinsically Disordered Protein</td>
</tr>
<tr>
<td>Lβ or Lβ’</td>
<td>Solid-Like Lamellar Gel Phase</td>
</tr>
<tr>
<td>Lα</td>
<td>Fluid Phase</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny Broth</td>
</tr>
<tr>
<td>Lc</td>
<td>Solid-Like Lamellar Phase</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>LEA</td>
<td>Late Embryogenesis Abundant</td>
</tr>
<tr>
<td>LSZ</td>
<td>Lysozyme</td>
</tr>
<tr>
<td>LUV</td>
<td>Large Unilamellar Vesicle</td>
</tr>
<tr>
<td>LysoPC</td>
<td>1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td></td>
<td>18.1-linoleoyl-2-hydroxy-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>LysoPE</td>
<td>Phosphoethanolamine</td>
</tr>
<tr>
<td>MC540</td>
<td>Merocyanine 540</td>
</tr>
<tr>
<td>MGDG</td>
<td>Monogalactosyl diacylglycerol</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic Acid</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PEG3350</td>
<td>Polyethylene glycol 3350</td>
</tr>
<tr>
<td>PG</td>
<td>Phosphatidylglycerol</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>T&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Melting Temperature</td>
</tr>
<tr>
<td>TMA-DPH</td>
<td>N,N,N-Trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl)phenylammonium p-toluenesulfonate</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

Cold exposure is a crucial consideration for all organisms living in temperate climates. Formation of ice in tissues causes drought, cell structural damage, and changes of osmolality within tissues; all are potentially lethal. Insects, mammals, and birds can migrate to escape the cold, but other organisms such as plants and fungi must be able to tolerate these temperatures. During warm months, plants have very little tolerance to cold (Thomashow 1999). Sensing the oncoming winter or sudden changes in temperature, plants alter their physiology in a process called cold acclimation. This ability to recognize environmental signals and acclimate to anticipate extreme temperatures is a requirement for any plant living in temperate climates.

Cold exposure can damage plant tissues in a variety of ways. One consequence of ice crystallization is the removal of water from inside the cell, and the subsequent dehydration of the membrane. When water freezes outside a cell, liquid water inside the cell diffuses through the membrane and freezes with the growing extracellular ice. Up to 90% of water within a cell can be lost this way, increasing the intracellular osmolarity and depressing the freezing point of the cytosol. The membrane then becomes the primary barrier between the exterior ice and the supercooled cytosol, and thus maintaining this barrier is critical for cold-tolerant organisms (Thomashow 1999). Without liquid water adjacent to the membrane, the packing and fluidity of the bilayer drastically changes. Even without significant dehydration, cold temperatures cause anomalies in the lamellar structure of a membrane, which can cause a breakdown of the
membrane itself and a complete loss in osmotic responsiveness (Gordon-Kamm and Steponkus 1984). In order to keep a functioning plasma membrane, plants must be able to moderate multiple factors which affect the lyotropic and chill-induced properties of the membrane including leaflet composition, presence of membrane proteins, hydrocarbon chain saturation, and cytosolic carbohydrate accumulation (Uemura and Steponkus 1999).

1.1 LEA Proteins

Plants produce significant quantities of small, hydrophilic proteins during cold exposures, and many of these are linked to enhancement of cold tolerance. The best-characterized of these proteins are called Late Embryogenesis Abundant (LEA) proteins. First discovered in high concentrations during late embryogenesis in plants, the proteins are also produced during dehydrating stresses such as cold, drought, and high salinity exposures (Ingram and Bartels 1996).

Notably, the majority of LEA proteins are intrinsically disordered (IDPs) (Hara 2010). The universal acceptance of the ‘lock and key’ paradigm of structured proteins by Emil Fischer over 100 years ago has meant that unstructured proteins have been largely overlooked, and only in the past few decades has there been an increased interest in this field. Their prevalence was revealed in 2001 when a bioinformatics group developed a computational method of predicting protein disorder (Dunker et al. 2001). It is now estimated that 23-30% of all eukaryotic proteins are mostly disordered (Oldfield et al. 2005), that more than half of eukaryotic proteins have significant regions of disorder
(Dunker et al. 2001; Oldfield et al. 2005), and that more than 70% of signalling proteins have large disordered regions (Iakoucheva et al. 2002). Intrinsically disordered proteins can take on four generally accepted conformations: native (ordered), molten globule, pre-molten globule, and coil-like (Fink 1995; Uversky and Ptitsyn 1996). Several comprehensive studies have demonstrated that IDPs carry out a wide variety of important biological functions (Ban 2000; Fong et al. 2009; Penkett et al. 1998), and often contain sites for post-translational modifications as well as regulatory proteolytic attacks (Dunker and Obradovic 2001).

One early study on LEA proteins exposed spinach leaves to relatively low temperatures (5°C) for prolonged periods of time (Guy et al. 1985). By comparing mRNA transcription in chilled leaves with un-chilled leaves, the researchers found unique high molecular weight mRNA species, apparently induced by cold. It was soon discovered that alfalfa (Mohapatra et al. 1989), Arabidopsis (Kurkela and Franck 1990), barley (Hong, Barg, and Ho 1992), wheat (Houde et al. 1992), and maize (Ceccardi, Meyer, and Close 1994) also had similar transcripts expressed during cold exposure. We now know that LEA proteins exist in a huge range of photosynthetic organisms, including algae, mosses, gymnosperms and angiosperms (Close 1997). To date, 51 LEA genes in Arabidopsis alone have been identified and confirmed, and they fall under nine classes of LEA proteins based on the arrangement and number of their domains, and the stimuli that cause their expression (Hundertmark and Hincha 2008). Of the proteins examined by Candat et al. in 2014, the majority (36/51) of Arabidopsis LEA proteins were found to be evenly dispersed throughout the cytosol and nucleus of the protoplast. The remaining
proteins were targeted to nearly every organelle – including peroxisome-encapsulating pexophagosomes – but none were visualized in the nucleus alone. During maximum expression, these proteins have been predicted to account for 0.1-5% of the total proteins and mRNA transcripts in the cells, and remain for the duration of the cold exposure (Close 1997; Guy et al. 1985).

1.2 Dehydrins

One family of LEA proteins called the dehydrins (also known as Group 2 dehydrins), are specific to plants. Typical of IDPs, dehydrins are rich in hydrophilic amino acids, and are thermostable, that is, they remain soluble in water up to 100 °C (Jepsen and Close 1995). They have been observed in the cytoplasm (Rinne et al. 1999), mitochondria (Borovskii et al. 2002), vacuoles (Heyen et al. 2002), and chloroplasts (Mueller, Heckathorn, and Fernando 2003), as well as nuclear regions such as euchromatin, heterochromatin, nucleoli, and nucleoplasm (Godoy et al. 1994; Schneider et al. 1993). By definition dehydrins have at least one canonical K-segment, roughly corresponding to the amino acid sequence EKKGIMDKIKEKLPG (Close 1997).

*Dehydrins can prevent freeze damage when over-expressed in plants*

Overlapping expression conditions between various LEA proteins make it difficult to determine whether or not dehydrins in particular are capable of improving tolerance to environmental stresses. However, researchers have developed transgenic lines of plants that over-express dehydrins, demonstrating that they do, indeed, have a significant effect. Many dehydrins are thought to be involved in plant resistance to
freezing, and indeed in vivo studies have proven this to be true. For example, cold-acclimated transgenic strawberry plants expressing WCOV410 from wheat could survive temperatures 5°C colder than cold-acclimated wild-type plants (Houde et al. 2004). Overexpression of RAB18 and Cor47 dehydrins in Arabidopsis was effective at increasing the plant’s freezing tolerance, and was even more effective when the plants were acclimatized for longer periods of time (Puhakainen et al. 2004). Transgenic tobacco plants expressing the spinach dehydrin CuCOR19 had enhanced germination at low temperatures (Hara et al. 2003). In addition, yeast cells and tobacco plants expressing the Brassica juncea dehydrins BjDHN2 or BjDHN3 demonstrated much greater recovery and less damage from freezing stresses (Xu et al. 2007).

Dehydrins reduce stress from high salinity

Dehydrins are expressed in wild-type plants when exposed to high salt conditions, which is another stressor that dehydrins can also protect against (Godoy et al. 1994; Xu et al. 2007). For example, transgenic Arabidopsis plants overexpressing wheat DHN-5 have an enhanced survival in high NaCl growth conditions, and transgenic seeds were able to germinate in substrate containing up to 200 mM salt, a concentration that none of the wild-type seeds could survive (Brini et al. 2007). A similar finding was found with transgenic tobacco overexpressing Rab16A from rice (RoyChoudhury 2007). Interestingly, these researchers also found that transgenic plants accumulated more Na+ and K+ ions than wild-type plants in their tissues (RoyChoudhury 2007).
Dehydrins can chelate metal ions

Metal ion chelation is thought to be another major function of dehydrins. In transgenic tobacco plants, spinach CuCOR19 reduced lipid peroxidation by scavenging free radicals at the mitochondrial membrane (Hara et al. 2003). This dehydrin was also shown to be able to scavenge hydroxyl and peroxyl radicals as well as Fe\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\), Cu\(^{2+}\) and Zn\(^{2+}\) ions, possibly hinting at a function as a radical scavenging protein during times of low water stress (Hara, Fujinaga, and Kuboi 2004; Hara, Fujinaga, and Kuboi 2005). Additionally, two Thellungiella salsuginea dehydrins TsDHN-1 and TsDHN-2 form ordered secondary structures when associated with zinc (Rahman et al. 2011).

Dehydrins can act as ‘molecular shields’

Dehydrins and LEA proteins act as molecular shields by localizing near enzymes that are susceptible to drought-induced unfolding and aggregation (Hughes and Graether 2011). In numerous studies, dehydrins have been more effective on a molar basis at preventing freeze-induced damage to the cold-sensitive enzyme lactate dehydrogenase (LDH) than other cryoprotective peptides and proteins (Peng et al. 2008; Boucher et al. 2010). For example, spinach COR85 was found to be three times more effective at protecting LDH from freeze inactivation than bovine serum albumin (BSA) (Kazuoka and Oeda 1994). A study performed on the mitochondrial enzymes rhodanese and fumarase also determined that PsLEAM (Group 3) is capable of protecting these enzymes from desiccation far more effectively than BSA (Grelet et al. 2005). Similar protection has been shown with other enzymes as well, such as citrate synthase (Goyal et al. 2005;
Sharon et al. 2009) and firefly luciferase (Nakayama et al. 2008). This shielding behaviour is also effective with non-plant proteins. Chakrabortee et al. (2007) demonstrated anti-aggregation effect by a LEA (VI) protein on a broad spectrum of nematode and mammalian proteins, including polyglutamine and polyalanine expansion proteins associated with neurodegenerative disease. The researchers also demonstrated a protective effect on human T-REx293 cells *in vitro* from dehydration (Chakrabortee et al. 2007). Cells over-expressing the AavLEA1 protein had significantly improved cell viability under dehydration stress induced by the addition of 100 mM NaCl to the aqueous medium.

While enzyme protection by these proteins has been shown experimentally, it is still questioned whether cold-protection is a specific, evolved function of these proteins, or an intrinsic feature of all hydrophilic, disordered proteins. In a recent publication, enzyme protection by dehydrins was tested against protection by disordered and ordered proteins of similar hydrodynamic radii (Hughes et al. 2013). The authors found that *Vitis riparia* dehydrin constructs were much more effective at protecting LDH from freeze-inactivation than similar sized ordered proteins, suggesting that dehydrins do have an evolved protective function.

Unlike other structured proteins with known binding partners or catalytic activities, no absolutely clear dehydrin role has been identified. The sheer number of proposed and experimentally demonstrated functions might indicate that they are broad-spectrum protectors, performing a number of generalized functions in any part of the cell required.
1.3 Dehydrin sequence motifs

*K-Segment*

The K-segment is the only required motif for a protein to be classified as a dehydrin, and is relatively conserved across all species. Early analysis of six LEA proteins by Baker et al. (1988) suggested that four of the proteins contained amino acid tracts that could exist as amphipathic helices. These tracts were later identified as K-segments, and many researchers have investigated the idea that these segments are capable of forming amphipathic α-helices. In the absence of any ligands, K-segments have a random-coiled structure. However, α-helix formation has been observed in the presence of membranes or membrane mimics. For instance, circular dichroism (CD) spectra reveal that a purified cow pea dehydrin is unstructured when free in aqueous solution, but demonstrates a spectrum typical of α-helices in the presence of sodium dodecyl sulphate (SDS) micelles (Ismail, Hall, and Close 1999). In another experiment, researchers removed the Y- and S-segments from a maize DHN1 and discovered that micelle binding by this truncated peptide still induces α-helicity as demonstrated by the CD spectrum (Koag et al. 2003). Subsequent removal of either (or both) K-segments from wheat DHN1 also attenuated the α-helix transition in the presence of the same vesicles (Koag et al. 2009). The authors also confirmed that the lipid-dehydrin interaction is electrostatic in nature, as DHN1 did not bind large unilamellar vesicles composed solely of phosphatidylcholine, a zwitterionic phospholipid.
The K-segment is also thought to be involved with the shielding of LDH during freezing. The K$_2$ DHN from *Vitis riparia* (composed of two K-segments separated by a φ-segment) demonstrated better cryoprotective activity than BSA. However, this protective activity was not associated with any strong α-helix formation or specific binding with the enzyme (Hughes and Graether 2011; Hughes et al. 2013).

