Dehydrins protect plant proteins and membranes from damage during drought and cold. *Vitis riparia* K1 is a 48-residue protein that can protect lactate dehydrogenase from freeze-thaw damage by preventing the aggregation and denaturation of the enzyme. To further elucidate its mechanism, we used a series of *V. riparia* Kc concatemers (K6, K8, and K10) and natural dehydrins (*V. riparia* YSK2, 60 kilodalton peach dehydrin [PCA60], barley dehydrin5 [Dhn5], *Thellungiella salsuginea* dehydrin2 [TsDHN-2], and *Opuntia streptacantha* dehydrin1 [OpsDHN-1]) to test the effect of the number of K-segments and dehydrin size on their ability to protect lactate dehydrogenase from freeze-thaw damage. The results show that the larger the hydrodynamic radius of the dehydrin, the more effective the cryoprotection. A similar trend is observed with polyethylene glycol, which would suggest that the protection is simply a nonspecific volume exclusion effect that can be manifested by any protein. However, structured proteins of a similar range of sizes did not show the same pattern and level of cryoprotection. Our results suggest that with respect to enzyme protection, dehydrins function primarily as molecular shields and that their intrinsic disorder is required for them to be an effective cryoprotectant. Lastly, we show that the cryoprotection by a dehydrin is not due to any antifreeze protein-like activity, as has been reported previously.

Drought and freezing can cause dehydrative stress in plants, which, if severe enough, can lead to their death. To lessen the negative effects of these environmental stresses, most, if not all, plants express dehydration proteins (dehydrins; Close, 1997; Allagulova et al., 2003; Rorat, 2006; Kosová et al., 2007; Eriksson and Harryson, 2011), a member of the group 2 LEA proteins. Dehydrins are thought to be involved in dehydration protection, since their transcription and translation are increased during dehydration, and a correlation exists between drought tolerance and the amount of dehydrin present. In vitro, dehydrins have been shown to protect enzymes from freeze-thaw damage (Lin and Thomashow, 1992; Kauzoku and Oeda, 1994; Momma et al., 2003; Goyal et al., 2005; Hughes and Graether, 2011) and heat denaturation (Kovacs et al., 2008), interact with and protect membranes from cold and dehydrative stresses (Rahman et al., 2010; Eriksson et al., 2011), and bind water (Tompma et al., 2006), ions (Alsheikh et al., 2003), and nucleic acids (Hara et al., 2009). Dehydrins have also been suggested to prevent the growth of ice crystals by functioning in a manner similar to antifreeze proteins (AFPs; Wisniewski et al., 1999; Simpson et al., 2005).

Dehydrin sequences are very modular in nature, containing a variable number and various types of segments. A dehydrin is defined by the presence of at least one copy of the K-segment in its sequence (Close, 1997). The K-segment is a Lys-rich region with the somewhat conserved sequence of EKKGIMDKIKEKLPG (Close, 1997). The only clear role shown to date for the K-segments is their ability to bind anionic membranes (Koag et al., 2009). It has been suggested that the K-segment may be an amphipathic helix, although NMR studies showed that the segment is only very weakly helical in solution (Hughes and Graether, 2011). Some dehydrins have two additional conserved amino acid motifs, known as the Y-segment and the S-segment. A dehydrin is dehydrated with K-segments, YnSK2,K n,K nS, SKn, and YnKn (Close, 1996), where n indicates a variable number of segments. The S-domain, composed of five to seven consecutive Ser residues, is found in many dehydrins. It has been shown to be a phosphorylation site (Goday et al., 1994), which may play a role in activating the segment’s ability to bind ions (Alsheikh et al., 2003) and/or translocating the dehydrin to the nucleus (Goday et al., 1994). The Y-segment has sequence similarity to the nucleotide-binding site of bacterial and plant chaperones [V/T DEYGNP] (Close, 1996), but as of yet, there is no experimental evidence that it binds nucleotides. Dehydrins also contain f-segments, which are rich in Gly, Ala, and small, polar amino acids. These motifs are poorly conserved in terms of their length and sequences and tend to be located between other segments (Close, 1996, 1997). Overall, dehydrin sequences are highly
hydrophilic and generally lack Cys or Trp residues, although one dehydrin has been discovered to have Cys residues (Peng et al., 2008; Reyes et al., 2008). Not surprisingly, the high polar content results in dehydrins being intrinsically disordered proteins (IDPs; Hughes and Graether, 2011), and as such, they do not have a single, well-defined protein structure (Lisse et al., 1996; Tompa, 2002; Uversky, 2002a). IDPs are generally highly flexible and have minimal secondary structure, although some undergo a disorder-to-order transition after binding a ligand (Tompa, 2002). IDPs often have specific functions in cell cycle control, the assembly of protein complexes, and the modification of protein activity (Uversky, 2002a).

