Structural and Functional Insights into the Cryoprotection of Membranes by the Intrinsically Disordered Dehydrins*

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Background: Genetic evidence supports a protective role for plant dehydrins against drought and cold.

Results: Dehydrins prevent membrane fusion and lower the transition temperature without altering membrane accessibility and fluidity.

Conclusion: The lysine-rich segments are important for protecting membranes from freeze-thaw damage.

Significance: An amphipathic helix with positively charged residues flanking the hydrophobic face may be a common motif for disordered stress proteins.

Dehydration can be due to desiccation caused by a lack of environmental water or to freezing caused by a lack of liquid water. Plants have evolved a large family of proteins called LEA (late embryogenesis abundant) proteins, which include the intrinsically disordered dehydrin (dehydration protein) family, to combat these abiotic stresses. Although transcription and translation studies have shown a correlation between dehydration stress and the presence of dehydrins, the biochemical mechanisms have remained somewhat elusive. We examine here the effect and structure of a small model dehydrin (Vitis riparia K2) on the protection of membranes from freeze-thaw stress. This protein is able to bind to liposomes containing phosphatidic acid and protect the liposomes from fusing after freeze-thaw treatment. The presence of K2 did not measurably affect liposome surface accessibility or lipid mobility but did lower its membrane transition temperature by 3 °C. Using sodium dodecyl sulfate as a membrane model, we examined the NMR structure of K2 in the presence and absence of the micelle. Biochemical and NMR experiments show that the conserved, lysine-rich segments are involved in the binding of the dehydrin to a membrane, whereas the poorly conserved ϕ segments play no role in binding or protection.

Water deficiency can be lethal because it leads to damage to DNA, membranes, and proteins, including an impairment of enzyme function. Many poikilothermic organisms express proteins that protect them from the detrimental effects of freezing and dehydration. In plants, this includes dehydrins (dehydration proteins) whose up-regulated transcription and translation has been associated with protection from several abiotic stresses, including drought, cold, and high salinity (for reviews on dehydrins, see Refs. 1–6). Dehydrins are a member of a large family of proteins, known as LEA (late embryogenesis abundant) proteins (7–9).

Dehydrins themselves have been found in a number of different intracellular locations and are often found in the cytoplasm and the nucleus. Other locations include at the mitochondrial, chloroplast, and plasma membranes. The diverse localization may also be reflected in the diverse protective effects of dehydrin in the plant. These include preventing electrolyte leakage across cold-stressed membranes (10, 11), preventing lipid peroxidation (11), and a reduction in stomatal density to prevent dehydration (12).

An examination of the dehydrin sequence reveals that they are modular in nature and contain a variable number of conserved sequence motifs. This modularity results in a range of dehydrin sizes (6–200 kDa). By definition, dehydrins must contain at least one K segment (1). This 15-residue, Lys-rich motif (EKKGIMDKIKEKLPG)n can be found in 1–11 copies in a single dehydrin sequence. The S segment, which consists of 5–7 Ser residues, is not present in all dehydrins. When present, it is normally present only once. The Y segment consists of the sequence motif (V/T)D(E/Q)YGNP and is usually present in one or two copies. Residues not found in one of these three motifs are said to be located in the ϕ segment. This segment is not conserved in terms of length but is rich in Gly and polar/charged amino acids such as Glu, Thr, Lys, and His (6). The lack of hydrophobic residues explains the lack of tertiary structure in dehydrins. Dehydrins are intrinsically disordered proteins (IDPs)2 (13, 14), a property that prevents them from being denatured during dehydration and has been used to purify them (15).

The roles of the various conserved motifs are not yet fully clear. Dehydrins that contain multiple copies of the K segment

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2 The abbreviations used are: IDP, intrinsically disordered protein; DPH, diphenylhexatriene; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; PC, L-α-lyso-phosphatidylcholine; PA, L-α-phosphatidic acid; PG, L-α-phosphatidylglycerol; PS, L-α-lysophosphatidylserine; TMA-DPH, trimethylammonium diphenylhexatriene; DSC, differential scanning calorimetry; DMPA, 1,2-dimyristoyl-sn-glycero-3-phosphate; HSQC, heteronuclear single quantum coherence.
tend to be more expressed during cold-stress, whereas the Y segment dehydrins tend to be correlated with desiccation and salt stress (5, 6, 16). In terms of localization within the cell, dehydrins containing K segments or K and S segments are found in the cytoplasm and at the membrane (17), with the possibility that residues in and near the S segment are responsible for nuclear localization (18).