*Y- and φ-Segments*

Dehydrin sequence motifs include the consensus-sequence N-terminal Y-segment, the serine-rich S-domain, and the poorly conserved φ-segment. The Y-segment consists of a string of amino acids with the sequence (V/T)D(E/Q)YGNP, and has no known function. The sequence is thought to resemble a bacterial nucleotide binding site (Close 1997), but no published data have validated the idea. The φ-segments are polar, highly variable, glycine-rich ‘spacers’ found interspersed between all segment types, and they appear to add conformational flexibility between the other domains (Close 1997). The most common amino acids found in φ -segments are Gly, Gln, and Thr, whereas Phe, Cys, and Trp are present ≤1% of the time (Graether and Boddington 2014).

*S-Segment*

The S-segment is a stretch of amino acids generally containing 5-7 serine residues in a row, and is often preceded by Ser-Asp (Graether and Boddington 2014). There is evidence that the S-segment is involved with nuclear localization when it is phosphorylated. Tomato dehydrin TAS14 is phosphorylated on a variety of S-segment serine residues *in vivo*, and maize Rab17 (DHN1) is phosphorylated prior to binding with
a nuclear localization signal peptide (Godoy et al. 1994; Vilardell et al. 1990). The maize Rab17 S-segment contains a serine cluster followed by a triplet of amino acids (EEE); a putative phosphorylation site for protein kinase 2, and a stretch of basic amino acids (RRKK) resembling nuclear localization signals found in mammalian Nopp140, yeast NSR1, and simian large T antigen-type nuclear localization signal (Rorat 2006). In vitro phosphorylation of two *T. thellungiella* dehydrins (TsDHNs) induced minimal structural change; however, phosphorylated TsDHNs demonstrated a much greater structural shift to an α-helical conformation upon binding to LUVs, suggesting that phosphorylation of the S-segment may enhance membrane localization (Rahman et al. 2011). The S-segment may also be primarily involved with metal ion binding activity. Heyen et al. (2002) have found that the dehydrin ERD14 possesses a calcium ion binding activity that is dependent on its phosphorylation state, and that S-segment phosphorylation increases during cold stress (Alsheikh, Heyen, and Randall 2003). Similar binding activity has been found with ERD14, ERD10, and COR47. This ion binding activity, and therefore, the S-segment, may be a critical component for dehydrin protection against drought and high salinity.

*Arrangements of dehydrin segments*

The Y-, S-, K- and φ-segments can be assembled in a wide variety of arrangements and patterns, and the “YSK” notation is used when naming and describing each species (Close 1997). Some of the various dehydrins and their configurations are shown in Table 1.1.
Table 1.1. Dehydrin architecture sub-families and unique characteristics. Adapted from Allagulova et al. 2003.

<table>
<thead>
<tr>
<th>Sub-family</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y&lt;sub&gt;2&lt;/sub&gt;SK&lt;sub&gt;2&lt;/sub&gt;</td>
<td>The most common dehydrin architecture, and thought to be induced by drought or ABA but not by cold temperatures. All barley DHNs have the YSK&lt;sub&gt;2&lt;/sub&gt; architecture.</td>
</tr>
<tr>
<td>K&lt;sub&gt;n&lt;/sub&gt;</td>
<td>Contain 2 to 9 K-segments, and are induced by cold temperatures, ABA, and dehydration. In freeze-tolerant wheat varieties exposed to 4°C for 24 h, the WCS120 accumulated to 20 times the concentration of non-chilled plants (Houde et al. 1992).</td>
</tr>
<tr>
<td>SK&lt;sub&gt;n&lt;/sub&gt;</td>
<td>Typically contain one S-segment and 2-3 K-segments, and are preferentially induced by cold temperatures. Accumulation of two SK&lt;sub&gt;n&lt;/sub&gt; type dehydrins from <em>Cichorium intybus</em> (Chicory) increased in wild trees in direct responses to field temperatures (Mingeot et al., 2009).</td>
</tr>
<tr>
<td>K&lt;sub&gt;n&lt;/sub&gt;S</td>
<td>The K-segment of this family begins with a (H/Q)KEG segment, which is unique to the family. Includes PtrDHN-6 from poplar and barley DHN13.</td>
</tr>
<tr>
<td>Y&lt;sub&gt;2&lt;/sub&gt;K&lt;sub&gt;n&lt;/sub&gt;</td>
<td>This class is associated with chilling tolerance of seed embryos. A Y&lt;sub&gt;2&lt;/sub&gt;K&lt;sub&gt;n&lt;/sub&gt; type dehydrin has been identified and sequenced from <em>Vigna unguiculata</em> (cow pea) (Ismail et al., 1999).</td>
</tr>
</tbody>
</table>
1.4 Regulation of dehydrin expression

Plants have multiple dehydrin genes that are induced by different stimuli. For instance, in *Prunus persica*, the dehydrin PpDhn2 is induced by water deficit, but not by low temperatures or by short photoperiods (Wisniewski et al. 2006). A second dehydrin called PpDhn1 is induced by low temperatures as well as by water deficit, but not by short photoperiods. In silver birch, short photoperiods alone do not cause significant induction of Bplti36, but greatly enhance its expression in response to low temperatures (Puhakainen et al. 2004).

Dehydrin family genes have many cis-acting elements, and deletion analysis in *Arabidopsis* plants indicates that some elements induce transcription upon exposure to specific types of environmental cues such as short photoperiods or drought, whereas others respond directly to the plant hormone abscisic acid (ABA). Of the dehydrins that have been studied, the Kₐ, SKₐ, and KₐS type dehydrins appear to be up-regulated by cold. In Table 1.2 is a list of dehydrins which have known localizations and environmental signals. Research on YₐSKₐ-type dehydrins is limited, with one up-regulated by salt and desiccation (Lin et al. 2012) and another by cold (Wisniewski et al. 1999). Cellular localization studies have hinted that Y-segment-containing dehydrins are more involved with desiccation and high-salinity stresses than with cold, and these proteins will be discussed in the next section.

Exactly how these environmental cues signal the transcription of DHN genes is not known, but some of the downstream pathways have been identified. Induction of *Arabidopsis* COR47 for example, begins with the transcription factor CBF (CRT/DRE
Table 1.2. Dehydrins and the environmental signals that up-regulate their transcription. Abbreviations: Cold, cold induced; Chloro, chloroplast; Const, constitutively induced; Cyt, cytoplasm; Desic, desiccation induced; Mito, mitochondrion; n/d, not determined; Nuc, nucleus; Salt, salt induced. From Graether and Boddington, 2014.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>YSK Architecture</th>
<th>Localization</th>
<th>Abiotic Stress</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV-dhn</td>
<td>K₅</td>
<td>Cyt</td>
<td>n/d</td>
<td>(Yakubov et al., 2005)</td>
</tr>
<tr>
<td>LTI30</td>
<td>K₆</td>
<td>n/d</td>
<td>Cold</td>
<td>(Nylander et al., 2001)</td>
</tr>
<tr>
<td>WCS120</td>
<td>K₅</td>
<td>Nuc/Cyt</td>
<td>Cold</td>
<td>(Houde et al., 1995)</td>
</tr>
<tr>
<td>DHN24</td>
<td>SK₃</td>
<td>Nuc/Cyt</td>
<td>Cold</td>
<td>(Szabala et al., 2014)</td>
</tr>
<tr>
<td>SpDHN1</td>
<td>SK₃</td>
<td>Cyt/Mem</td>
<td>Desic</td>
<td>(Yang et al., 2014)</td>
</tr>
<tr>
<td>CpDHN</td>
<td>SK₂</td>
<td>n/d</td>
<td>Cold</td>
<td>(Porat et al., 2004)</td>
</tr>
<tr>
<td>Peudhn1</td>
<td>SK₂</td>
<td>n/d</td>
<td>Cold</td>
<td>(Caruso et al., 2002)</td>
</tr>
<tr>
<td>COR47</td>
<td>SK₃</td>
<td>n/d</td>
<td>Cold</td>
<td>(Nylander et al., 2001)</td>
</tr>
<tr>
<td>ERD14</td>
<td>SK₂</td>
<td>n/d</td>
<td>Cold, Salt</td>
<td>(Nylander et al., 2001)</td>
</tr>
<tr>
<td>LTI29</td>
<td>SK₃</td>
<td>n/d</td>
<td>Cold, Salt</td>
<td>(Nylander et al., 2001)</td>
</tr>
<tr>
<td>PgDHN1</td>
<td>SK₄</td>
<td>n/d</td>
<td>Cold, Desic</td>
<td>(Richard et al., 2000)</td>
</tr>
<tr>
<td>WCOR410</td>
<td>SK₃</td>
<td>Mem</td>
<td>Cold, Desic</td>
<td>(Dany luk et al., 1998)</td>
</tr>
<tr>
<td>CuCor19</td>
<td>K₅S</td>
<td>Mito</td>
<td>Cold</td>
<td>(Hara et al., 2003)</td>
</tr>
<tr>
<td>AmDHN1</td>
<td>YSK₂</td>
<td>Nuc/Cyt</td>
<td>Desic, Salt</td>
<td>(Mehta et al., 2009)</td>
</tr>
<tr>
<td>VrDHN1a</td>
<td>YSK₂</td>
<td>n/d</td>
<td>Cold, Desic</td>
<td>(Xiao and Nassuth, 2006)</td>
</tr>
<tr>
<td>TAS14</td>
<td>YSK₂</td>
<td>Nuc/Cyt</td>
<td>Salt</td>
<td>(Godoy et al., 1994)</td>
</tr>
<tr>
<td>Rab17</td>
<td>YSK₂</td>
<td>Nuc/Cyt</td>
<td>n/d</td>
<td>(Goday et al., 1994)</td>
</tr>
<tr>
<td>Rab21</td>
<td>YSK₂</td>
<td>Cyt</td>
<td>Desic</td>
<td>(Mundy and Chua, 1988)</td>
</tr>
<tr>
<td>VrDHN1</td>
<td>Y₂K</td>
<td>Nuc/Cyt</td>
<td>Desic, Salt</td>
<td>(Lin et al., 2012)</td>
</tr>
<tr>
<td>PCA60</td>
<td>Y₂K₉</td>
<td>Nuc/Cyt</td>
<td>Cold</td>
<td>(Wisniewski et al., 1999)</td>
</tr>
<tr>
<td>52 &amp; 53 kDa dehydrins</td>
<td>n/d</td>
<td>Mito</td>
<td>Cold</td>
<td>(Borovskii et al., 2005)</td>
</tr>
<tr>
<td>31 kDa dehydrin</td>
<td>n/d</td>
<td>Chloro</td>
<td>Const</td>
<td>(Mueller et al., 2003)</td>
</tr>
<tr>
<td>24 kDa Dehydrin</td>
<td>n/d</td>
<td>Nuc/Cyt</td>
<td>Cold</td>
<td>(Karlsson et al., 2003)</td>
</tr>
<tr>
<td>24 kDa Dehydrin</td>
<td>n/d</td>
<td>Nuc/Cyt</td>
<td>Cold</td>
<td>(Rinne et al., 1999)</td>
</tr>
</tbody>
</table>
Binding Factor), which is induced by cold temperatures (Shinozaki and Yamaguchi-Shinozaki 2000; Stockinger et al. 1997). CBF binds to a DNA regulatory element, the CRT/DRE (C-Repeat/Dehydration Response Element), which stimulates transcription of rd29A gene (Yamaguchi-Shinozaki and Shinozaki 1994). There can be up to 3 CRT/DRE elements which can be used to increase transcription (Thomashow et al. 2001). Other stress-related dehydrins are up-regulated in response to increased levels of ABA. The presence of ABA recruits bZIP transcription factors to bind the upstream ABA Response Element (ABRE), and also involves the MYC-element (CACCTG) as well as the MYB-element (TAACTG) (Busk and Pagès 1998; Rock 2000; Leung and Giraudat 1998; Finkelstein, Gampala, and Rock 2002). The ABA-independent induction of dehydrin genes appears to be a rapid response, and conversely ABA-dependent induction is slow. Many combinations of conditions can induce transcription for entire regulons of genes; each potentially specialized for the stressor (Jaglo-Ottosen 1998).

1.5 Dehydrin tissue and cellular localization

Some dehydrins are mainly found in mature seeds. The RAB17 and RAB18 dehydrins from Zea mays are found in the embryo and endosperm of mature seeds (Goday et al. 1994; Lång and Palva 1992). The YSK2-type pea dehydrin DHN-COG accumulates in mid-to-late embryogenesis in cotyledons in dehydrin-stressed seedlings (Robertson and Chandler 1994), and when mature, DHN-COG is approximately 2% of the total seed protein. Other dehydrins are commonly found in vegetative tissue under
ordinary growth conditions and in floral organs (Rorat 2006). *Arabidopsis* ERD14 and ERD10 have been found in the root tips, and the vascular tissues of roots, stems, and flowers (Nylander et al. 2001). In peaches, PCA60 DHN accumulated in all of the root tissues (Wisniewski et al. 1999). In addition, many dehydrins are found in specific cell types, such as guard cells, pollen sacs, root meristematic cells, or plasmodesmata (Rorat 2006).

Dehydrins are found in even greater quantities when the plants are under stresses such as drought, salinity, and cold. Wheat DHN (WCOR410, SK3), for example, accumulates preferentially during cold exposure in vascular transition areas of roots, leaves, and crown (Rorat, 2006). *Arabidopsis* LTI30 (K6) is not expressed in tissues during normal growth conditions, but does accumulate when the plants are grown in the cold (Nylander 2001).