We have previously characterized the Vitis riparia K2 dehydrin (Findlater and Graether, 2009; Livernois et al., 2009; Hughes and Graether, 2011). This protein appears to be a splice variant of YSK2 mRNA whose transcription is up-regulated after cold stress (Xiao and Nassuth, 2006). K2 represents a minimal dehydrin model that can be used to better understand the structure-function relationships of this family of proteins. Using NMR chemical shifts and relaxation data (Hughes and Graether, 2011), we have shown that in solution the K-segment is very weakly α-helical and flexible, while the φ-segment has no secondary structure preference whatsoever and is highly flexible. Despite its small size (48 residues), the V. riparia K2 was very effective at protecting lactate dehydrogenase (LDH) from freeze-thaw damage (Hughes and Graether, 2011), a model enzyme used in many cryoprotective studies (Lin and Thomashow, 1992; Kazuoka and Oeda, 1994; Momma et al., 2003; Goyal and Brooks, 1994). Unlike the weak, negative shoulder at 222 nm for K2 and the K-segment peptides, the Kn constructs showed a shift of the minimum at 198 nm, showing that the secondary structures of the peptides, K2 and Kn, concamers, were examined by circular dichroism (CD). The CD spectrum of K2 (Fig. 2A) shows that it is disordered, with no significant secondary structure. The spectrum has a molar elliptical minimum centered at 198 nm and a weak minimum at 222 nm. Neither truncation of the protein to one K-segment (K-peptide) nor deletion of the φ-segment (KK-peptide) results in a shift of the minimum at 198 nm, showing that the peptides have a similar type of disorder compared with K2.

In the case of the longer dehydrin concamers, it is possible that with the larger size the proteins may begin to form some secondary structure due to different regions interacting with each other, especially since the large number of positively charged residues in the K-segments could interact with some of the negatively charged Asp and Glu residues located in the φ-segments. This is not the case, since the CD spectra on these proteins show that the Kn concamers remain highly disordered, with an unshifted minimum at 198 nm for all of the proteins studied here (Fig. 2B). Unlike the weak, negative shoulder at 222 nm for K2 and the K-segment peptides, the Kn constructs showed a range of molar ellipticities, with the shoulder being the least negative for K10, and demonstrated increasing values as the protein became longer. Generally, a decrease in the CD signal at 222 nm is ascribed to the loss of helical content, although in this case it more likely represents the increased separation of the weakly helical segments rather than the disruption of the helical secondary structure (Hirst and Brooks, 1994).

We also examined whether the various constructs were becoming more compact, and hence less disordered, in the absence of a change in secondary
structure. We used the following equation from Uversky (2002b):

$$\log(R_h) = y_0 + a \cdot \log(M_r)$$  \hspace{1cm} (1)

where $R_h$ is the hydrodynamic radius of the protein in Å, as measured by gel filtration, $y_0$ is the $y$ intercept of the fit, $a$ is the slope of the fit, and $M_r$ is the $M_r$ of the construct. The relationship between hydrodynamic radius and $M_r$ will hold true for an IDP; however, deviation from the line can indicate that the protein is gaining structure through compaction. The log-log plot of the $M_r$ versus the hydrodynamic radius of the K-segment constructs used in this study is shown in Figure 2C. The linear fit ($r^2 = 0.997$) of the data points demonstrates that there is no compacting of the larger dehydrin constructs compared with the peptides. The CD and hydrodynamic data show that the creation of the concatamers has not resulted in any sort of artificial structuring of the proteins.