To better characterize the physiological role of dehydrins, a number of in vitro assays have been performed. One of the most extensively performed assays is the enzyme cryoprotection. The most commonly used enzyme is lactate dehydrogenase (19, 20), although the starch degradation enzyme α-amylase has also been used (21). The repeated freezing and thawing of lactate dehydrogenase results in its loss of activity, most likely because of aggregation (22). The addition of dehydrin results in the recovery of activity, with the longer the dehydrin the more efficient the recovery (23). Dehydrins have been shown to bind metals, which may prevent the formation of reactive oxygen species. These proteins have also been found to bind DNA and RNA in a nonspecific manner (24), which was postulated to help protect nucleic acids from desiccation damage. The localization studies showing that dehydrins can be located near the membrane (25) prompted several studies to examine whether binding can occur in vitro, using both liposomes and micelles (5, 26–30).

Many IDPs can gain structure upon binding to a ligand. The same is true for dehydrins, because CD studies showed that the protein became α-helical in the presence of SDS micelles and liposomes consisting of phosphatidylcholine and a second lipid (phosphatidic acid, phosphatidylglycerol, or phosphatidylserine) that must be negatively charged (27, 30). Dehydrins have also been shown to interact with liposomes whose lipid composition mimics that of the plasma membrane (31). Deletion studies with the maize dehydrin ZmDH11 showed that the K segments are likely responsible for binding to these different membranes, although which residues were responsible was not determined, nor if there is a weaker interaction with the φ segment (28). A need to closely examine the role of the φ segment is driven by a study of the Arabidopsis dehydrin Lti30, which is able to bind liposomes, whereas the K peptide (that is, a peptide consisting of only the 15-residue K segment) failed to bind (32).

The mechanisms by which dehydrins may protect the membrane from desiccation and cold stress also require further study. A study on the Arabidopsis dehydrins ERD10 and ERD14 used diphenylhexatriene (DPH) to examine the mobility of a membrane in the presence of these two dehydrins (30). No effect was observed, but this may not be surprising because this probe partitions in the acyl chain region of the liposome, whereas the polar dehydrins are likely to interact at the liposome surface. The study using Arabidopsis Lti30 dehydrin also examined membrane fluidity by measuring the phase transition temperature of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC):1,2-dimyristoyl-sn-glycero-3-phospho-1-α-serine liposomes (32). The protein was able to lower the transition temperature of the liposomes, which would allow the plant to maintain membrane fluidity at lower temperatures (32). However, this does not address the problem of how dehydrins could protect membranes at subzero temperatures.

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We examine here the ability of the minimal dehydrin K2 to protect liposomes from freeze-thaw and cold stress damage, what effect binding has on some membrane properties, and what effect binding has on K2 structure. We also see whether the gain in α-helicity is restricted to the K segments and whether the φ segments play any role in binding to the liposome.

Experimental Procedures

Expression, Purification, and Labeling of the Dehydrin—The Vitis riparia K2 protein was expressed and purified as described previously (15, 22), with the following modifications for isotopically labeled proteins: Escherichia coli BL21(DE3) cells transformed with pET-22B expression vector containing the K2 gene were grown in Luria-Bertani medium until an A600 of ~0.8 was reached. Cell cultures were centrifuged at 5,000 × g for 30 min, and the cell pellet was resuspended in 1 liter of modified M9 minimal with [13C]glucose (3 g) and/or [15N]NH4Cl (1 g) for protein labeling. The protein was extracted by boiling the resuspended pellet in water for 20 min; sodium acetate, pH 5.0 buffer was added to give a final buffer concentration of 20 mM. Purification by cation exchange and desalting by reversed phase HPLC was performed as previously described (22). The protein was lyophilized to dryness and stored at −20 °C until use. K peptide was synthesized and purified as described previously (23).

Preparation of Liposomes—L-α-Lyso-phosphatidylcholine (egg PC), L-α-phosphatidic acid (egg PA), L-α-phosphatidyglycerol (egg PG), and L-α-lysophosphatidylserine (brain PS) were obtained from Avanti Polar Lipids (Birmingham, AL). The lipids were dissolved in a 4:1 chloroform:methanol solution at a concentration of 100 mg/ml. To form a lipid cake, 12.5 μl of both PC and PA, PG, or PS were dispersed in the bottom of a glass vial, mixed, and subsequently dried under a stream of nitrogen gas. After 1 h of further evaporation of solvents under vacuum, the lipids were resuspended in 50 mM phosphate buffer, pH 7.4. After five liquid nitrogen freeze-thaw cycles, the solution was extruded 21 times through a 100-nm polycarbonate membrane that had been warmed to 45 °C (33). Liposome size was confirmed using dynamic light scattering (described below), and preparations were used for experiments within 4 days.

Liposome Freeze-Thaw Damage and Recovery by K2—For the fusion assays, liposome solutions were diluted to a concentration of 0.4 mg/ml in 5 mM phosphate buffer, pH 7.4, with and without K2, lysozyme (egg white, white crystalline, ~100% pure; Fisher Scientific), or PEG 3350 (~99.95% pure, M, 3,000–3,700; Sigma-Aldrich). Samples (100 μl) were chilled to −20 °C in an enzyme cooler box for ~2 min. To nucleate the flash-freeze process, the base of each tube was then firmly pressed against a metal spatula that had been chilled by liquid nitrogen. Once frozen, tubes were stored at −20 °C for 12–16 h. Tubes were then thawed at room temperature, and 400 μl of buffer was added before size measurement by dynamic light scattering. Size distributions for liposomes were measured using a ZetaSizer Nano S (Malvern Instruments, Worcestershire, UK). Analysis was done in the Malvern Dispersion Technology software suite, version 3.3, using the default settings. Measure-
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ments were made at 25 °C, and the data were analyzed using the discrete peak calculation method with polystyrene latex as the reference standard.