At the cellular level, dehydrins have been identified in nearly every part of the cell, including the nucleus, cytosol, plasma membrane, mitochondria, and vacuoles (Godoy et al. 1994; Danyluk et al. 1998; Borovskii et al. 2002; Rinne et al. 1999). As mentioned earlier, dehydrins without a Y-segment are unlikely to be involved in membrane protection during cold conditions, since *Y*SKn and *Y*Kn proteins have not been observed near the membrane (Wisniewski et al. 1999; Lin et al. 2012). The fact that different dehydrins appear to localize to different parts of the cell for different species means that dehydrins are specialized to their target location, but still may have a common function.
1.6 Introduction to membranes

Common to all living organisms are membranes composed of lipids. Almost all biological lipids are amphiphiles, composed of a polar molecule attached to a hydrocarbon chain (Ceve 1993). Human red blood cell membranes contain approximately 100 species of lipid, and most biological membranes are composed of more than 600 species (Ceve 1993). Lipid heterogeneity exists across all animal kingdoms, species, cell types, and organelles. In eukaryotes, cholesterol molecules add size diversity to the hydrophobic interior of the membrane, allowing just enough spacing for small molecules to pass through, but making it tight enough so that proteins and large molecules cannot escape. The striking variety of lipid molecules underpins the finely-tuned, specialized nature of membranes.

Lipids provide strong, flexible barriers and enclosures between adjacent solutions. These barriers rely on weak chemical interactions between lipid molecules, in addition to rotational flexibility of hydrocarbon chains, which allow lipid molecules to arrange themselves into flexible, yet tightly sealed barriers and enclosures (Cho and Stahelin 2005). When pure lipids are mixed in an aqueous environment, they self-assemble into lamellar sheets of bilayers, with hydrocarbon chains assembling together to minimize their interaction with water. Some lipid types spontaneously form into continuous hexagonal phases or micelles, depending almost entirely on the size and shape of the lipid molecule itself.
1.7 Protein–membrane interactions

On the basis of their interaction with membranes, peripheral membrane proteins can be categorized into three major classes: S-type (surface) proteins come into contact with the lipid head groups and the interfacial region, and interact mainly with the phosphate head-groups (Cho and Stahelin 2005). I-type (intermediate) proteins penetrate into the head-group region and interact strongly with the glycerol phospholipid backbone. H-type (hydrophobic) peripheral membrane proteins penetrate into the hydrophobic core but still interact with the phospholipid head-groups (Cho and Stahelin 2005). Some proteins and peptides are targeted to the membrane and have a reversible interaction with the bilayer, and others such as gramicidin and alamethicin are fully integrated into the bilayer. These proteins are referred to as integral membrane proteins. Other integral proteins such as magainin and dermaseptin are located within the hydrophobic core, interacting with the inner perimeter of the polar head groups and not with the external solvent.

Protein-membrane interactions are diverse and multi-functional. For instance, many membrane-spanning proteins recruit or enrich the membrane in their vicinity to specific types of lipids. This recruitment may be done by lipid head-group interactions with peripheral proteins, or fatty acid tail requirements by integral membrane proteins. These selectively-enriched vicinal lipids form what is called a lipid annulus (Gil et al. 1998). Some membrane-spanning proteins will undergo conformational changes when the curvature of the membrane changes. These conformational changes play an important part in the protein’s function (Marsh 2007; Phillips et al. 2009). Other proteins will
facilitate the association and fusion of membranes, and are key to extracellular signalling and hormonal excretion. Others still can manipulate individual lipid molecules, maintaining lipid composition disparity between bilayers by flipping or flopping lipids, or modifying lipids depending on the extracellular environment and needs of the cell (Sharom 2011).

Electrostatic interactions are often the first step of protein targeting to membranes as they enhance the association rate constant \( (k_a) \) with the membrane and promote non-specific complexes (Cunningham and Wells 1993). Phosphorylation of the membrane-targeted protein or the activation of electrostatic switches by association of \( \text{Ca}^{2+} \) often accelerate these associations (Sprong et al. 2001). The burying of side-chains or entire hydrophobic domains then often requires a protein re-structuring, which allows proteins to have a high degree of lipid and membrane type specificity. Hydrophobic mismatch can occur when the width of a protein’s hydrophobic region does not perfectly patch with the width of the membrane interior. To compensate for this, the protein or the membrane might undergo slight expansion or compression to satisfy hydrophobic requirements (Duque et al. 2002). This mismatch also aids in protein-organelle specificity. The thickness of membranes in different organelles varies depending on their constitution (Silvius and McElhaney 1978), and integral proteins which span the membrane region have been shown to match the thickness of their intended membrane (Bretscher and Munro 1993). The membrane, while seemingly a static barrier, is truthfully a dynamic, constantly adapting organelle in itself.
1.8 Membrane phase transitions

Plants and other ectotherms must regulate membrane phase behaviour at all environmental temperatures to prevent critical cell damage. The vast majority of functional biological lipid membranes exist in a state of having “liquid-ordered” (or fluid lamellar, or liquid-crystalline) molecules (Cevc 1993). In this state, the hydrocarbon tails contain many gauche rotational conformers, the cross-sectional area of the lipid molecules increases, the bilayer thins and lipids move in an unpredictable, fluid manner on the horizontal plane (McMullen et al. 2004). When sufficiently harsh conditions such as cold temperatures, dehydration, or high salinity are present, a membrane will undergo often lethal transitions into other, less functional states. Some of the most biologically relevant states are described in Table 1.3.

1.9 Damage to membranes caused by cold temperatures

Plant membranes in temperate climates must maintain a fluid state even at low temperatures, and must lower the $L_\alpha \rightarrow L_\beta$ transition temperature to survive. When temperatures drop below the $L_\alpha$ transition temperature, disordered lipid-lipid associations are shifted into an ordered state to allow for a rearrangement and re-distribution of water (Quinn 1985). The transition involves the nucleation of gel lipid domains in those regions followed by domain growth (Cevc 1993). A cooperative domain expansion can occur over a relatively short temperature range, and for biological systems, maintaining the lamellar fluid phase ($L_\alpha$) is paramount.
Table 1.3. Common lyotropic mesophases. Dimensions indicate the cross-sectional restraints on the phase. Because it is flat, 1-dimensional membrane phase can be depicted in the X plane only. Whereas a tubular or hexagonal, 2-dimensional phase requires the X and Y plane to demonstrate its shape. Adapted from Cevc 1993.

<table>
<thead>
<tr>
<th>Phase Description</th>
<th>Dimensions</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solid-like lamellar Gel phase (Lβ or Lβ′)</strong></td>
<td>1-Dimensional, (tilted or un-tilted)</td>
<td>Hydrocarbons are well ordered; extended mostly in the trans-state. This phase can be seen in any biological membrane below its transition temperature. The rippling form is considered 2-dimensional.</td>
</tr>
<tr>
<td><strong>Fluid Phase (Lα):</strong></td>
<td>1-Dimensional, Fluid Lamellar</td>
<td>When the temperature is high enough, the hydrocarbon chains undergo a transition from the β or β′ conformation to a liquid-like conformation. There is an expansion of 15-30% in the interfacial area per molecule. Maintained by living organisms.</td>
</tr>
<tr>
<td><strong>Inverse Hexagonal (HII) Phase</strong></td>
<td>2-Dimensional, Fluid</td>
<td>This phase occurs in biological membranes exposed to extreme cold conditions and is common in phospholipids with small, weakly hydrated head groups, or phospholipids with strong head group-head group interactions. This form manifests itself in a hexagonal packing of cylinders, with hydrocarbon tails facing outwards and head groups forming the inner tubes.</td>
</tr>
<tr>
<td><strong>Solid-like Lamellar crystal Phase (Lc)</strong></td>
<td>3-Dimensional lamellar crystal</td>
<td>Induced by low temperatures by all phospholipids. Possess long and short-range order in 3 dimensions, and are “true crystals”. Dehydrated, pure lipids will often be in this form.</td>
</tr>
</tbody>
</table>
A gel-state biological membrane is susceptible to fusion, loss of osmotic responsiveness, loss of membrane-protein interactions, loss of lamellar integrity, and more. On the cellular scale, membrane damage is caused by a number of effects including the volumetric contraction of enclosed fluids, the concentration of intra- and extracellular solutes, pH changes due to different solubilities of buffering compounds, eutectic crystallization, and the dehydration of macromolecules (Gordon-Kamm and Steponkus 1984; Wolfe and Bryant 1999). Mechanical failures in protoplast membranes are caused by expansion-induced lysis, loss of osmotic responsiveness, altered osmotic behaviour, and intracellular ice formation (Dowgert and Steponkus 1984). Eutectic ice formation forces solutes into small volumes of liquid water, dehydrating and concentrating them. These hypertonic solutions at the membrane surface cause a rearrangement of lipid polar heads to maximize contact with water. When this occurs, the membrane is in a gel-crystalline phase, which is characterized by tightly packed polar lipid heads and elongated hydrophobic fatty acid tails (Kornberg and McConnell 1971). Further dehydration of the membrane causes an inverse-hexagonal (HII) transition, which forces the lipid polar heads to reorient around small volumes of liquid water into hexagonal cylinders. This transition has been demonstrated in biological membranes at temperatures as high as -4 °C to -8 °C (Steponkus et al. 1998a), a range that most plants in temperate climate zones must tolerate. Such drastic lipid rearrangements cause the membrane to lose its vital function: the ability to retain molecules and form a continuous barrier.

Fluids enclosed by lipids can present other challenges during freezing exposures, and various types of freezing damage caused on liposomes and protoplasts have been
explored (Dowgert and Steponkus 1984; Uemura et al. 2006). Generally, movement of water from protoplasts causes significant volume loss, which leaves excess membrane. When this occurs, endocytotic vesicles can pinch off to remove the excess. Upon thawing, the influx of water into the protoplast with a reduced membrane surface area causes the protoplast to rupture. This type of damage is called expansion-induced lysis (Figure 1.1). At even colder temperatures, when the vesicle-forming and frozen protoplast is severely dehydrated, HII phase transitions occur in the membrane, causing a loss of osmotic responsiveness (Steponkus and Lynch 1989). In this scenario, water does not re-enter the protoplast upon thawing.

1.10 Plant membranes and cold temperature acclimation

During warm months, plants have little tolerance to cold. Sensing shorter photoperiods and lower temperatures, plants begin to acclimatize, making physiological changes to tolerate low temperatures. For instance, the cold tolerance of commercial rye can increase from -5°C to -25 °C when exposed after being acclimatized (Uemura and Steponkus 1989). Plant cells have many types of membranes, each tailored to suit the function of the organelle. Composition is regulated by lipid transport systems, and bilayer asymmetry is maintained in the plasma membrane, endosomes, and trans-Golgi network membranes by flipping lipids across leaflets (Sharom 2011). Bulk lipids provide the general or most basic barrier, and include phosphatidylcholine, cholesterol, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and sphingomyelin. Low abundance lipids such as phosphatidic acid and diacylglycerol often act as signalling lipids that can recruit lipid-associating proteins and other peripheral proteins.
Figure 1.1. Types of freeze-induced dehydration damage on isolated protoplasts. (A) Expansion induced lysis (EIL). Vesiculation occurs upon volumetric contraction of the protoplast, resulting in a reduction in total membrane surface area. Subsequent influx of fluid upon rehydration, with a reduced membrane surface ruptures the protoplast. (B) Severe dehydration causes the membrane to undergo an $H_{II}$ phase transition, causing a loss of osmotic responsiveness (LOR). $H_{II}$ damage is not reversed upon thawing, and the protoplast does not expand. Fusion events called fracture-jump lesions may subsequently occur. (C) Cold acclimated protoplasts undergo exocytotic extrusions upon contraction, and these extrusions are continuous with the membrane. This prevents lysis; however $H_{II}$ transitions still eliminate osmotic responsiveness and cause fracture-jump lesions. Image adapted from Wolfe & Bryant, 1999.
For plant cells under normal conditions, consider the lipid species of *Arabidopsis*. Comparing non-acclimated plants and sub-lethally chilled plants demonstrates that the most dramatic alterations to membrane head-groups occurs after the plant undergoes a high stress cold treatment (Table 1.4). Uemura and Steponkus (1994) also observed that lipid ratios in plasma membranes respond to cold-treatments, but new lipid species are not introduced. For the three plant species tested, the amount of phosphatidylethanolamine (PE) increases, while phosphatidic acid (PA), phosphatidylinositol (PI) and phosphatidylserine (PS) levels appear to decrease in response to cold. These changes are not simply generic transcriptional stress responses; they specifically improve the membrane’s resilience in sub-zero temperatures. Not included in this particular table is the hydrocarbon chain distribution. An increase in the proportion of unsaturated phospholipids in the plasma membrane early in the cold exposure is common for many cold acclimated plants (Uemura et al. 2006). Interestingly, liposomes made from non-acclimated tissue can be made cold resistant by adding di-unsaturated phosphatidylcholine, which reduces vesicle formation and H_{II} transitions during freezing (Cudd and Steponkus 1988; Sugawara and Steponkus 1990). Liposomes composed of lipid extracts from cold-acclimated leaf tissue demonstrate exocytotic extrusions, which maintain membrane continuity after freezing (Dowgert and Steponkus 1984; Gordon-Kamm and Steponkus 1984). Unlike the endocytotic vesicles previously described, these extrusions can then be re-incorporated into the cell upon thawing; however, damage incurred by H_{II} phase transitions is irreversible.
Table 1.4. Total lipid amount based on the dry weight of cold and non-cold-acclimated Arabidopsis tissue. Plants were cold acclimated by placing 35 day old plants at 4°C for 3 days. Non-acclimated plants were grown at 23°C and harvested on the same days that cold acclimated plants were. For the -8°C treated plants, cold-acclimated plants were subjected to a temperature drop of -2°C at 4°C per hour. When the temperature reached -2°C, ice crystals were placed onto the soil to encourage ice crystal formation. The temperature was then lowered to -8°C and tissue was harvested after 2 h at this temperature. Adapted from Welti et al (2002).