**Cryoprotection of LDH by Dehydrin Peptides and Concatamers**

We subsequently tested the ability of the K-segment peptides to protect LDH from freeze-thaw damage compared with $K_2$. The recovery of LDH activity was measured over a range of protein concentrations, from which the molar concentration required to achieve 50% recovery of LDH activity ($PD_{50}$) was calculated. A lower $PD_{50}$ value represents a protein that is more efficient at protecting LDH. If only the K-segments are involved in cryoprotection, one would expect the efficiency of the K-peptide and $K_2$, represented by the $PD_{50}$ of 122 versus 459 $\mu M$. The lack of the predicted 2-fold difference between the $PD_{50}$ of the K-peptide and $K_2$, and the large difference in $PD_{50}$ between the K-peptide and $K_2$, would suggest that all parts of the protein, and not just the K-segments, are involved in protecting LDH from damage in our assay. Since all parts of the protein are involved in the enzymatic cryoprotective function of dehydrins, we next examined the $K_n$ constructs. As can be seen in Figure 3B, the same pattern is observed as for the peptides, in that as the construct becomes longer, there is an increase in the efficiency of protection. Taken together, it is clear that the larger the dehydrin, the more effective the cryoprotection, and that both K-segments and $\phi$-segments contribute to the protection. The dehydrins act most likely as molecular shields, essentially preventing the collision of one LDH molecule with another (Wise and Tunnalcliffe, 2004; Chakrabortee et al., 2012).

**Hydrodynamic Radius, Disorder, and Cryoprotective Efficiency**

The results show that as the dehydrins become longer, the $PD_{50}$ value decreases. We used the hydrodynamic radii as a measure of protein size and plotted this value against the $PD_{50}$. The plot (Fig. 3C) shows that there is a rapid decrease of $PD_{50}$ as the hydrodynamic size increases between the K-peptide and $K_2$, representing the increase in efficiency as the protein becomes larger. For constructs larger than $K_4$, the $PD_{50}$ decreases less rapidly, even though the hydrodynamic radius is still increasing (compare Figs. 2C and 3C). This shows that the smaller dehydrins such as $K_2$ and $K_4$ are good at protecting LDH, but increasing the size of the dehydrin beyond that no longer provides a proportional increase in the efficiency of the protection.

Figure 1. Sequences of the dehydrin constructs. The sequences and architectures of $K_2$ and variants are shown using the single-letter amino acid code. The complete sequences are shown for $K_2$, K-peptide, and KK-peptide. The insert used to generate the $K_n$ constructs is shown as the base repeating unit that is added to the C terminus of $K_2$, where $n = 1$ attached to the C terminus of the $K_2$ sequence would be the sequence of the $K_4$ construct.
As noted in the introduction, dehydrins consist mainly of three types of conserved sequence elements (K-, Y-, and S-segments) interspersed with polar and small amino acid-rich f-segments. The Kn concatemers are experimental constructs and do not occur in nature. We performed the cryoprotective assay with five natural dehydrins (V. riparia YSK2, 60 kilodalton peach dehydrin [PCA60], barley dehydrin5 [Dhn5], Thellungiella salsuginea dehydrin2 [TsDHN-2], and Opuntia streptacantha dehydrin1 [OpsDHN-1]) that have various segmental architectures. Figure 4A shows that those natural dehydrins are also able to protect LDH in a way similar to the K-segment constructs. The PD_{50} values of the natural dehydrins overlapped with those of the Kn constructs (Fig. 4B; Supplemental Table S2). The natural dehydrins and dehydrin constructs appear to be similarly effective in their ability to protect LDH, although because of the asymptotic nature of the PD_{50} values in this region, it is difficult to say whether the levels of protection are identical.

We then examined whether the molecular shield is simply a generic protein effect (i.e. a volume-exclusion effect) or whether it is specific to disordered stress proteins such as dehydrins. The cryoprotective assay was repeated using a series of ordered proteins (lysozyme, carbonic anhydrase, ß-casein, ovalbumin, bovine serum albumin [BSA], and phosphorylase) that have a similar range of hydrodynamic radii to the dehydrins. The results of the cryoprotection assay are shown in Figure 5A, with a plot of the hydrodynamic radius versus PD_{50} shown in Figure 5B and tabulated in Supplemental Table S3. Unlike the dehydrins, ordered proteins do not show any relationship between size and the efficiency of protection. In fact, two ordered proteins (lysozyme and carbonic anhydrase) have hydrodynamic radii similar to that of K2 (on the order of 20 Å) yet have PD_{50} values that vary considerably.