Measurement of Lipid Accessibility and Mobility—Surface accessibilities in the presence of K2, lysozyme, and PEG 3350 were assessed using the dye merocyanine 540 (34). Liposomes (0.3 mg/ml) were prepared in 50 mM phosphate buffer, pH 7.4. K2, lysozyme, or PEG 3350 were added to the liposome containing solutions, and samples were equilibrated for 30 min. A stock solution of merocyanine 540 was added to a final concentration of 10 mg/ml. The mixtures were allowed to incubate for 30 min and with high gain feedback mode enabled. High gain feedback was used because of the relatively low signal intensity observed at these lipid concentrations. Because the noise was generated in this mode, the filtering period was set to 5 s to provide a more reliable signal. To account for the “thermal history” of a sample, 15 scans were performed, with only the final scan kept for analysis.

K2-Liposome Binding—The binding of K2 to liposomes was assessed by separating bound and unbound protein by centrifugation (36). PC:PA liposomes in 5 mM sodium phosphate, pH 7.4, were added to protein to create a range of 0.5:1 to 10:1 of liposome:protein (w/w) concentrations. Samples (200 µl) were incubated with and without K2 (0.2 µg/µl) at room temperature for 1 h before centrifugation at 80,000 × g for 30 min, after which the supernatant and pellet were separated. Pellets were resuspended in the same binding buffer, and both supernatant and pellets were subjected to SDS-polyacrylamide gel electrophoresis. Quantification of the bound and unbound protein was made using Bio-Rad Quantity One software, trace quantity command. The amount of protein was quantified by comparison to a gel with known amounts of K2. To determine the affinity of the protein-liposome interaction and to estimate the stoichiometry, the data were fit according to the equation (37),

\[
\frac{[K2]_b}{[K2]_{total}} = \frac{B_{max}}{1 + (K_{d,app}L)}
\]

where \([K2]_b\) is the amount of bound K2, \([K2]_{total}\) is the total K2 concentration, \(B_{max}\) is the maximal bound signal, \(K_{d,app}\) is the apparent dissociation constant, \(L\) is the total PA concentration, and \(h\) is the Hill coefficient.

Secondary Structure Analysis—CD data of the dehydrin proteins were collected using a Jasco-815 CD spectropolarimeter (Easton, MD). All protein samples were dissolved in 10 mM sodium phosphate, pH 7.4, at a protein concentration of 0.16 mg/ml (30 µM). A quartz cuvette with a 2-mm pathlength (Hellma, Concord, Canada) containing the protein sample with and without liposome (0.65 mM) or SDS (10 mM) was scanned from 250 to 190 nm. In the SDS titration experiment, the concentration of the detergent was varied between 0 and 1,000 µM. For scans containing liposomes, the spectra were averaged over 25 accumulations. For scans containing SDS, the spectra were averaged over 8 accumulations. All CD experiments were performed at 25 °C.

NMR Experiments—Lyophilized 15N-K2 or 13C/15N-K2 was resuspended in 600 µl of NMR buffer (20 mM sodium phosphate, pH 6.0, 10 mM NaCl, 0.01% sodium azide, 0.1 mM dimethyl-4-silapentane-1-sulfonic acid, and 10% D2O (v/v)). Data were collected on a Bruker Avance DRX600 spectrometer equipped with a cryogenic triple resonance probe with the temperature set to 300 K. 1H, 13C, and 15N referencing was performed relative to dimethyl-4-silapentane-1-sulfonic acid as described previously (38). For micelle containing samples, 2H-SDS was added to a final concentration of 50 mM.