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Non cold-acclimated plants</th>
<th>Cold-Acclimated Plants</th>
<th>Plants subjected to -8°C Treatment</th>
<th>Change in Saturation (non-acclimated to acclimated)</th>
<th>Change in Saturation (acclimated to -8°C Treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount of lipid per dry weight in wild-type plants (nmol/mg)</td>
<td></td>
<td></td>
<td>More, Less, or No Change in Saturation</td>
<td></td>
</tr>
<tr>
<td>PI</td>
<td>5.1 ± 0.7</td>
<td>5.4 ± 0.3</td>
<td>5.9 ± 0.6</td>
<td>NC</td>
<td>L</td>
</tr>
<tr>
<td>PG</td>
<td>22.3 ± 1.9</td>
<td>24.0 ± 1.2</td>
<td>18.8 ± 2.6</td>
<td>L</td>
<td>M</td>
</tr>
<tr>
<td>PE</td>
<td>11.7 ± 1.4</td>
<td>15.5 ± 0.8</td>
<td>9.7 ± 1.7</td>
<td>L</td>
<td>M</td>
</tr>
<tr>
<td>PC</td>
<td>22.8 ± 0.7</td>
<td>26.0 ± 0.2</td>
<td>14.4 ± 1.1</td>
<td>L</td>
<td>M</td>
</tr>
<tr>
<td>PA</td>
<td>1.2 ± 0.1</td>
<td>1.9 ± 0.5</td>
<td>11.5 ± 1.0</td>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td>MGDG</td>
<td>71.4 ± 5.1</td>
<td>77.0 ± 6.0</td>
<td>71.5 ± 8.3</td>
<td>L</td>
<td>NC</td>
</tr>
<tr>
<td>DGDG</td>
<td>30.7 ± 2.8</td>
<td>35.9 ± 0.9</td>
<td>40.2 ± 4.9</td>
<td>L</td>
<td>NC</td>
</tr>
<tr>
<td>LysoPE</td>
<td>0.12 ± 0.03</td>
<td>0.06 ± 0.02</td>
<td>0.8 ± 0.11</td>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td>LysoPC</td>
<td>0.14 ± 0.01</td>
<td>0.09 ± 0.09</td>
<td>0.71 ± 0.14</td>
<td>L</td>
<td>L</td>
</tr>
</tbody>
</table>
1.11 Membrane vitrification

Ice formation should not be mistaken for vitrification in the context of membranes and protein damage. While ‘bulk’ ice crystals adjacent to membranes can dehydrate them by sequestering liquid water molecules at decreasing temperatures, vitreous or glass-state water might protect membranes from further damage. Solutes in water prevent the nucleation of ice crystals in supercooled water by reducing the probability that a crystal of critical volume will form, and by increasing the viscosity of the fluid (for a comprehensive review, see Wolfe and Byrant 1999). At sufficiently high concentrations of solute, ice cannot form, and the solution will form glass at ambient temperatures (Koster 1991). Despite lack of evidence for vitrification occurring in vivo, glass formation might protect membranes by a) limiting further dehydration, b) reducing the chance of crystallization, and c) allowing the membranes to remain in fluid lamellar phases even at extremely low temperatures. Glass-state water can exist inside macromolecules, between membranes, and can include solutes. In this form, it can continue to hydrate at sub-zero temperatures. A type 3 LEA protein from *Typha latifolia* pollen was capable of stabilizing a glassy state at higher temperatures in sucrose (Shimizu et al. 2010), and so perhaps some dehydrins and LEA proteins are expressed to encourage vitrification over crystallization of water (Sun and Leopold 1997; Wolkers et al. 2001).
1.12 Dehydrins and membranes

*In vivo*

The majority of dehydrin research has been done *in vitro*, but there is evidence that membrane-bound dehydrins enhance cold tolerance. For instance, wheat WCOR410 is found in much higher concentrations in lipid fractions of cell lysates (Danyluk et al. 1998). Immunolocalization and electron microscopy of wheat tissue demonstrates that up to three quarters of detectable dehydrins are located at the plasma membrane (Danyluk et al. 1998). When this dehydrin was expressed in strawberries, WCOR410 provided a significant enhancement of cold tolerance. Leaf tissue of transgenic plants could withstand temperatures up to 5°C colder than wild-type strawberries. However, tolerance in these plants was only demonstrated in leaf tissue and not in root organs (Houde et al. 2004).

Another research group produced transgenic *Arabidopsis* plants expressing two different dehydrins (Puhakainen et al. 2004). In non-acclimated plants, approximately one-third of the dehydrin LTI29 was localized to membranes (plastid envelopes, plasma membrane, tonoplast). In cold-acclimated plants, approximately two-thirds of the total dehydrin could be found associating with the membranes (Puhakainen et al., 2004). These over-expressing plants had significantly enhanced freeze and cold tolerance, in both cold acclimated and non-acclimated plants. In a study by Artus et al. (1996), COR15a was constitutively expressed in transgenic *Arabidopsis* plants. By measuring variable/maximal fluorescence of chlorophyll in frozen and unfrozen chloroplast membranes from these plants, the researchers could quantify the amount of absorbed
light energy directed towards photosynthetic processes, and therefore determine how well the membrane functions. The researchers found that chloroplast membranes from transgenic plants are 1°C to 2°C more tolerant of in vitro freezing, which is significant, considering that the combined effects of the Arabidopsis’ natural acclimation response enhances its cold tolerance by about 6°C (Artus et al. 1996).

The current body of knowledge agrees that the interaction between membranes and dehydrins effectively enhances the freeze tolerance of cells and protoplasts; however there have been a handful of studies which refute the idea that these proteins have in vivo efficacy. Craterostigma plantagineum is a resurrection plant which tolerates extreme desiccation, and a transgenic tobacco over-expressing a C. plantagineum dehydrin did not have enhanced drought or freezing hardiness (Iturriaga et al. 1992). The likelihood that a single dehydrin species acts alone in maintaining the cells of C. plantagineum is low, considering the variety of dehydrins and the various expression pathways found in less cold-hardy plants.

**In vitro**

Hydrophilic polypeptides can stabilize lipids during freezing conditions (Thomashow 1998), and it is thought that the α-helix transition in the K-segment facilitates this interaction. Investigations into the binding effects of α-synuclein – a disordered protein with helical tendencies – revealed that the protein binds anionic micelles to form α-helix structure (Davidson et al. 1998). The similarities between α-synuclein and dehydrins may have inspired membrane-dehydrin studies. A dehydrin from cowpea acquired α-helical structure when bound to SDS micelles (Ismail, Hall, and Close
The dehydrins GmDHN and rGmD-19 from soybean indeed gain some (8%) helical structure in the presence of SDS micelles, but were unchanged in the presence of zwitterionic and anionic LUVs (Soulages et al. 2002; Soulages et al. 2003). Change in secondary structure in the presence of SDS micelles has also been observed with a DHN from wild grape (Graether et al., unpublished data).

Liposomes are great tools for simulating biological membranes because they are composed of lamellar bilayers and can be composed of any lipid or lipid mixture the researcher chooses. A fair number of studies have used liposomes to measure dehydrin-membrane interactions, and in numerous cases, acidic dehydrins have been shown to bind anionic membranes. In a gel filtration analysis, Koag et al. (2003) demonstrated that maize DHN1 is found in elution fractions containing anionic liposomes. This binding was also correlated with an increase in α-helicity as measured by CD (Koag et al. 2003). Studies performed with *T. salsuginea* TsDHN-1 and TsDHN-2 on plant-membrane-mimicking liposomes also demonstrated that these dehydrins undergo an α-helix transition upon membrane binding (Rahman et al. 2010). The dehydrins ERD10 and ERD14 were also found to bind anionic phospholipid vesicles, but binding in this case did not show any significant structural change by CD (Kovacs et al. 2008). Those researchers also observed that membrane fluidity was not altered by binding of either of these proteins. In another study, both Cor15A and Cor15B shifted the thermotropic phase transition temperature of dry, 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) lipid membranes to lower temperatures, but had no effect on similar membranes that contained egg phosphatidylethanolamine (EPE) (Thalhammer et al. 2010). Eriksson and colleagues
have used surface plasmon resonance as another method of measuring the binding of the dehydrin Lti30 to bound vesicles (Eriksson and Harryson 2011). Lti30 was found to bind both anionic and zwitterionic membranes, while in the same study, solid-state NMR measurements revealed that the binding of Lti30 occurs through electrostatic interactions.

Membrane binding by dehydrins appears to prevent membrane damage by cold temperatures. Supercooled membranes can form H_{II} structures in adjacent or contacting membranes, which is thought to bridge and fuse them (Cudd and Steponkus 1988). The intrinsic curvature of the surface and the type of membrane are both factors which influence this H_{II}-induced fusion. By constitutively expressing Cor15am in Arabidopsis cells, Steponkus et al. (1998) reduced H_{II} formations in closely-associating membranes, evidence for protection of a specific type of damage by a dehydrin.

1.13 K_{2} Dehydrin

A recently discovered wild-grape plant dehydrin has a very simple K-ϕ-K architecture. It is a 48 residue potential splice-variant of YSK_{2} mRNA from Vitis riparia, and is possibly expressed during dehydrating stress (Xiao & Nassuth 2006). Designated DHN1-U for the reason that it is unspliced, the gene has two open reading frames, but cannot be transcribed into one continuous dehydrin. The first reading frame is a YS polypeptide which is unlikely to directly impact cold stress tolerance. The second reading frame is a KϕK (K_{2}) polypeptide which is functional when transcribed and expressed. Because the K_{2} portion is in a second open reading frame on the DHN1-U transcript, it is unlikely that it is transcribed unless a second translational start-site is present. It is
uncertain whether or not K2 DHN1 protein (referred to as K2 from here on) is actually present in V. riparia tissue. Nevertheless, K2 represents a minimal dehydrin construct that provides an excellent opportunity to study dehydrin K-segment function with a simple model protein.

Previous studies have shown that K2 protects LDH from freeze inactivation (Hughes and Graether 2013). Sufficiently high concentrations of K2 can completely protect LDH activity from diminishing after a freeze-thaw cycle. In addition, $^{15}$N-HSQC NMR experiments performed by Findlater and Graether (2009) determined the chemical shifts of $^1$H and $^{15}$N of isotopically-labelled K2. These data determined that K2 dehydrin does not undergo a disorder-to-order transition in the presence of LDH, so the interaction between the dehydrin and LDH is most likely a non-specific shielding interaction. More recent $^{15}$N HSQC NMR experiments performed on K2 in the presence of SDS micelles have also shown that K2 has a moderately weak affinity for SDS micelles and does not undergo an $\alpha$-helix transition upon binding them (Clarke et al., unpublished results).

Dehydrins belong to a well-studied but not fully characterized family of proteins. It is becoming clear that there are likely many different functions for dehydrins, and they are expressed differently according to environmental conditions. There is mounting evidence that dehydrins interact with membranes to protect cells from cold, drought, and high salinity (Godoy et al. 1994; Xu et al. 2007; Houde et al. 2004; Puhakainen et al. 2004). So far, K2 has demonstrated binding to anionic liposomes, and undergoes a disorder-to-order transition in the process (Barker and Graether, unpublished results). This binding has been shown to protect these liposomes from fusion after a freeze-thaw
cycle (McKenna and Graether, unpublished results). The helix-formation and subsequent activation of natively disordered proteins occurs throughout all organisms, and these processes are not always well understood. Freeze-induced damage on cells is also a highly complex and multi-faceted process, which has implications in crop protection to cryopreservation. Nearly all temperate-climate plants express these proteins in extremely high amounts, and yet very little is known about them. Characterizing the effects and interactions between dehydrins and membranes can provide a broad model for one facet of this plant protection mechanism, and K$_2$ provides an attractive subject for study with a broad range of applications.

**CHAPTER 2: MATERIALS AND METHODS**

2.1 Purification of K$_2$ Dehydrin

The purification protocol used for dehydrins was previously described in Livernois et al., (2009). Briefly, E. coli BL21 (DE3) competent cells (Invitrogen) were transformed with a pET22b(+) plasmid previously cloned with the K$_2$ dehydrin insert. Freshly transformed cells were grown on Lysogeny Broth (LB) agar plates containing 100 μg/mL ampicillin, and small cultures were inoculated with a single colony from these plates in 5 mL LB with ampicillin. After growing overnight, 1 mL of small-scale culture was added to 500 mL of LB media, and cells were grown at 37°C with shaking at 180 rpm. Once the culture reached an optical density of 0.8, protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG). Following a 3 hour induction period, cells were pelleted for 15 minutes at 6,000 x g and re-suspended in water. EDTA
was added to a final concentration of 0.5 mM, and one protease inhibitor cocktail tablet (Roche Diagnostics, Germany) was added. Cells were lysed by boiling for 20 minutes, and hand-agitated approximately every 5 minutes. Due to the unstructured and hydrophilic nature of dehydrins, they remain in solution even after boiling. After cooling the suspension to room temperature on ice, sodium acetate was added to a final 20 mM concentration, and cellular debris was pelleted at 70,000 x g for 30 minutes. Soluble protein in the supernatant was collected and filtered through a 0.22 μm PVDF membrane, and then purified by FPLC using a cation exchange column (5 mL HiTrap SP FF, GE Life Sciences). The protein was loaded onto the column with a buffer consisting of 20 mM sodium acetate (pH 5.0), and eluted with a NaCl elution gradient from 0 to 1 M. After the protein-containing fractions were collected, the sample was desalted by reversed-phase HPLC through a Biobasic C18 column (Thermo Scientific, Waltham MA) using Buffer A (0.1% Trifluoroacetic acid) and Buffer B (0.1% trifluoroacetic acid in acetonitrile). Elution was done using a linear gradient of 1 to 100% Buffer B over 1 h at a flow rate of 1 mL/min. Fractions containing K$_2$ were identified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and were pooled. The pooled fractions were then lyophilized for storage in the freezer at -20°C until use.