The previous results show that it is not just any protein that protects LDH from freeze-thaw damage. To examine whether disorder is a key factor in LDH protection, we used the polymer PEG covering a similar size range to the dehydrins in the assay. PEGs are similar to dehydrins in the sense that they are polar polymeric chains with no appreciable structure. The hydrodynamic radii of the various PEGs were calculated using the following formula from Devanand and Selser (1991):

\[ R_h = 0.145M_r^{0.571} \]  

where \( R_h \) is the hydrodynamic radius in Å and \( M_r \) is the \( M_r \) of the PEG.

Figure 6A shows the cryoprotection assay results, where the pattern and extent of the recovery of enzyme activity are similar to those of the dehydrins. The hydrodynamic radii versus PD_{50} data for PEG are shown overlaid with the dehydrin data in Figure 6B, with the data shown in Supplemental Table S4. The polymer appears to be slightly more efficient at...
protecting LDH than dehydrins at shorter radii, but at longer radii the two compounds show similar efficiencies.

Potential AFP Activity of Dehydrins

AFPs are proteins that have been discovered in many different organisms and are thought to function by binding to the ice surface and inhibiting further ice growth (Jia and Davies, 2002; Graether and Sykes, 2004). Two previous reports suggested that dehydrins also have the ability to shape ice crystals and prevent their growth at moderate subzero temperatures (Wisniewski et al., 1999; Simpson et al., 2005). This activity was suggested to come from the dehydrin present in peach (Prunus persica) bark (PCA60; Wisniewski et al., 1999) and from a partially purified and characterized Forsythia suspensa protein that may contain K-segments (Simpson et al., 2005). The anti-freeze activity could explain the cryoprotective effects of dehydrins in plants. It is possible that part of the dehydrin’s ability to protect LDH from freeze-thaw activity may come about from its ability to alter ice crystal growth. Our previous study, however, had shown that the V. riparia K2 and YSK2 proteins were unable to inhibit ice growth (Livernois et al., 2009). We had ascribed that to the fact that the peach PCA60 dehydrin contained 11 K-segments, whereas our proteins contained only two. To determine if this activity is found in dehydrins, we examined whether bacterial recombinant Dhn5, K10 (a dehydrin similar in size to PCA60), and PCA60 would be able to inhibit ice crystal growth. Rather than measuring thermal hysteresis using a nanoliter osmometer (Ramlov, 2011), we looked for the presence of AFP-like activity using the ice recrystallization inhibition assay (Knight et al., 1995). In this method, the presence of AFP activity will keep the ice crystals small, while in its absence small ice crystals shrink and disappear while the larger ice crystals will grow. Although these two techniques measure different ice growth inhibition effects, we chose the ice recrystallization inhibition assay because it is highly sensitive and able to detect activity in the nanomolar range for a flounder AFP (Knight et al., 1995).

The results of the assays are shown in Figure 7. The 30% Suc is a negative control, and as can be seen the ice crystals grow considerably larger after 60 min at −6°C. In contrast, the positive control of type I AFP (Patel and Graether, 2010) shows that the ice crystals remain small. Figure 7 also shows, in contrast to previous reports, that none of the recombinant proteins (Dhn5, K10, or PCA60) have any ice recrystallization inhibition activity, suggesting that dehydrins lack AFP-like activity; therefore, this effect would not contribute to enzyme cryoprotection.