Chemical Shift Assignments—The chemical shifts of K2 alone have been previously measured and are available under Biological Magnetic Resonance Data Bank entry 16445 (39). The backbone and side chain 1H, 15N, and 13C chemical shifts in the presence of 50 mM SDS were assigned by using 1H, 15N HSQC, HNCA, CBCA(CO)NH, HNCO, HNCACO, and 15N-ed-
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The Protective Effects of K2 on Stressed Liposomes—Several previous reports have shown that dehydrins are able to interact with membranes (5, 26–30). We wished to examine the ability of the minimal dehydrin K2, to protect from cold stress. Liposomes of 100 nm in diameter were created using an equal weight mixture of PC and PA. The freezing and thawing of a membrane can cause different problems that all lead to a loss of membrane integrity. In our case, we examined the fusion of liposomes by measuring the distribution of lipid diameters before and after freezing in the presence of increasing concentrations of K2 (Fig. 1, A and B). The unfrozen liposomes have a range of sizes as expected using the extrusion method, with an average diameter of ~100 nm. In the absence of any dehydrins, the freeze-thaw-treated liposomes became very large, with diameters averaging ~800 nm. K2 was added over a range of concentrations before freezing. In the presence of the dehydrin, at low to mid-concentrations of protein (5–50 μM), the average diameter first increased to 1,000 nm, but at higher concentrations (75 and 100 μM), there was a downward shift in the diameter. At the highest concentration (250 μM), there is a distribution of sizes clustered around the original diameter (Fig. 1A, left panel). To determine whether the protective effect is specific to PA, the fusion assays were repeated using PC:PS and PC:PG. The results are shown in Fig. 1A (center and right panels) and indicate that K2 is able to protect liposomes that contain a mixture of neutral and negatively charged lipids.

To examine whether the protective effects of K2 are specific to dehydrins or are a generic effect that can occur with any protein or disordered polymer, the fusion assay was repeated in the presence of lysozyme and PEG 3350. Neither lysozyme nor PEG 3350 showed any protective effects whatsoever, even at a concentration of 250 μM (Fig. 1B). Lysozyme was chosen as a control because it is a small protein with approximately the same hydrodynamic radius as K2 (23) and is also known to bind to liposomes and promote their aggregation (44). As with dehydrins, lysozyme has previously been shown to bind to negatively charged liposomes (45). This protein did not prevent fusion at moderate concentrations (25–100 μM), and at concentrations of 250 μM, the liposomes began to show visible signs of aggregation (data not shown), demonstrating that lysozyme did not provide any protection from freeze-thaw damage (Fig. 1B, left panel) and that the binding of a protein to liposomes could promote aggregation. PEG 3350 was used because it is a disordered polymer with a similar hydrodynamic radius to K2. With PEG 3350, the average diameter of the liposomes becomes
~1,000 nm. Even at the higher concentrations of PEG 3350, the liposomes remained large, and fusion was not prevented (Fig. 1B, right panel).

The effect of K2 on membrane surface properties was examined next. We examined whether K2 coats the surface of a liposome to prevent another liposome from coming in close contact with the surface (i.e. by causing some protective steric hindrance). Surface accessibility was measured using the dye merocyanine 450, which partitions itself between the hydrophilic and hydrophobic phases (34). The presence of 50 or 250 μM K2 or PEG 3350 did not measurably alter accessibility (Fig. 2A, left and right panels). However, the presence of lysozyme did lower the accessibility of the liposomes as evident in the 4.5-fold decreased fluorescence ratio, possibly because of its ability to cause liposome aggregation (44).

A previous report showed that an Arabidopsis dehydrin was able to lower the transition temperature (Tm) of a liposome (32). However, the authors found that dehydrin membrane binding required His-His sequences flanking the K segment. To see whether this is also required for a minimal dehydrin like K2, we measured the Tm by DSC. The acyl chain diversity in the egg PC and PA preparations prevents the detection of a distinct transition peak (data not shown); to overcome this problem liposomes were made with DMPC and DMPA to ensure cooperative transition processes that are easily observed by this technique. Liposomes alone exhibited a sharp gel fluid phase transition at 39.5 °C (Fig. 2B). The addition of 75 μM K2 (Fig. 2B, left panel) caused the Tm to shift to 36.6 °C, with a premelt peak at 33.7 °C, whereas the addition of PEG 3350 caused the Tm to remain at 39.7 °C (Fig. 2B, right panel). The addition of lysozyme in the DSC measurements prevented a major enthalpy transition and caused the formation of precipitate, suggesting that the liposomes may have been damaged by this protein (Fig. 2B, middle panel). We examined whether the presence of the K2 dehydrin altered the mobility of lipids in the liposome using DPH and TMA-DPH probes using steady-state fluorescence anisotropy (35). DPH, being a highly hydrophobic molecule, probes the mobility in the alkyl chain region of the bilayer, whereas the TMA-DPH probes mobility near the lipid headgroups. The same concentration range for the compounds was used as in the fusion assay. As can be seen in Fig. 2C, none of these compounds (K2, lysozyme, or PEG 3350) significantly altered the mobility of the lipids.

Lipid Binding, Disorder to Order Transition, and the Importance of Charge—The cryoprotective effects of K2 on the enzyme lactate dehydrogenase was previously shown to occur by a molecular shield effect (23). To see whether the same is true for the cryoprotection of liposomes, we first examined whether K2 is able to directly bind to the lipid vesicles using a differential centrifugation binding assay (36). The results in Fig. 3A (left column) show that K2 bound to liposomes consisting of

**FIGURE 1.** The K2 dehydrin protects highly negatively charged membranes from the detrimental effects of cold stress. A, size distribution of various liposomes before and after freeze-thaw treatment in the presence and absence of K2. Untreated liposomes are marked as Unf. (unfrozen). Compounds were added to a final concentration of 0–250 μM before liposomes were frozen and thawed. The lipid composition is shown above each graph. B, size distribution of liposomes before and after freeze-thaw treatment in the presence and absence of lysozyme and PEG 3350.
PC:PA and that molecular shielding is unlikely to be the mechanism because it requires the protein to remain unbound. Lysozyme was used as a control because other studies have shown that it binds to negatively charged lipid (44), and BSA was used as a negative control because it only interacts weakly with negatively charged liposomes (46). Fig. 3A (left column) shows that lysozyme did bind to the liposomes under our assay conditions, whereas BSA did not.