2.2 Measuring the critical micelle concentration of sodium dodecyl sulfate

The fluorescent dye 1-anilinonaphthalene-8-sulfonic acid (ANS) partitions into any available hydrophobic regions, causing its fluorescence emission peak to shift from 545 nm to 490 nm. The critical micelle concentration thus can be measured by
plotting fluorescence emission (490 nm) against concentrations of SDS. Below CMC levels, the change is fluorescence as the SDS concentration increases is linear and has a shallow slope. When the CMC of the solution is reached, fluorescence emission of ANS dye increases, providing a steeper regression. By interpolating the SDS concentration value at the intersection between these two linear regressions, an estimate for the CMC can be made. To perform the fluorescence experiments, the detergent was dissolved in 20 mM phosphate buffer, pH 6.0. Protein was added, and an aqueous solution of 1-anilinonaphthalene-8-sulfonic acid (ANS) was then added to each sample to a final concentration of 20 μM, and allowed to equilibrate for 30 minutes. Fluorescence excitation was performed at 370 nm with an emission measurement wavelength of 495 nm using a PTI fluorimeter (Photon Technology International, London, ON). Data were recorded in FeLiX software version 1.41.

For conductivity measurements, a stock solution of 10 mM sodium dodecyl sulfate (SDS) was prepared by dissolving dry SDS powder in 20 mM phosphate buffer, pH 6.0. All measurements were made using a CDM210 Conductivity Meter (Radiometer Analytical, Villeurbanne, France). Initial calibration was first done by drawing up approximately 700 μL of calibration solution. After approximately 1 minute, the conductivity measurement was recorded. The sample was dispensed and the apparatus rinsed thoroughly with buffer. Each measurement was repeated in triplicate and results were plotted in SigmaPlot 10.0. Linear regression analysis was used to fit two lines to
each linear region of the curve, and the x-axis intersect of the lines was taken as the critical micelle concentration (CMC).

2.3 Reconstituting liposomes

Lipids (egg phosphatidic acid, PA; egg phosphatidylcholine, PC; 1,2-dimyristoyl-sn-glycero-3-phosphocholine, DMPC; 1,2-dimyristoyl-sn-glycero-3-phosphate, DMPA) were obtained from Avanti Polar Lipids (Birmingham, AL) in lyophilized form. Lipid solutions were prepared by dissolving pure lipids in 4:1 chloroform:methanol (v:v) at a concentration of 100 mg/mL. To prevent oxidation of lipid fatty acids, nitrogen gas was used to displace excess oxygen from vials of lipids before storing at -20°C. To prepare liposomes, a film of lipids was formed in the bottom of a glass test tube by depositing 12.5 μL of both PC and PA solutions, mixing by gently swirling, and then drying under a gentle stream of dry nitrogen gas. The remaining solvents were removed by placing the tube under vacuum for a minimum of 1 hour. The dry lipid film was then re-suspended in 5 mM phosphate buffer, pH 7.4, by vortexing thoroughly and incubating at 37°C for 30 minutes. This process produces large, partially hydrated heterogeneous sheets of lipids. The lipids were further homogenized by performing five cycles of flash freezing in liquid nitrogen and thawing at 45°C. Hydrated sheets of lipids and large multilamellar vesicles were then homogenized into large unilamellar vesicles by the extrusion method (Olson et al. 1979). Briefly, this method requires an apparatus which holds a polycarbonate membrane between two opposing syringes. The membrane has pores of defined sizes, and passing the hydrated lipid emulsion through it multiple times provides a homogenous
population of vesicles. To produce large unilamellar vesicles (LUVs), we used an Avanti mini-extruder (Avanti Polar Lipids, Alabama) with a membrane pore size of 100 nm.

Dynamic light scattering was then used to analyze liposome size and ensure successful reconstitution. Using a Malvern Zetasizer Nano S (Malvern Instruments, Worcestershire, UK) a particle size distribution curve was generated, and preparations containing average diameters of greater than 120 nm were not used.

2.4 Liposome fusion assays

Liposome size distribution was used to quantify membrane damage after freezing. Distributions were measured by DLS. Fusion assays were performed on 1:1 (w/w) PC:PA liposomes. These lipids were chosen to maintain congruence with previous experiments, as K₂ has been shown to bind with liposomes composed of this type of membrane. Sample size was 120 μL with a lipid concentration of 0.4 mg/mL, control protein concentrations ranging from 0-250 μM, in 5 mM phosphate buffer, pH 7.4. Before performing the freeze treatment, the samples were incubated at room temperature in microcentrifuge tubes for 30 minutes. They were plunged into liquid nitrogen, and stored at -80°C overnight. The tubes were then thawed at 37°C for 5 minutes, and 400 μL of buffer was added for dynamic light-scattering measurements. Measurements were made at 25°C using the discrete peak calculation method, with polystyrene latex as the material standard. Analysis was done in the Malvern Dispersion Technology Software Suite version 3.3, and plotted with SigmaPlot 10.0. Fusion experiments were repeated with
identical conditions using bovine serum albumin (BSA), polyethylene glycol 3350 (PEG3350), and lysozyme (LSZ).

2.5 Surface accessibility assay

To quantify the accessibility of the membrane surface when bound by K₂ DHN, the dye merocyanine 540 (MC540) was used. When partitioned into the hydrophobic interior of a membrane, the absorbance of the dye at 570 nm increases, while its absorbance at 530 nm decreases. Anionic liposomes at a concentration of 0.3 mg/mL were suspended in 50 mM phosphate buffer, pH 7.4 and were prepared with the required concentration of protein and allowed to equilibrate for 30 minutes. Samples were transferred into quartz cuvettes, and the light absorbance ratio (A₅₇₀/A₅₃₀ nm) for each sample was measured using a CARY Bio 100 UV-Visible Spectrophotometer (Agilent Technologies, California, USA). A stock solution of MC540 was then added to a concentration of 4.1 μM, and allowed to equilibrate for 5 minutes. Absorbance measurements were made again. Surface accessibility was then defined as

\[ A = \left( \frac{A_{570}}{A_{530,MC540}} - \frac{A_{570}}{A_{530,Initial}} \right), \]

and plotted against protein concentration in SigmaPlot.

2.6 Measurement of membrane fluidity using diphenylhexatriene and trimethylammonium diphenylhexatriene probes

Liposomes were reconstituted in the usual manner, with either diphenylhexatriene (DPH) or trimethylammonium diphenylhexatriene (TMA-DPH) added to the lipid mixture to produce a lipid:probe ratio of 200:1. The probed lipid cake was dissolved in 50 mM
phosphate buffer, pH 7.4 and extruded as described previously. Lipids were then diluted to an experimental concentration of 0.1 mg/mL for measurement in a PTI QuantaMaster C-61 steady-state fluorimeter with an electronic polarized emission filter and two manual excitation polarizing filters (Photon Technology International, London, ON). The G-factor was obtained first for each sample using the formula: \( G = \frac{I_{HV}}{I_{HH}} \), where \( I_{HV} \) and \( I_{HH} \) are the fluorescence intensities produced by the vertical and horizontal excitations, respectively, with the emission polarizer set in the horizontal position. Measurement of the G-factor is necessary in order to account for the relative efficiencies of each detector. Once these measurements were made, the emission polarizer was set in a vertical position and the measurements repeated. Anisotropy was calculated by the equation \( r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2G \cdot I_{VH}} \). Data were analyzed and plotted in SigmaPlot.

2.7 Differential scanning calorimetry

The thermotropic phase behaviours of the membranes were measured using a MicroCal VP differential scanning calorimeter (DSC) (MicroCal, Northampton MA). Due to the heterogeneity of egg lipid extract phospholipid tails, and the near 0 °C melting temperature, it was not possible to use egg PC/PA extracts used in the previous experiments. All liposomes used in the calorimetry experiments were composed of 1:1 DMPC:DMPA (w:w) to ensure that discrete melting transition could be measured. Head groups were chosen to maintain consistency with liposome fusion assays, and to ensure that K₂ would be bound to the liposomes. These liposomes were reconstituted using the same method as described previously, but the process was done at 55°C to keep the lipids
fluid and allow for extrusion. Sample sizes were 600 μL, with lipids at 0.4 mg/mL and a protein concentration at 75 μM. Liposomes were first allowed to incubate with the experimental protein for 30 minutes before measurement began. Samples and reference solutions were degassed in the ThermoVac system for approximately 15 minutes under a vacuum before use. The reference cell was filled with buffer only. To account for the heat capacity of the buffer alone, the buffer was scanned against a water reference. DSC was performed with up-scans at a rate of 45°C per hour, using a 5 second filtering period, a 15 minute pre-scan equilibrium, and with high-gain feedback mode enabled. High-gain feedback was used due to the relatively low signal intensity observed at these lipid concentrations. Due to the noise being generated in this mode, the filtering period was set to 5 seconds to provide a steadier signal. To account for the sample’s “thermal history”, fifteen scans were performed, and only the final scan was kept for analysis. Data from these experiments were exported into SigmaPlot and plotted.

2.8 Liposome leakage assays

In order to quantify leakage of liposome contents by freeze damage, a fluorescent probe which self-quenches at high concentrations was used. By encapsulating carboxyfluorescein (CF) at a self-quenching (100 mM) concentration, membrane damage could be observed by measuring the relative fluorescence of released dye. Powdered, 95% pure 5(6)-carboxyfluorescein dye (Sigma-Aldrich) was dissolved in 50 mM phosphate buffer by adding 1 M NaOH drop-wise to solubilize. The resulting solution was then used to hydrate a lipid film composed of 1:1 PC:PA. Dye-filled liposomes were
prepared using the extrusion method described above. Unencapsulated dye was removed with an Econo-Pac 10DG desalting column (Bio-Rad) using the minimal dilution protocol provided by the manufacturer. The liposomes were used in a 0.4 mg/mL concentration in a sample size of 120 μL.

Samples were treated by incubating with the protein for 30 minutes, and then frozen by plunging into liquid nitrogen. After being left overnight at -80°C, samples were thawed at 37°C and then transferred to a quartz UV cuvette. Released CF was measured by fluorimetry using an excitation wavelength of 470 nm and an emission wavelength of 515 nm. All remaining intact liposomes were then lysed by adding a solution of Triton X-100 at a concentration of 0.5% (v/v), and the fluorescence was measured again to determine the total CF in the sample. Liposome leakage was then calculated as

\[
\frac{\text{Sample fluorescence}}{\text{Maximum Fluorescence (post-lysis)}}
\]
CHAPTER 3: RESULTS

3.1 Introduction

The primary goal of the project was to examine the effect of K2 on membranes, and to determine whether or not membrane interactions with K2 provided a cold-protective effect. The idea that K2 might stabilize membranes in vitro was first investigated in this thesis by using SDS micelles as a membrane mimetic. It has been proposed that the binding of SDS molecules by charged, disordered proteins might reduce electrostatic repulsion and lower the energetic barrier to micelle formation (Necula 2003). By measuring the critical micelle concentration of SDS with conductivity and fluorimetric techniques, K2’s impact on micelle formation was determined. Bilayer membrane protection experiments were then performed using large unilamellar liposomes composed of egg PA and PC. First, the leakage of encapsulated, self-quenching carboxyfluorescein dye was measured after freezing with and without K2 to quantify the extent of membrane damage. Liposome fusion was also monitored by dynamic light scattering to examine how well K2 could prevent alteration of liposome structures. Lipid behaviour and membrane surface coverage by K2 was explored next. The effect of K2 on membrane fusion was thought to be a result of significant membrane coverage, and this was tested by probing membrane surface accessibility with merocyanine 540 dye. Lipid viscosity was then measured, using the fluorescence anisotropy of two DPH and TMA-DPH probes. Finally, differential scanning calorimetry
was used to measure major phase transition temperatures for membranes alone and those membranes that were bound to K$_2$ or control proteins.

3.2 Critical micelle concentration

Initial experiments using ANS dye established that there were drastic changes in the critical micelle concentration when the ionic strength of the buffer alone was modified. Similar results have been published by Esposito et al. (1998), who observed that high ionic strength buffers (0.5 M) reduced the CMC by up to ten-fold. In order to achieve a CMC value similar to that reported in the literature, a 20 mM phosphate buffer was used. With this buffer, the CMC of SDS is $3.9 \pm 1.4$ mM (Figure 3.1). Next, the effect of K$_2$ on the CMC was examined. Anticipating a reduction in the CMC, more data points were collected for lower SDS concentrations. With K$_2$ present, fluorescence increased dramatically in the low SDS concentration range (Figure 3.1) but then dropped at approximately 1 mM SDS. Despite the fluorescence spike, the CMC occurred at $4.3 \pm 1.5$ mM, as demonstrated by the increase in slope.