Figure 3. Cryoprotection of LDH by K2 and the various constructs. The ability of the dehydrins to protect LDH from freeze-thaw damage was plotted as percentage recovery of LDH activity versus additive concentration. The data were fitted to Equation 3. The error bars represent SD of four measurements. A, K-peptide, black squares; KK-peptide, white squares; K2, black circles. B, K2, black circles, K4, white triangles; K6, gray triangles; K8, black triangles; K10, white diamonds. C, Comparison of PD50 versus hydrodynamic radius. The plot shows the relationship between the size of the protein and the cryoprotective efficiency, which is expressed as the PD50. K-peptide, black squares; KK-peptide, white squares; K2, black circles, K4, white triangles; K6, gray triangles; K8, black triangles; K10, white diamonds.
DISCUSSION

Residual Structure of K-Segment Peptides and K\(_n\) Constructs

While IDPs are defined as being disordered and lacking significant secondary structure in solution, their structures can be broadly classified as random coil or premolten globule depending on their CD spectra profiles (Uversky, 2002a). Specifically, disordered proteins with molar elliptical values between \(-22 \times 10^3\) and \(-16 \times 10^3\) deg cm\(^2\) dmol\(^{-1}\) near 200 nm, and between \(-2.4 \times 10^3\) and \(-1 \times 10^3\) deg cm\(^2\) dmol\(^{-1}\) at 222 nm, are said to have a random coil structure (a less compact structure with little secondary structure), whereas proteins with \(-15 \times 10^3\) and \(-12 \times 10^3\) deg cm\(^2\) dmol\(^{-1}\) near 200 nm, and values between \(-5 \times 10^3\) and \(-2.8 \times 10^3\) deg cm\(^2\) dmol\(^{-1}\) at 222 nm, are said to have a premolten globule structure (a moderately compact structure with some secondary structure; Uversky, 2002a). Using this classification system, the K-segment peptides, K\(_2\) or K\(_4\), cannot be clearly classified into the category of coil-like or premolten globule but instead fall between these two classifications (Fig. 2, A and B). The CD spectra did reveal that the K\(_6\) and longer constructs are becoming coil like, with their large minimum at 198 nm and the shoulder at 222 nm becoming slightly more negative (Fig. 2B). CD studies by Mouillon et al. (2006) on K-segment peptides from several Arabidopsis (Arabidopsis thaliana) dehydrins showed similar results, and the authors also stated that these proteins do not

Figure 4. A, Cryoprotection of LDH by natural dehydrins. The ability of the larger dehydrins to protect LDH from freeze-thaw damage was plotted as percentage recovery of LDH activity versus protein concentration. The error bars represent the SD of four measurements. The dotted line indicates 50% recovery of LDH activity. K\(_2\), black circles; YSK2, yellow circles; TsDHN-2, purple circles; OpsDHN-1, blue circles; PCA60, red circles; Dhn5, green circles. B, Plot of PD\(_{50}\) versus hydrodynamic radius. Symbol colors are as in A, with the data from the K\(_n\) constructs overlaid as black circles. [See online article for color version of this figure.]

Figure 5. Cryoprotection of LDH by structured proteins. A, The ability of the ordered proteins to protect LDH from freeze-thaw damage was plotted as percentage recovery of LDH activity versus protein concentration. The error bars represent the SD of four measurements. The dotted line indicates 50% recovery of LDH activity. B, Comparison of PD\(_{50}\) with the hydrodynamic radius. Lysozyme, gray squares; carbonic anhydrase, white squares; \(\beta\)-casein, white upside-down triangles; ov-albumin, white circles; BSA, black upside-down triangles; phosphorylase, gray upside-down triangles. For comparison purposes, the PD\(_{50}\) of K\(_2\) is included as black circles.
clearly fall into either category. They suggested that this proves that the individual K-segments of dehydrins do not interact with one another, which helps to keep them highly flexible in solution (Mouillon et al., 2006). Our previous comparison of *V. riparia* K2 with YSK2 by NMR demonstrates the same thing for the entire protein. That work showed that the chemical shifts of residues in the K2 region were identical in the presence and absence of the YS region, indicating that there is no notable interaction between these two regions. The data shown in Figure 2C here extend this observation to the larger dehydrins, since the longer Kn concatemers did not show any compaction. We propose that this lack of even a weak interaction helps to keep the dehydrin hydrodynamic radii quite large compared with their sequence length.