Two studies on other dehydrins had shown that negatively charged lipids are important for the interaction (27, 28). To test whether this is also true for K2, we examined binding in the presence of PC-only liposomes. As can be seen for K2 in Fig. 3A (right column), neither K2 nor lysozyme were able to bind to these zwitterionic lipids because almost all of the protein is found in the unbound supernatant fraction. This shows that the presence of phosphatidic acid, most likely caused by the nega-

FIGURE 2. Liposome properties are altered in the presence of K2. A, surface accessibility of liposomes. Liposomes were incubated with K2 and the control compounds for 30 min before merocyanine 540 was added. The partitioning of the dye was determined by measuring the absorbance at 530 and 570 nm. Results are n = 3 with the error bars showing the standard deviation. B, lipid melting temperature (T_m). The T_m of DMPC:DMPA liposomes were measured using differential scanning calorimetry in the presence (solid line) and absence (dashed line) of the three compounds. C, lipid mobility in liposomes. The probes diphenylhexatriene (filled circles) and TMA-DPH (open circles) were used to measure lipid mobility at the end of the acyl chains and near the lipid headgroups, respectively, using fluorescence anisotropy. The results are n = 3 with the error bars showing the standard deviation.

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tive charge of the phosphate headgroup, is important for the binding of K2 to the membrane surface. Because PA needs to be present for K2 to bind to the liposome, the importance of electrostatic forces in the binding interaction was directly examined with increasing amounts of NaCl. As the concentration of salt was increased, the amount of K2 bound to the liposome decreased (Fig. 3B). The largest drop in the percentage of protein bound occurred between 0 and 200 mM NaCl (30% decrease), after which there is a further drop but in much smaller decrements (3–10% decreases). To further biochemically characterize the interaction, the apparent binding affinity was measured using the pulldown method (Fig. 3C). The ligand concentration is expressed in terms of total PA concentration because K2 does not interact with PC-only liposomes. Because of the sigmoidal shape, the binding curve was fit to Equation 3. From the fit, the apparent Kd was determined to be 19 μM with a cooperativity coefficient of 6.8.

Many IDPs gain structure when they bind to a ligand. To examine what changes may have occurred in K2, we compared the CD spectra of this dehydrin in the presence and absence of PC:PA liposomes (Fig. 3D). In the absence of lipids (Fig. 3D, filled circles), the spectrum shows the typical pattern of a disordered protein with a strong negative ellipticity at 198 nm and a weak shoulder near 222 nm. After the addition of a 70 molar excess of lipid, it can be seen that the minimum at 198 nm has shifted to 205 nm and become less negative and that the ellipticity at 222 nm has decreased (Fig. 3D, open squares). These changes suggest that the K2 protein has lost ~6% coil structure and gained ~2% α-helicity in the presence of a membrane (47). Based on the CD data from previous dehydrin deletion studies...
that suggested that K segments have a role in membrane binding (28) and the importance of electrostatics in the K2/liposome interaction we observed here, we present a possible helical model of the K segment sequence and how it may interact with the surface of a membrane. Using a combination of the helix/coil transition prediction algorithm Agadir (48–50) (Fig. 3E) with the NMR data from K2 in the presence of micelles (see below), we propose a model where 11 residues of the K segment interact with the membrane surface such that there are four hydrophobic residues (two Met, Leu, and Ile) that weakly interact with the acyl chains and four flanking Lys residues that have favorable electrostatic interactions with the phosphate head groups (Fig. 3F).

**Interaction of K2 Dehydrin with a Micelle Membrane—**Our goal was to determine the site-specific secondary structure of K2 when bound to the membrane using several biophysical techniques, including solution state NMR. Because of the large size of a liposome, NMR studies on bound proteins can be challenging because of the large molecular weight of the liposome that causes a dramatic increase in the tumbling rate of the bound protein. SDS micelles with their smaller size are especially suitable to the study membrane proteins by NMR (51, 52). To that end, we used SDS micelles as a membrane because it is regarded as a good membrane substitute to mimic the interactions that occur between proteins and lipids (53).

We acquired the CD spectrum in the presence and absence of SDS micelles (Fig. 4A) to see whether the structural changes of K2 upon binding were similar to those observed in the presence of liposomes. As with K2/liposome interaction shown in Fig. 3D, the K2 signal minimum shifted from 198 to 205 nm with a concomitant decrease in the CD signal, and a significant gain in the CD signal was observed at 222 nm. The latter shows that K2 has lost ~15% coil structure and gained ~7% -helicity when bound to the micelle surface (47).