Solution conductivity measurements were then performed to validate the fluorescence experiments with a different technique. When SDS is dissolved in an aqueous solution at increasing concentrations, the conductivity data produce a positive linear regression. The post-micellization slope is always less steep than the pre-micellization slope, and the intercept between the regressions is the critical micelle concentration. Using these properties, the approximate CMC in 20 mM phosphate buffer
Figure 3.1. Effect of K$_2$ on micellization of sodium dodecyl sulfate as measured by ANS fluorescent probe. Control, ●; SDS with 10 μM K$_2$, ○. All samples were measured in 20 mM phosphate buffer, with an ANS concentration of 20 μM. Errors represent the standard deviation of three replicates from one experiment, and a minimum of three independent experiments were performed.
was measured, with and without K$_2$ present. In agreement with the ANS fluorescence data, solution conductivity experiments indicate that the CMC of SDS is $4.0 \pm 1.4$ mM in 20 mM phosphate buffer (Figure 3.2). In addition, with K$_2$ present at a concentration of 10 μM, the CMC was observed to be at $4.3 \pm 0.8$ mM. This set of experiments thus confirmed that K$_2$ DHN does not decrease the CMC.

3.3 Liposome fusion

In the liposome fusion experiments, the PC/PA LUVs or vesicles (1:1) were frozen and thawed in the presence of K$_2$ and control proteins BSA, LSZ, and PEG3350. Conditions such as buffer strength, the inclusion of salt, and the type of freeze-thaw had a major influence on the extent and variability of membrane damage. A method described by Hincha et al. (1999) requires chilling the samples to below zero without freezing and then inducing crystallization by touching the base of the tube with a liquid-nitrogen chilled spatula. This method was effective at nucleating the crystallization consistently, and appeared to be more controlled than the liquid nitrogen plunge. The samples were then held at -20°C for various periods of time (up to 24 hours). Even after five cycles, the method was ineffective at destroying PC/PA liposomes, and provided inconsistent results as measured by DLS (data not shown).

In another effort to damage liposomes in a using freezing temperatures closer to possible natural environments, samples were incubated at -20°C, and allowed to equilibrate over a number of time periods of 3-12 hours. In this case, freeze damage measured by DLS was also inconsistent and unpredictable. The inclusion of salt at a
Figure 3.2: Effect of K$_2$ on micellization of sodium dodecyl sulfate as measured by solution conductivity. Control, ●; SDS with 10 μM K$_2$, ○. Samples were prepared in phosphate buffer at 20 mM.
concentration of 100 mM and using a buffer concentration of 50 mM also reduced the amount of damage done to the liposomes. With the combined effects of salt, high buffer strength, and a gentle freeze-thaw process, PC/PA liposomes are fairly resistant to freeze-induced stress. Consistency of freeze damage, defined as having at least three sets of data in a row with nearly identical results, was achieved only at low buffer strength (5 mM phosphate), no salt, freezing by liquid nitrogen, and thawing at 37°C.

When 100 nm PC:PA liposomes alone are frozen in liquid nitrogen and thawed at 37 °C, size distributions shift from a bell-curve shape centered at 100 nm, to a bimodal distribution, with the greatest intensity centered at diameter of approximately 1000 nm (Figure 3.3, A-D). This result indicates that most of the liposomes are fused and have merged into large particles. Lower intensity peaks centered on 100 nm also indicate that some vesicles remain undamaged, and some very small particles (~10 nm) are a product of the freeze-thaw process. To gauge the relative ability of K₂ to protect liposomes from freezing, control proteins were used in the freeze-thaw experiments. Bovine serum albumin (BSA) was tested as it is known to have cryoprotective activity with LDH-protection assays (Hughes and Graether 2011). Lysozyme and PEG3350 were used as structured proteins and unstructured polymers, respectively, both with similar hydrodynamic radii as K₂. All proteins were used with identical lipid:protein molar ratios under identical experimental conditions.

At low concentrations, K₂ is ineffective at protecting the liposomes from fusion. Size distribution is still centered on 700-1000 nm with some particles of approximately 60 nm in size remaining (Figure 3.3 A). At a K₂ concentration of 75 μM, there is a
Figure 3.3. Size distributions by intensity of frozen liposome solutions with protein and control compounds. A: K$_2$, B: Bovine serum albumin, C: Egg white lysozyme D: PEG3350. Measurements were made with a lipid concentration of 0.4 mM. All fusion experiments were performed a minimum of 3 times, with representative data being shown.
dramatic shift in particle size distribution. Nearly half of the liposome population remains at 100 nm in size, while the other half are fused into large particles. At the highest protein concentration of 250 μM, particle sizes produce a bimodal distribution with peaks at 70 nm and 190 nm. At this K2 concentration, the higher radius peak has shifted significantly, leaving no particles with sizes greater than 400 nm.

BSA appears to provide liposome freeze protection also, as seen by the shift in peak intensity as both proteins increase concentrations (Figure 3.3 B). Rather than clearly separated 2-peak distribution of sizes, liposome diameters are continuous across a large spectrum, even at relatively high concentrations of BSA (i.e., at a concentration of 250 μM, many large fused particles remain at 1000 nm). The control compounds, PEG3350 and lysozyme, demonstrated no protective effect on the liposome populations at the concentrations used (Figure 3.3 C-D).

3.4 Liposome leakage

To perform liposome leakage assays, PC/PA liposomes encapsulating 100 mM CF were prepared. After treating liposomes with the freeze-thaw process, fluorescence released by treatment damage was first measured, and then compared with the total fluorescence of the sample after lysing the liposomes with detergent. As a positive control for dye encapsulation and protection, a generic cryoprotectant, 5 mM D-sucrose, was added to fresh liposomes, and the sample was frozen and thawed. Significant decreases in liposome leakage in the presence of D-sucrose confirmed that the liposomes were successfully formed with dye inside (data not shown).
Initial experiments indicated that liposomes incubated with K₂ released less dye after being frozen (not shown). It was thought that K₂ was demonstrating a good protective effect; however, post lysis fluorescence was also decreasing (Figure 3.4). The phenomenon of decreasing CF fluorescence was then investigated further. Simple fluorescence measurements using CF dye in buffer with various concentrations of K₂ determined that K₂ alone does not quench CF dye (Figure 3.4). Despite the acidic nature of K₂, it had no effect on CF fluorescence. There was also the possibility that an interaction between the free lipids, and dye was occurring. Lysing CF-filled liposomes and then adding K₂ confirmed that fluorescence was the same in these samples with and without K₂. When the experiment was attempted with calcein instead of CF, identical results were observed (not shown). The only remaining possibility is that during freezing, K₂ facilitates the formation of dye-quenching structures that are not lysed by Triton X-100.

3.5 Liposome surface accessibility

Liposome fusion protection had been demonstrated by K₂, and it was thought that K₂ might provide a blanket-like cover which keeps adjacent membranes from coming into contact with one another. One way to measure the extent of membrane coverage is by measuring the light absorbance of a dye called merocyanine (MC540), which partitions itself into the membrane-aqueous interface and orients itself parallel to the glycerol backbone. This partitioning into a more hydrophobic environment increases its absorbance at 570 nm, while decreasing its absorbance at 530 nm (Bakaltcheva et al. 1994). It then becomes a semi-quantitative probe for membrane surface accessibility, as
Figure 3.4. Effect of K$_2$ on pre and post-lysis fluorescence intensity of carboxyfluorescein dye, encapsulated by PC/PA liposomes. Carboxyfluorescein was encapsulated at a concentration of 100 mM. Fluorescence emission was first measured for each sample, and then samples were split, and K$_2$ was added to half of them. All samples were lysed with a final concentration of 0.5% Triton X-100 and total fluorescence was measured. K$_2$ was then added to the other half of the samples. Means ± SD of three parallel samples are shown. Samples before detergent-lysis, □; Samples where K$_2$ is added first, and then detergent-lysed, ■; Samples that were detergent-lysed, and then K$_2$ added after, ▪.
the change in these absorbance ratios would be proportional to membrane surface accessibility.

For these measurements, PC/PA liposome and protein samples were incubated for 30 minutes. The absorbance ratio ($A_{570}/A_{530}$) for each sample was measured, and a solution of merocyanine (MC540) was added and allowed to equilibrate for 30 minutes. Absorbance measurements were made again, and the absorbance ratio from the untreated sample was subtracted from treated absorbance ratio. This value was plotted as a function of $K_2^+$, BSA, lysozyme, or PEG 3350 concentrations (Figure 3.5). No significant change in the ratios was observed, even at the highest concentration of $K_2^+$ used, suggesting that there is no significant coverage. In addition, BSA and PEG3350 provide no measurable membrane coverage.

The drastic decrease in $A_{570}/A_{530}$ indicates that lysozyme provided significant coverage at a concentration of 50 μM. Increasing the LSZ concentration did not cause greater surface coverage, suggesting that the liposome surfaces were already saturated by lysozyme at a concentration of 50 μM.

3.6 Fluorescence anisotropy

After determining that $K_2^+$ binds anionic membranes, preventing them from fusing but not protecting them by binding the phosphate head-groups, the physical properties of the membranes in the presence of $K_2^+$ were explored. Lipid dynamics can be examined by measuring the degree of fluorescence depolarization of molecular probes embedded in the membrane. The probe 1,6-diphenyl-1,3,5-hexatriene (DPH) is hydrophobic, and is used to measure the fluidity of membrane hydrophobic core. The extent of depolarization
Figure 3.5. Coverage of liposome surfaces by K₂ and control compounds measured by the A₅₇₀/A₅₃₀ ratio of merocyanine dye. Samples were incubated with protein for 30 minutes prior to the initial absorbance measurement. Merocyanine dye was then added, and UV absorbance was again measured after 30 minutes. K₂, □; LSZ, ■; BSA, ¥; PEG3350, ★. Means ± SD of three parallel samples are shown.
of the excited DPH molecules is related to the degree to which a population of
photoselected molecules loses their initial orientations and becomes randomized (Dale et
al. 1977). When the tumbling of DPH is slowed by a less fluid, more densely packed
membrane, a higher proportion of emitted light is in the same polarity as the excitation
beam. Measurement of the degree of polarization of emitted light from a probe such as
DPH is called steady-state fluorescence anisotropy. Probing liposomes with DPH was
done by adding DPH to lipid formulations before hydrating with buffer. A lipid to probe
ratio was kept at 200:1, which is consistent with experiments by Hincha et al. (1999).
Confirmation that DPH had inserted into the membrane was done by heating the
liposomes and observing the decrease in steady-state anisotropy. All DPH measurements
were done at 25°C, which is above the estimated transition temperature for the
membranes reported by Cevc (1993). Steady state anisotropy was measured for probed
PC/PA liposomes in the presence of K₂, BSA, LSZ, and PEG3350 at a range of
concentrations. Excitation polarization was first set to a horizontal position, with
emission measurements being made in both the horizontal and vertical configurations.
The excitation beam was then rotated 90 degrees and the measurement repeated. The K₂
dehydrin and control proteins were tested to observe any changes in the viscosity of the
inside of the bilayers. Interestingly, K₂ had no effect on inner membrane viscosity, at any
concentration. As shown in Figure 3.6, anisotropy remained constant regardless of the
protein concentration. Higher concentrations of K₂ were not possible due to aggregation
of liposomes by the protein. In addition, PEG 3350 and lysozyme had no measurable
effect on membrane interior microviscosity. The addition of BSA did cause an apparent
Figure 3.6. Steady-state anisotropy of DPH-probed PC/PA liposomes in the presence of K₂, BSA, LSZ, and PEG3350. Probed liposomes were incubated for 30 minutes at 25 °C before measurements were made. Means ± SD of three parallel samples are shown. K₂: ●; BSA: ○; LSZ: ▼; PEG3350: Δ.
significant increase in anisotropy, suggesting an increase in membrane viscosity. However, BSA is known to bind DPH (Trotter and Storch 1989) so the results are considered to be artifactual.

A second fluorescent molecule was then used to probe the phospholipid interfacial area. We used N,N,N-Trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl) phenylammonium p-toluenesulfonate (TMA-DPH) which has a charged trimethylammonium group, causing it to embed itself into the lipid-water interface. It is widely used to monitor the mobility of the lipid head-groups. Measurements of surface microviscosity in the presence of K₂, PEG3350, LSZ, and BSA are shown in Figure 3.7.

Again, K₂ does not appear to affect the fluidity of the membrane, with very small increases and decreases in anisotropy likely resulting from experimental error. Binding by BSA does not appear to occur with this probe, perhaps due to the change in charge and shape of the molecule. Lysozyme and PEG3350 exert no visible effect.

3.7 Differential scanning calorimetry

DSC is a powerful, non-perturbing technique which allows the user to determine the temperature, enthalpy, and cooperativity of phase transitions. For these studies, pure lipid compositions are required. Egg lipid extracts have been used here for all previous experiments; however, the high acyl chain diversity created smooth enthalpy DSC thermograms with no distinct transition peaks or periods (Figure 3.8). To overcome this problem, dimyristoyl acid tails with phosphatidic acid and phosphatidylcholine head
Figure 3.7. Steady-state anisotropy of TMA-DPH-probed PC/PA LUVs in the presence of K2, BSA, LSZ, and PEG3350. Means ± SD of three parallel samples are shown. K2, ●; BSA, ○; LSZ ▼; PEG3350, Δ.
Figure 3.8. Heating differential scanning calorimetry thermograms of PC/PA liposomes. Scans were performed at a rate of 45°C/hour, using 5 mM phosphate buffer in both cells and 1 mg/mL lipids in buffer for the sample cell. The melting temperature of was predicted to be approximately 8°C.
groups were used. Having a single species of acyl chain with only two head-group types favoured short, cooperative transition processes which are easily observed by DSC.