Enzymatic Cryoprotection

Numerous studies have reported on the ability of dehydrins to protect LDH from losing activity after being frozen and thawed (Lin and Thomashow, 1992; Kazuoka and Oeda, 1994; Houde et al., 1995; Hara et al., 2001; Momma et al., 2003; Goyal et al., 2005). Previously, we had shown that the 48-residue K2 protein is able to protect the enzyme from freeze-thaw damage despite its small size (Hughes and Graether, 2011). Here, we first examined the importance of the conserved K-segments by quantitatively comparing the PD$_{50}$ of K2 with that of the K-segment peptides (Fig. 3A). As noted in “Results,” the large difference between the PD$_{50}$ of the KK-peptides and K2 dehydrin demonstrates that the entire protein is involved in the cryoprotection of LDH and not just the K-segments.

The role of the conserved K-segments was also examined by Reyes et al. (2008) using deletion mutants of Arabidopsis ERD10 dehydrin and the *Rhododendron catawbiense* RcDhn5 dehydrin. With ERD10, the deletion of three K-segments resulted in the recovery of some LDH activity above that of the negative control, although deletion of two K-segments in RcDhn5 resulted in the same level of activity as the negative control (Reyes et al., 2008). Based on these observations, the authors suggested that the K-segments must be important for the recovery of LDH activity. A recently published paper on wheat (*Triticum aestivum*) DHN-5, also using deletion mutants, suggested that the K-segments are critical for dehydrin’s protective functions (Drira et al., 2013).

Our alternative interpretation is that the differences in activities are due to differences in the hydrodynamic radii of the truncated ERD10, RcDhn5, and DHN-5 dehydrins rather than being specifically due to the loss of the conserved K-segment motifs. This could explain the apparently contradictory results where the loss of three K-segments in one dehydrin had a less deleterious effect than the loss of only two K-segments in another (Reyes et al., 2008). A similar relationship between the level of protection from abiotic stresses and the size of the protein has been reported for several LEA group 3 proteins using assays such as LDH freeze-thaw (Honjoh et al., 2000), salt tolerance in *Escherichia coli* (Liu and Zheng, 2005; Wang et al., 2012), and aggregation of *α*-casein (Furuki et al., 2012). The group 2 dehydrins and the group 3 proteins share little sequence similarity other than having low sequence complexity, suggesting that some of the protective effects of these proteins may be due to their polar and disordered nature rather than to the presence of specific sequence motifs.

We examined the role of dehydrin size in enzyme protection by increasing the length of K2 to make the Kn series of concatemers. We defined the size of the dehydrins by their hydrodynamic radius and examined what relationships may exist between the hydrodynamic radius and PD$_{50}$. Since the entire protein is involved in protecting LDH, we theorized that the longer the dehydrin, the lower the PD$_{50}$. The PD$_{50}$ values in Figure 3C show that, as the protein construct
Dehydrin Size and Cryoprotective Efficiency

becomes larger, the protection becomes more efficient. Similarly, the natural dehydrins show the same pattern (Fig. 4B). As can also be seen in Figure 4B, all of the dehydrins, with the exception of the K-peptides and K$_2$, have a similar PD$_{50}$ (range, 10–30 $\mu$M). This may suggest that, after a certain length, dehydrins are no longer able to provide considerably more efficient protection from enzyme denaturation.

The possibility exists that the observed protection of LDH by dehydrins is a simple volume-exclusion effect and that any protein will effectively protect LDH from freeze-thaw damage. Therefore, we used a series of structured proteins and PEG in the assays (Supplemental Table S4). Previous assays have used molarity and weight concentration to compare PD$_{50}$ values. The results presented here show that using the hydrodynamic radius is a more accurate way to compare them. They also suggest one reason why abiotic stress proteins are often disordered: an IDP will have a considerably larger hydrodynamic radius than a structured protein with the same number of residues. The second reason is that the structure of IDPs is not affected by the abiotic stress, since they are only very weakly structured.

The assay results with the structured proteins are shown in Figure 5. Here, it can be seen that the efficiency of protection is quite scattered: some proteins provide relatively efficient protection, while others do not. Interestingly, enzymatic proteins are rather poor at protecting LDH, whereas nonenzymatic proteins function at levels similar to K$_2$ but are 3- to 10-fold less efficient than the larger dehydrins (Fig. 5B, black circles).