To facilitate an analysis of the changes in the structure and dynamics that occur when the dehydrin binds to a membrane, we determined the chemical shifts of K2 in the presence of SDS micelles. The changes that occurred are highlighted by the overlap of a 15N HSQC of K2 in the presence (blue peaks) and absence (orange peaks) of SDS (Fig. 4B). The K2 alone spectrum shows very little dispersion in the amide proton and nitrogen dimensions, with all of the backbone HN resonances located between 8.0 and 8.5 ppm. This observation is typical for intrinsically disordered proteins. The addition of SDS shows the increased dispersion of many resonances, with HN resonances now spread over 7.3 to 8.7 ppm. These findings are in agreement with the CD data in that there is some structuring and/or interaction of the protein because of the micelles. Some resonances show larger changes than others, suggesting that specific regions of K2 are interacting with the detergent.

To determine which K2 residues were affected by SDS binding, we plotted the chemical shift differences of the backbone
protein and nitrogen amide atoms of the bound and unbound forms of K2 on a per residue basis (Fig. 4C). As this figure shows, the largest changes occurred in residues 4–11 and residues 37–44. These residues are found in the K segments (defined in K2 as residues 1–11 and 32–46). The largest chemical shift changes (>2 ppm) are located in the ends of the K segment (residues 6–9 and 41–44). The ϕ segments, located between residues 12–31 and 47–48, showed generally little chemical shift change (0.5 ± 0.3 ppm). These results suggest it is the conserved K segments and not the ϕ segments that are interacting with the SDS.

These localized chemical shift effects in the presence of SDS are also reflected in gains in the secondary structure in K2. The HN, Hα, Cα, Cβ, C’, and backbone N chemical shifts of K2 alone and in the presence of SDS were analyzed using the program D2D (54). The method uses these chemical shifts to predict the presence of transient secondary structures in disordered proteins. The program discriminates between α-helices, β-strands, type II polyproline helices, and coil on the basis of the combined chemical shifts. The top panel of Fig. 4D shows the secondary structure probability (Pα) for K2 alone in buffer. The structure is predominantly coil, with a small amount of polyproline II helix located in the residues 4–6, 16–20, 27–30, and 36–43, and a small amount of β-strand in residues 9, 24–26, and 44–45 (i.e. adjacent to the three proline residues). In the presence of SDS micelles (Fig. 4D, bottom panel), one can see that a considerable amount of α-helicity has been gained in residues 3–9 and residues 34–44, with remaining residues in the K segments showing a small amount of polyproline II helix structure, whereas a small amount of strand is still seen in residues 24–25.

To examine the K2–micelle interaction in greater detail, a 15N HSQC titration experiment was performed (Fig. 5A). Resonances that were very well separated from all others (Glu-7, Arg-14, Thr-21, Glu-44, and Ala-47) were used to further characterize the protein–detergent micelle binding interaction (Fig. 5, B–D). The residues cover the three different segments of K2 (first K segment, central ϕ segment, second K segment, and C-terminal ϕ segment). In addition, these various residues were undergoing exchange on three different time scales (slow, intermediate, and fast). The data from residues undergoing fast exchange were fitted to the binding equation (Equation 5) to determine the affinity and apparent stoichiometry of K2 binding to SDS. The results show that the apparent Kd is 16 μM (i.e. a moderately weak affinity), and the stoichiometry is 7. We mapped the different exchange rates on the K2 sequence (Fig. 5E) depending on how the peaks changed during the titration (i.e. the single peak that shifted without a loss in intensity is considered to be a residue in fast exchange; peaks with decreasing intensity in one location and appearing in another location are considered to be in slow exchange; and peaks that both displayed a change in intensity and shifted are considered to be in intermediate exchange). Proline residues and residues that could not be followed in the titration because of spectral overlap remain uncolored in the figure panel. Residues located in the middle of the K segments were in slow exchange. This suggests that they are likely binding to the micelles and changing structure (gaining α-helicity). Residues in the central ϕ segment and at the C-terminal ϕ segment (the two C-terminal residues of the protein) were in fast exchange, suggesting that they do not interact with the membrane surface. Residues located at the interface between fast and slow exchanging regions were undergoing intermediate exchange because they represent transitioning from parts of the protein that are strongly interacting with the micelle to those that have no interaction.