Phase transitions were monitored during heating scans rather than cooling. This was done to reduce supercooling effects, which have been described by Yi and MacDonald (1973). This effect causes a transition shift by up to 1°C, and often causes much less pronounced pre-transition periods.

Liposomes composed of DMPC:DMPA (1:1, w/w) in 5 mM phosphate buffer exhibit a gel-fluid phase transition at 39.5°C (Figure 3.9A). This transition occurs over a period of approximately ΔT = 4°C, and is almost half-way between the predicted transition temperatures for each of the two species of lipid alone. The highly endothermic, single peak demonstrated by these liposomes indicates that the process is highly cooperative with a good degree of lipid purity. The cooperativity of the transition is 1.2, and was calculated by locating the temperature range at half-height of the heat absorption peak (McElhaney 1986). A cooperativity value of zero would indicate the theoretical maximum amount of participation with all the molecules available. This would be seen as a high peak with zero width, demonstrating a transition occurring in all molecules at a single temperature.

Once consistency had been attained for transitions with liposomes alone, the process was repeated with liposomes incubated with K$_2$. A concentration of 75 μM was chosen because it was an effective concentration for liposome fusion assays, and due to equipment time restrictions, it was not possible to examine a range of protein concentrations. Here, K$_2$ was added to liposomes and incubated for 30 minutes. At this
Figure 3.9 Differential scanning calorimetry thermograms of 1:1 DMPC : DMPA liposomes in the presence of K2, BSA, LSZ, and PEG3350. Heating scans were performed at 1°C/minute with high feedback and a 5 second filtering period. Samples were scanned against a reference cell containing buffer only. A: Control; B: K2; C: PEG 3350; D: BSA; E: Lysozyme.
lipid:protein ratio, the liposomes appeared to aggregate and precipitate out of solution. Earlier liposome leakage assays indicate that despite this aggregation, liposomes are not damaged, so measurements were continued at this concentration. As seen in Figure 3.9B, the transition period in the presence of K$_2$ is widened to an approximate $\Delta T = 6^\circ$C, and is centered at 36.6 $^\circ$C with a pre-melt peak at 33.7$^\circ$C. The heat of transition is also significantly reduced to less than half of the control, and has a cooperativity of 4.1. After 15 heating scans and subsequent cooling, the samples were removed from the cells. Interestingly, the protein: lipid aggregates were no longer present after this treatment. BSA also reduces the phase transition temperature of the DMPC/DMPA Liposomes (Figure 3.9 D). The transition is split into two peaks centered at 33 $^\circ$C and 36.5$^\circ$C, with a smaller release of heat and the lowest cooperativity, with a value of 6.5.

In the presence of PEG3350, the transition is centered at 39.7$^\circ$C, nearly identical to that of liposomes without any protein. Cooperativity is slightly reduced, with a value of 1.6, and the heat of the transition is also significantly less, with a peak height approximately 40% of the size of the control sample.

Lysozyme appeared to have the most dramatic influence on the transition of DMPC/DMPA liposomes (Figure 3.9 E). Lysozyme caused a remodelling of the liposomes, resulting in a white cloudy precipitate which persisted even after the heating and cooling process. No major enthalpy transition can be observed, though a slight downward shift is seen throughout the phase transition period of the lipids alone. The lack of a distinct endothermic transition peak suggests that DMPC/DMPA liposomes were destroyed by lysozyme, and non-bilayer structures may have been formed.
CHAPTER 4: DISCUSSION

4.1 $K_2$ does not reduce the critical micelle concentration of SDS

Exploring the effect of $K_2$ binding of micelles was first done to determine whether or not $K_2$ could stabilize SDS micelles at lower concentrations. Micelle formation is spontaneous at a threshold concentration, often reported to be approximately 8 mM for SDS (Esposito et al. 1998). The process requires electrostatic and hydrophobic barriers to be overcome, allowing a cluster of SDS molecules to come together with hydrophilic head-groups projected towards the aqueous solution, and hydrophobic dodecyl tails projecting into the center, which would exclude water molecules. Disordered, charged proteins have been shown to have a strong effect on the micellization of anionic detergents by significantly reducing the critical micelle concentration (CMC) as measured by fluorescent probes (Necula et al. 2003; Rivers et al. 2008).

The result that the CMC did not decrease in the presence of $K_2$ was not particularly surprising. There are broad similarities between $K_2$ dehydrin and $\alpha$-synuclein: the presence of many repeated charged residues, hydrophilic properties, and a lack of intrinsic structure. Necula and colleagues (2003) observe a dramatic decrease in CMC in the presence of $\alpha$-synuclein protein; however, micelle formation also induced a fibrillization behaviour for $\alpha$-synuclein, a cooperative aggregation behaviour which dehydrin or LEA proteins do not perform. As suggested by the aforementioned authors, the detergent micelles perhaps encourage bound, partially structured $\alpha$-synuclein molecules to come into close association, lowering the activation energy for amyloid
aggregate formation. As far as current literature goes, dehydrins also have some structural propensity when bound to anionic micelles, but no data support the idea that dehydrins form fibrils under these conditions (Ismail et al. 2009; Koag et al. 2003; Soulages et al. 2003; Koag et al. 2009).

The low SDS concentration fluorescence spike observed upon the K₂-SDS interaction is unusual in that it returns to basal fluorescence levels (Figure 3.1). One possible explanation is that a small amount of anionic SDS molecules is being corralled by multiple dehydrins with net positive charges, creating a water-excluding aggregate. Addition of more SDS molecules provides more binding sites for dehydrin interactions and disperses dehydrins over a greater number of SDS molecules, dissipating these protein aggregates. A theoretical model for this phenomenon is proposed in Figure 4.1.

Although binding of K₂ to SDS micelles has been demonstrated here, that the CMC is not reduced in the presence of K₂. This observation indicates that there is no cooperativity between the stable formation of SDS micelle aggregates and the transition from random coil to α-helix like structure in K₂. It is tempting to suggest that the CMC is slightly raised in the presence of K₂, however a high level of error in CMC calculations makes it impossible to draw this conclusion with confidence. If it were true, perhaps it is due to individual SDS molecules being preferentially bound to K₂, effectively reducing the number of SDS molecules able to participate in micelle formation. In effect, K₂ might enhance the hydration of SDS by reducing the concentration of free SDS molecules able to participate in micelle formation. This hydrating property of K₂ might be the mechanism behind some of the results that will be discussed in more detail later.
Figure 4.1. Proposed model for the spike in hydrophobicity at low SDS concentrations in the presence of K$_2$. K$_2$ molecules are shown as wide grey ribbons, while SDS molecules are depicted as small dark lines. (1) At SDS concentrations of ~0.5 mM, SDS-K$_2$ complexes are dispersed homogeneously. (2) A threshold SDS:K$_2$ concentration ratio is reached and K$_2$ molecules compete for electrostatic binding with SDS molecules. Close association of SDS molecules facilitates hydrophobic regions produced by dodecyl hydrocarbon tails. (3) An SDS concentration of 1 mM is high enough to allow for equilibrium binding and free exchange of SDS molecules. Homogeneous distribution of molecules removes hydrophobic regions. (4) Micelle formation forms hydrophobic pockets surrounded by K$_2$.
4.2 PC/PA liposomes are protected from freeze–thaw–induced fusion by $K_2$

Membranes are very susceptible to freezing damage (Dowgert and Steponkus 1994). When liposomes are frozen in liquid nitrogen and then thawed to room temperature, the average liposome diameter increases ten-fold. This increase in size requires a re-arrangement of lipid head group packing and a breaching of the continuity of the membrane, followed by a merging and gathering of large, broken vesicle aggregates into fusion products. Dynamic light scattering can be used to measure the average diameters of the entire population of particles in a sample, and size distributions and their intensities are an indicator of the products and the extent of liposome damage when samples are measured before and after freezing. Intensity peaks at the different sizes are interpreted as an approximate measure of membrane damage, with the assumption that a change in size represents “freeze damage”, even though I acknowledge that the final measured sample may be a result of a series of damaging processes from the combined effects of freezing, expansion-induced lysis, thawing, and temperature-induced lipid rearrangements.

The liposome fusion assay provided some detail of the extent of membrane damage and liposome population remodelling. Both $K_2$ and BSA are known to protect enzymes from freeze-thaw damage (Sanchez-Ballesta et al. 2004), and dehydrins have frequently demonstrated superior protection ability. We found that $K_2$ and BSA also have profound impacts on the end-result of the freeze-thaw process. As the amount of $K_2$ bound to liposomes increases, a greater proportion of post-freeze aggregates remains at
approximately 100 nm in size. This result is in agreement with data collected previously by Christine Barker in our laboratory (unpublished results). Although BSA also demonstrates this possible protective effect, in terms of molarity, K$_2$ is more effective at keeping liposomes at the 100 nm diameter size. At a concentration of 250 μM, the majority of liposomes are still in aggregated, large particles in the presence of BSA. A clear pattern emerges from this size distribution data: the more K$_2$ that is bound to anionic liposomes, the more liposomes “survive” a freeze-thaw process.

One of the main problems with measuring liposome fusion in the presence of K$_2$ was managing aggregation. At high protein (>500 μM) concentrations and low buffer strengths, it was impossible to measure the size distributions of the liposomes due to insoluble precipitates forming. In order to prevent this from occurring, protein concentrations had to be kept low. We observed that K$_2$ binding to the liposomes does not burst liposomes open because K$_2$ added to dye-filled liposomes does not result in dye release (data not shown). In addition, thermotropic phase behaviour can still be observed on aggregated liposomes bound to K$_2$ that have been treated by multiple cycles of heating and cooling. In vivo, this is likely not an issue, as membranes would be much larger than liposomes and not pulled together by electrostatic forces. Despite being limited to low protein concentrations for the experiments, the concentrations of two different LEA proteins in cotton embryos has been calculated to be 226 μM and 283 μM (Roberts et al. 1993). Coincidentally the maximum concentration we were able to use was 250 μM, so the protein range used in these experiments is perhaps physiologically relevant. Another limitation to the liposome fusion data, which is important to note, is that all freeze-thaw
procedures were done by plunging samples into liquid nitrogen. The technique itself is not physiologically relevant, but was necessary, as several other methods were attempted and resulted in poor consistency (data not shown). So, while freeze-damage methodology may not be physiologically relevant, it is required to produce consistent baseline data.

A few hypotheses could explain the protective effect by K₂. One idea is that lipid damage induced by extreme cold and dehydration stress causes the formation of H_{II} phases. As discussed in the Introduction, these phases are formed by an arrangement of hydrophilic head groups to remain hydrated with a very small amount of water. The resulting hexagonal lipid tubes have hydrophobic tails projecting outwards. Upon rehydration, it would be very easy for two H_{II} phase membranes from different liposomes to merge rather than flip the fatty acid tails back over and re-integrate to their parent liposomes. The ability of K₂ to maintain hydration of the lipid membrane, even when water molecules are being sequestered into ice, might prevent H_{II}-formation and subsequent fusion. Alternatively, sections of H_{II} membrane might be contained within a single liposome during thawing due to crowding by K₂.

Another possibility is that bound K₂ can provide a molecular film, creating a space between two potentially interacting membranes. The interesting juxtaposition to this idea is that if K₂ were electrostatically bound, the neutralization in overall membrane charge would allow membranes to associate more closely. This might cause an increase in liposome fusion, due to a collective reduction in repulsive forces between liposomes. The collision of adjacent membranes would be increased until all the membranes were un-charged, at which point further addition of K₂ might begin to prevent the direct
contact between adjacent liposomes. This might explain why liposome damage appears to increase with low concentrations of K₂ before decreasing (Figure 3.3A). In order to test the extent of membrane coverage and shielding, surface accessibility was examined using MC540 dye (Refer to Chapter 3.5 and 4.4 for Results and Discussion).

4.3 K₂-liposome interaction may form detergent resistant capsules upon freezing

Carboxyfluorescein (CF) is a dye that self-quenches at concentrations greater than 100 mM. When encapsulated in liposomes at quenching concentrations, fluorescence emission at 518 nm is almost non-existent. Rupturing or damaging the liposomes with Triton X-100 releases the dye, causing a drastic increase in fluorescence due to dye dilution. The extent of membrane damage can thus be determined by measuring the amount of CF released by liposomes during freeze-thaw treatment compared to the detergent-treated control. It was expected that liposomes would retain more soluble fluorescent dye in the presence of K₂ because other LEA proteins had demonstrated similar protective effects (Tolleter et al. 2010; Uemura et al. 1996). LEAM protein from pea demonstrates significant enhancement of leakage protection with POPC:EPE:CL liposomes (Tolleter et al. 2010), and COR polypeptides are effective at protecting DOPC:DOPE:FS-SUVs (Uemura et al. 1996). Several LEA and LEA-like proteins, such as soybean GmPM6/GmPM16 (Yang et al. 2011) and yeast HSP-12 (Sales et al. 2000),
have also demonstrated a significant enhancement of CF retention during dehydration stress.

Unfortunately, the $K_2$-liposome interaction during freezing produced particles which prevented accurate measurement of CF retention. I speculate that prior to freeze-thaw, the highly positively charged lysine residues in $K_2$ strongly attract phosphatidic acid, causing a localized enrichment of PA on a liposome surface. These PA-enriched microdomains may be less structurally stable due to the balance between close association of the phosphatidic acid head-groups repelling each other, and $K_2$ pulling them in. Boundaries of these unstable domains perhaps become nucleation points for freeze-induced membrane damage. I further speculate that these microdomains may bulge outwards, with an enrichment of PA molecules on the outer leaflet, in order to satisfy electrostatic interactions with $K_2$ while maximizing the amount of interfacial head-space area. Membrane destruction by freezing might then cause a separation of these PA-enriched domains, followed by a spontaneous re-closing of the membranes around small amounts of CF dye. Due to the high charge density of these fission products, significant coverage by $K_2$ molecules would make them resistant to detergent lysis.

4.4 Bound $K_2$ does not reduce membrane surface accessibility

Membrane surface coverage by $K_2$ was not demonstrated in the MC540 experiment (Figure 3.5), and due to the flexibility of $K_2$ and its lack of structure, this was not surprising. In a random coil or helix conformation, the protein still occupies a very
small volume, which combined with its potential ability to “slither” over a surface would mean that dye molecules competing for the surface would have no trouble accessing it. Surface accessibility data also indicate that BSA, despite being a structured molecule, does not compete for surface space. These data are consistent with experiments done by Jeff Madge, which show that BSA does not bind PC/PA liposomes, as they are not detected in centrifuged liposome pellets (data not shown). Also of note is that PEG3350 does not provide any surface coverage, but lysozyme does. Lysozyme, being structured and known to bind negatively-charged phospholipids (Gorbenko et al. 2007), likely provided a barrier for MC540 accessibility. Despite its small volume, it is the only protein capable of reducing liposome surface accessibility.

We can conclude that a molecular shielding for anionic membranes is not being provided by K₂, even though K₂ does bind them. The association must then be more flexible, and be intended to allow membrane interaction by other small molecules. This scenario would be beneficial in a plant cell, as the transport of solutes, as well as the ability of membrane-associated proteins to access the membrane is a requirement for all cells.

4.5 Lipid packing and viscosity are unaffected by K₂ binding

Lipid packing and membrane fluidity are properties of membranes which affect solute permeability and membrane phases. A dense packing of the acyl chain interior could be facilitated at low temperatures by crowding of small molecules such as cholesterol, or by increasing the saturation of the acyl chains. An increase in viscosity
reduces the probability of ice nucleation and growth during freezing (Wolfe and Bryant 1999). The result is that rather than freezing, vitrification or glass formation occurs, a state which maintains membrane bilayer structure and prevents further dehydration. On the other hand, a decrease in membrane viscosity can lower the membrane phase transition temperature and allow the membrane to maintain a higher level of fluidity at a reduced temperature. If K<sub>2</sub> is decreasing or increasing the viscosity of membranes, this effect could provide a significant contribution to freeze-protection. Using two fluorescent probes, I monitored the behaviour of the membranes within the hydrophobic core and at the interfacial area between the hydrophobic acyl chains and the hydrophilic head groups.

The likelihood of K<sub>2</sub> dehydrin completely inserting itself within the hydrophobic core is low due to its high proportion of hydrophilic residues, but K<sub>2</sub> does have three isoleucines and four methionines which might be capable of projecting their side chains into the hydrophobic core to secure the protein to the membrane surface. There is also the possibility that lysine side chains can “snorkel” by keeping the charged amino group in the phosphate head-group area, while burying the remainder of the side chain into the hydrophobic space (Strandberg and Killian 2003). One or multiple of these side-chain configurations might be required to satisfy the dehydrin α-helix conformations that has been observed for K<sub>2</sub> bound to the liposomes. It was hypothesized that membrane microviscosity would be reduced in the presence of K<sub>2</sub> dehydrin, since a lowering of the viscosity would allow the membrane to compensate for lipid crowding from decreases in temperature or dehydration stresses. However, steady-state anisotropy data indicate that K<sub>2</sub> has no influence on the viscosity of the membrane deep within the hydrophobic core,
or at the interfacial area (Figure 3.7). Similar findings have been reported by Papahadjopoulos et al. (1975), who observed no change in membrane permeability in anionic phospholipid vesicles in the presence of a polylysine peptide. The authors concluded that the interaction was an electrostatically-induced surface adsorption.

Similar electrostatic adsorption might be occurring between K$_2$ and PC/PA liposome membranes. The adsorption would allow for a binding and neutralization of negative charges by lysine side chains on K$_2$, and also allow for unhindered motion of all unbound lipids. Lipids not associating with K$_2$ would likely be PC molecules, which would be partially excluded from a PA-enriched phase. Due to the wide diversity of acyl chains in egg phospholipid extracts, even if a two-phase lipid mosaic is being formed – where head groups are restrained by K$_2$ in the PA-enriched phase – the overall rotational motion and homogenization of the hydrophobic interiors is likely unaltered.

4.6 K$_2$ lowers the thermotropic phase transition of anionic membranes

One of the most important aspects of a biological membrane is its relative fluidity. At low temperatures, membranes thermodynamically favour non-fluid states such as the L$_\beta$ or gel-state. Non-fluid states are extremely detrimental to the well-being of the cell due to numerous reasons, including loss of osmotic function and alteration in protein-membrane interactions. The main goal of the experiment was to identify the L$_\beta$ $\rightarrow$ L$_\alpha$ (gel to fluid) transition temperature of the membrane system, and to see if this value was altered in the presence of K$_2$. This transition has been reported to be a fast, high enthalpy, and highly cooperative process, making it suitable for measurement by differential
scanning calorimetry (DSC) (Dopico 2007). DSC also allows one to record the entire transition process without introducing foreign probes or molecules that might interact with the membrane or the protein of interest. Many factors influence the transition enthalpy (ΔH). Träuble and Eibl propose that the transition enthalpy is equal to the sum of terms arising from gauche isomer formation, expansion against van der Waals forces, disruption or alteration of inter-facial and head group structures, and the difference in the electrical double-layer energies (ΔT) (Träuble and Eibl 1974). Each term represents the difference in energy between the ordered and disordered state. Matters are further complicated when one considers that a membrane can have multiple domains containing varying levels of order, and also that the extent of ordering can depend entirely on the species of lipid within the domains.

The Gouy-Chapman theory of the electric double layer predicts that an increase in charge density causes a decrease in the membrane transition temperature (Chapman et al. 1974). Melting an anionic membrane leads to an expansion of surface area, lowering the density of bound cations and decreasing the apparent pKₐ value of the anionic phospholipids. The transition therefore also affects lipid ionization state and the adsorption of ions (Cevc 1993), and by raising the pH of solution containing anionic liposomes, the transition temperature can be significantly lowered. It is surprising, then, that K₂ induced a significant reduction in the transition temperature of PC/PA bilayers, as acidic K₂ should raise the apparent pKₐ value according to this theory and cause an increase in the transition temperature.
Literature on lipid/protein interactions indicates that K$_2$ should participate in a “type 1” interaction, one that is usually found between hydrophilic, charged proteins and charged membranes (Papahadjopoulos et al. 1975). The canonical type 1 interaction is characterized by electrostatic binding, minimal protein penetration into the hydrophobic membrane interior, and an increase in the major transition temperature (Papahadjopoulos et al. 1975). Effects of type 1 interactions have been studied using Poly(L-Lysine) peptides of varying length. These peptides are similar to K$_2$ in that they are highly charged, disordered, relatively small, and of course contain many lysines. They also are capable of forming helices when bound to membranes (Hartmann and Galla 1978). When 1,2-dipalmitoyl-sn-glycero-3-[phospho-rac-(3-lysyl(1-glycerol)]( DPPG) membranes were studied by Papahadjoulos (1975), poly(L-lysine) caused a marked increase in the thermotropic phase transition and the enthalpy of this transition increased, indicating that the membrane was being stabilized by the poly(L-lysine). This was thought to be an effect of decreasing the electrostatic repulsion between head groups, allowing for greater lipid packing and ordering. These results were further confirmed by Boggs and Moscarello (1982), who then used ESR spectroscopic studies to show that the hydrocarbon chains of DPPG membranes are being disordered by poly(L-lysine) in the gel state, but ordered in the liquid crystalline state. To make matters more complex, in egg PG and egg PA membranes, poly(L-lysine) also orders acyl chains to increase the T$_m$ value of the membrane (Boggs and Moscarello 1982). Furthermore, Carrier and Pezolet (1986) found that low ratios of polylsine:lipid increased the transition temperature by 3-4 °C, which was caused by an intra-chain molecular ordering in the gel and liquid states.
for DPPG bilayers. However, when membrane interiors were probed with DPH, they found that increasing the poly(L-lysine) concentration increased membrane interior fluidity, contrary to their previous findings. It is surprising that K$_2$, being a basic protein capable of performing similar charge neutralization and binding with helix propensity do not exert the same effect as polylysine. In a study which used ox-brain phosphatidylserine, Chapman et al. (1974) found that poly(L-lysine) also reduces the T$_m$, and lowers the degree of cooperativity of transitions. Perhaps K$_2$ provides enhanced hydration at the membrane surface which is not effective in the hydrophobic spaces below it.

By visual appearance alone, the lower peak height and a smaller transition curve of the membrane in the presence of K$_2$ indicates that it lowered the enthalpy ($\Delta$H) of transition, suggesting that the presence of K$_2$ increased the fluidity or disorder of the lipid head-groups. Microviscosity data from previous experiments (Figures 3.7 and 3.8) suggests that this is not the case, but perhaps the fluorescence experiments used were not sensitive enough to detect these changes. A small pre-transition peak also occurred, which might be due to the tendency of the polar lipid head groups to achieve a certain level of fluidity and solvation while the hydrocarbon chains still remain ordered (Zasadzinski et al. 1988). Poly(L-lysine) has been shown to broaden the transition period while dramatically reducing the transition enthalpy in at least one other experimental system (Boggs and Moscarello 1982). Electron micrographs show that polylysine binds to dipalmitoyl phosphatidic acid bilayers and induces formation of crystalline domains, something K$_2$ could be doing with egg PC/PA membranes. For physiological relevance,
Isothermally induced phase separations are perhaps a requirement to facilitate the formation of enriched lipid microdomains suitable for specific protein/enzyme function.

Varying the length of the poly(L-lysine) chain also appears to be a have an effect on membrane transition temperature. Short lysine tracts (MW ≈ 4000) failed to significantly change the membrane melting temperature, whereas long (MW ≈ 60 000) poly(L-lysines) were able to produce three distinct melting transition periods in DPPG membranes (Carrier et al. 1985). Despite the similarity in size between K₂ (MW 5381) and the “short” polylysine tracts (MW ≈ 4000), K₂’s effect is noticeable, and has the opposite effect of poly(L-lysines) used in the aforementioned studies. However, although I have observed a decrease in the DMPC/DMPA transition temperature by K₂, altering conditions such as protein concentration, ion and salt concentration, as well as dehydrin length could invariably change the outcome of the experiments.

As a final hypothesis, K₂ might simply be providing enhanced hydration to phosphate head-groups. This enhancement allows for an increase in interfacial head group area, and might reduce the free energy of the gel-state. Dehydration of membranes is known to elevate the membrane transition temperature by up to 40°C in a wide range of lipids (Wolfe and Bryant 1999). This effect is called the Clausius-Clapeyron effect (Bryant and Wolfe 1992) and explains that in a dehydrated phase, lipid ordering incurs an extra energy cost, which stabilizes the gel phase. By providing hydration through its strong water association, or by its polar side-chain projections, K₂ might be capable of destabilizing the gel-state and therefore reducing the gel to liquid crystalline transition.
4.7 Future Directions

Some areas of this investigation could benefit from studies to satisfy questions raised in the discussion section. Fusion experiments were difficult to analyze in great detail, as the exchange and mixing of lipids, as well as the association of membranes cannot be observed by dynamic light scattering. One method which quantifies the extent of membrane fusion is to measure resonance energy transfer of the fluorescent lipids N-(7-nitro-2,1,3-benzoxadiazol-4-yl) phosphatidylethanolamine and N-(lissamine Rhodamine B sulfonyl) phosphatidylethanolamine as they mix with non-probed membranes. A set of liposome fusion experiments using these lipid probes is perhaps one future direction for this investigation.

The nature of the CF-encapsulating, detergent resistant particles are also something that might yield interesting results. Currently, the phenomenon has not been documented with dehydrins or any other disordered proteins. These proposed detergent-resistant CF capsules could perhaps be re-created by preparing dye-filled liposomes using 100% PA, and then saturating with K₂.
CHAPTER 5: CONCLUSIONS

Dehydrins are disordered proteins which are expressed in significant amounts in plant tissues during low-water stresses, including cold, drought, and high salinity. A growing body of research indicates that membranes are a major location of plant cell destruction, as membranes are exquisitely complex and delicate structures that separate the living components from the outside world. Interactions between dehydrins and membranes have been observed, and many studies in vivo and in vitro demonstrate a good correlation between dehydrin expression and the improved survival of membranes (Houde et al. 2004; Steponkus et al. 1998). The K-segment is thought to be key to the dehydrin-membrane binding interaction, and the purpose of this research project was to investigate the potential cryoprotective effects of a simple dehydrin – K2 from V. riparia – on membranes. Experiments using SDS micelles as a membrane mimetic indicated that K2 does not have a micelle-stabilizing effect, which α-synuclein is known to exert. With liposomes, K2 can however protect opposing, negatively charged membranes in liposomes from colliding during freeze-thaw. This effect is apparently not a direct result of significant surface coverage, or the alteration of the membrane interior and surface viscosity. However, the binding of K2 caused a 3°C reduction in a DMPC/DMPA membrane’s melting transition temperature. This observation suggests that increased lipid hydration, and decreased lipid ordering and packing is occurring in the presence of K2, possibly with a heterogeneous distribution.
CHAPTER 6: REFERENCES CITED


Rahman LN, et al. (2011) Phosphorylation of Thellungiella salsuginea Dehydrins TsDHN-1 and TsDHN-2 Facilitates Cation-Induced Conformational Changes and Actin Assembly.