To further characterize the dehydrin membrane surface interaction dynamics, we measured the R1, R2, and 1H NOE relaxation parameters of K2 in the presence and absence of SDS (Fig. 6, A–C). In the absence of micelles (filled circles), the two K segments showed slightly higher R1 values than the mean (R1 average 1.3 ± 0.1 s−1), whereas the center of the ϕ segment showed slightly lower values. This suggests that the K segments are slightly more rigid than the ϕ segment, which may be reflected in their propensity to form secondary structure in the presence of a membrane surface (see below and Ref. 28). The R2 and NOE values showed similar patterns with the ϕ segment being more flexible (Fig. 6, B and C). Note that the R2 plot (R2 average 2.0 ± 0.2 s−1) appears flat (Fig. 6B, filled circles), but an expanded y axis scale reveals the same pattern of increased flexibility in the ϕ segment region (see Fig. 2 in Ref. 22). In the absence of SDS, the NOE values were negative over the entire protein (<−0.25), with the ϕ region showing NOE ratios of −1.5 to −2.

In the presence of micelles (open squares), not all three parameters show the same pattern. For the R1 data (Fig. 6A; R1 average 1.5 ± 0.1 s−1), the plot is rather featureless, showing only a small increase in flexibility at the C terminus. R1 is not affected by conformational exchange, so the gain in helicity is not reflected in this relaxation parameter. In contrast, the NOE ratio (Fig. 6C) and the R2 data (Fig. 6B) showed a difference once again between the different segments, with the NOE ratio approaching zero for the ϕ region and 0.5–1.0 for the K segments. The largest range of values are in the R2 measurements (R2 average of 7 ± 3 s−1), where the core of the K segment residues have R2 values of 10–13 s−1, whereas the ϕ segment residues flanking the K segments have R2 values of ~7 s−1. The center of the ϕ segment has R2 values of ~4 s−1, approaching the R2 values of K2 alone in solution.

**FIGURE 5. Characterization of the binding between K2 and SDS micelles.** A, an overlap of the 15N HSQC spectra showing the titration of SDS into an NMR sample of K2. Peaks are shown in a rainbow gradient color, with the lowest concentration of SDS shown in red and the highest concentration shown in purple. Peak assignments are shown as the residue number followed by its single-letter amino acid code. B–D, binding curves of K2/micelle interactions of several residues undergoing different exchange regimes. In the three panels, the closed circles represent the normalized, observed change in chemical shift (Δδobs). E, E, fast exchange; C, slow exchange; D, intermediate exchange. Residues undergoing fast exchange were fitted to Equation 5. Open squares, normalized peak intensity at the beginning of the titration; open triangles, normalized peak intensity at the end of the titration. E, map of residues undergoing chemical shift change at the different exchange rates. Slow exchange, blue; intermediate exchange, pink; fast exchange, red. Residues shown in white are residues for which insufficient assignments were available (i.e. proline residues or overlapped resonances).
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**FIGURE 6. The K segments become rigid upon binding SDS micelles.** Graphs show the $R_1$ (A), $R_2$ (B), and (C) heteronuclear $^{15}$N NOE relaxation data of K$_2$ in the presence (open squares) and absence (filled circles) of SDS micelles. The error bars represent the error in fitting the relaxation decay curves as described in CCPNMR (42). The diagram at the top of the figure shows the location of the various segments in the K$_2$ sequence.

**Discussion**

Our results show that even a minimal dehydrin such as K$_2$, with only two K segments and a central $\phi$ segment, is able to prevent membrane damage during cold stress. The type of cold protection provided appears to be 2-fold: by preventing freeze/thaw-induced fusion (Fig. 1A) and by keeping membranes fluid at a lower temperature (Fig. 2B). These two effects have also been observed for two other plant stress IDPs. The dehydrin Lti30 from *Arabidopsis* has also been shown to lower the $T_m$ (32). In that report, the authors suggested that the decreased $T_m$ allows the membrane to remain fluid at lower temperatures, a property that is important for proper membrane function (32, 55). A mitochondrial LEA protein (LEAM) was also shown to lower the $T_m$ of model liposomes, and to reduce fusion caused by dehydration (56). The yeast Hsp12 protein, a yeast stress IDP, in contrast, increased the $T_m$ of a model liposome (57). Another difference between Hsp12 and K$_2$ is that Hsp12 decreased membrane fluidity as measured by fluorescence anisotropy, which the authors say is the cause of the increased $T_m$ (57). Functionally this makes sense, because Hsp12 is a heat shock protein that would need to keep the membrane at the same fluidity at higher temperatures. Hsp12 has been shown to gain four helices in the presence of SDS micelles. Although helix 4 is similar to the K segment sequence, the other three helices are different, with helix 2 having a Lys residue located on the hydrophobic face (see Fig. 3 in Ref. 57). It is possible that a different arrangement of the residues in these amphipathic helices and/or a different insertion depth could decrease lipid mobility rather than keep it fluid.

The dramatic flexibility of the $\phi$ segment even in the presence of a membrane surface (Fig. 6B) led us to hypothesize that the $\phi$ segment could act as a steric shield to protect the membrane from fusion. This could prevent two liposomes from coming close enough together to fuse. Such a model would be similar to what we proposed with K$_2$ preventing the aggregation of lactate dehydrogenase after freeze-thaw denaturation (22, 23). To test this, we used the fusion assay with the PC:PA liposomes and measured the $T_m$ of DMPC:DMPA liposomes using a K peptide (Fig. 7). The 15-residue peptide is identical to the second K segment found in *V. riparia* K$_2$. For both experiments, the peptide was used at twice the concentration to compensate for the presence two K segments in K$_2$. The prevention of fusion and the decrease in $T_m$ are very similar to what is seen for the intact K$_2$, showing that it is the K segments that are important for membrane protection and that the $\phi$ segment has no role.

Our binding assay shows that the presence of a negative charge on the liposome lipid is important for K$_2$ binding (Figs. 1A and 3B). The somewhat weaker protection with the PC:PS liposomes is likely a function of the dehydrin binding more weakly to the PS (27). Although it is true that PA is a relatively low abundant lipid in normal plant membranes (58), there are several reasons as to why it may be important in the protective function of dehydrin from abiotic stresses (27). Freezing temperature treatment of *Arabidopsis* plants was shown to cause a 6-fold increase in PA content of the plasma membrane (58), and the authors speculated that during cold-stress the increased PA can cause an increased chance of membrane fusion. In *vivo*, fusion can be a problem during cold stress; it has been shown that plasma membranes can fuse at lower temperatures (59). A contributing factor may be that PA can form hexagonal II phase membranes, where the acyl tails point outwards and the dehydrated headgroups face inwards (60). Outward pointing tails would likely increase the chance of membrane fusion. Dehydration of the membrane headgroups is therefore linked with an increase in the probability of fusion (61). In addition, dehydration of headgroups has also been linked with an increase in $T_m$ (62, 63). Previous work by Tompa et al. (64) showed that dehydrins are able to bind a large amount of water compared with a BSA control and keep this water in an unfrozen state even at subzero temperatures. A possible mechanism to explain how K$_2$ functions to both reduce $T_m$ and reduce fusion may be that dehydrins could bind to the periphery of the membrane, whereas their high level of hydrophilicity ensures that many water molecules are present to help hydrate the headgroups.
In addition to the common protective effects shown by several cold and desiccation stress IDPs (i.e. reduced fusion and a lowering of the $T_m$), evidence points to a common structural motif that allows these proteins to bind to the membrane. Several stress IDPs, including K2 discussed here and other dehydrins, gain $\alpha$-helical structure in the presence of a membrane (28, 55, 57, 65). The helical wheel projections for some of these stress IDPs are shown in Fig. 8. The amphipathic helices have a similar pattern of arrangement of amino acid types: hydrophobic residues are clustered on the face that points toward the membrane surface, positively charged residues, predominantly Lys, flank the hydrophobic face, whereas the negative residues (Asp and Glu) are located opposite the hydrophobic face. In all of the examples the proteins were found to preferentially interact with the negatively charged phosphates on lipids and/or negatively charged sulfates on SDS, involving Lys residues (28, 55, 57, 65). These results also show that it is not necessarily true that any flexible, cationic peptide could provide membrane protection. First, it is Lys and not Arg that is found flanking the hydrophobic face. We speculate that this difference is due to the ability of Lys to snorkel on a membrane surface (66), although this proposal requires further experimentation for confirmation. Second, the use of polylysine polymers has been shown to increase the $T_m$ of a liposome rather than decrease it (67).

FIGURE 7. K peptides have a similar effect as intact K$_2$ on membrane protection. A, size distribution of liposomes before and after freeze-thaw treatment in the presence and absence of K peptide. The peptide was added to a final concentration of 0–500 $\mu$M before liposomes were frozen and thawed. Data marked as Unf. (unfrozen) are the untreated control; Kpep, K peptide. B, liposome melting temperature ($T_m$) in the presence and absence of K peptide. The $T_m$ of DMPC:DMPA liposomes was measured using differential scanning calorimetry in the presence (solid line) and absence (dashed line) of K peptide. $C_p$, specific heat capacity.

FIGURE 8. Several stress proteins have similar helical wheel projections. Helical wheel representation of disordered proteins that bind negatively charged membranes. The beginning and end of the sequence are marked with a red N and a red C, respectively. The arrow indicates the hydrophobic moment $\langle \mu^+ \rangle$. Hydrophobic residues are colored yellow, basic residues are blue, acidic residues are red, serine and threonine are purple, asparagine and glutamine are pink, and alanine and glycine are gray. A, dehydrin K segment. B, Hsp12 helix 4. C, LEAM helix motif. D, CDeT11–24 lysine-rich sequence element.
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Author Contributions—M. W. C. and S. P. G. designed the study and wrote the paper. M. W. C. and K. F. B. collected the liposome fusion data, membrane accessibility, and fluidity data and measured the transition temperatures. J. M. W. and J. A. assigned the NMR data. S. M. and C. H. B. collected the CD data and conductivity data. J. M. collected the protein-liposome binding data. All authors analyzed the results and approved the final version of the manuscript.

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