It is not clear why enzymes are worse. It cannot be a question of stability, since lysozyme and BSA both heat denature at approximately 65°C. While quantitative cold temperature denaturation data are not available, lysozyme is used in freeze-thaw lysis of bacteria, and BSA is often added as a cryoprotectant to frozen proteins, suggesting that they should be similarly stable. In addition, the surface hydrophobicity of these two proteins is similar (Cardamone and Puri, 1992), showing that the poor protection by lysozyme is not due to interaction with apolar residues on its surface. An explanation for the very different PD$_{50}$ values between enzymatic and nonenzymatic proteins will require further study.

The inability of many ordered proteins to provide protection of LDH as efficiently as dehydrins or other polar polymers such as PEG is most likely due to how dehydrins function in cryoprotection. Dehydrins prevent the aggregation of LDH (Hughes and Graether, 2011), a property also observed for group 1 and group 3 LEA proteins (Goyal et al., 2005). If the ordered protein itself also denatures, it will add to the aggregation problem rather than prevent it. Even the more effective structured proteins, such as BSA and ovalbumin, have PD$_{50}$ values that are on the order of 100 $\mu$M compared with 10 $\mu$M for the large dehydrins. This difference may be explained by a few structured proteins still losing some structure during the freeze-thaw treatment, and therefore contributing to the aggregation problem, whereas IDPs such as the dehydrins cannot denature.

Since disorder is important for effective cryoprotection, we examined whether PEG might be a substitute for dehydrins, since it is a polar polymer (Fig. 6). The PEGs, the K$_c$ concatemers, and the natural dehydrins have the same relationship of a decreasing PD$_{50}$ as the hydrodynamic radius increases. The PEGs appear to be slightly more efficient at protection at smaller hydrodynamic radii, although it is not clear why. Nevertheless, the data show that disorder and length are important for dehydrins to function as effective molecular shields.

A similar protective effect was seen between LDH and another polar polymer, polyvinylpyrrolidone (Anchordoquy and Carpenter, 1996), and the Suc polymer Ficoll70 was shown to reduce the protein aggregation of soluble cell protein extracts (Chakrabortee et al., 2012). This brings up the question of why plants do not simply synthesize a polar polymer instead of a protein for cryoprotection, and why the dehydrins have conserved sequence motifs. One possible answer is that dehydrins are also able to protect against other stresses. The K$_c$, S$_c$, and Y-segments are conserved so that they may carry out their other functions, such as binding ions (Alsheikh et al., 2003; Tompa et al., 2006), nucleic acids (Hara et al., 2009), and membranes (Rahman et al., 2010; Eriksson et al., 2011).
Ice Recrystallization Inhibition by Dehydrins

 AFPs (Jia and Davies, 2002; Patel and Graether, 2010) are a family of proteins that protect organisms from damage caused by freezing. One of these protective activities is ice recrystallization inhibition, which refers to a process whereby large ice crystals grow as small ones shrink (Knight et al., 1995). Previously, we showed that V. riparia K2 and YSK2 do not have any AFP activity (Livernois et al., 2009). In contrast, two other studies showed that PCA60 (a peach Y2K9 dehydrin; Wisniewski et al., 1999) and a putative dehydrin from *F. suspensa* (Simpson et al., 2005) have AFP activity. At the time, we thought that the two K-segments may have been insufficient to bring about AFP activity. A second possibility is that the other two studies were contaminated by trace AFPs, since the dehydrins were purified from natural sources while our proteins were from recombinant bacteria. We examined whether the K10 concatemer, recombinant PCA60, or DHN5 had ice recrystallization inhibition activity (Fig. 7). This assay is exquisitely sensitive (Knight et al., 1995), but we purposely chose a relatively high concentration of 1 mg mL⁻¹ to see if there was even weak activity. The type I AFP is a positive control and causes the ice crystals to remain very small in the absence of ice recrystallization. K10 did not have any ability to inhibit recrystallization. Since the K₉ constructs are not found in nature, we also examined the peach dehydrin PCA60 (Wisniewski et al., 1999) and the winter rye (Secale cereale) dehydrin Dhn5 (Bravo et al., 1999). PCA60 has been shown previously to have thermal hysteresis (separation of equilibrium melting and freezing points), which would indicate that it would also have ice recrystallization inhibition, but as seen in Figure 7, neither natural dehydrin has the ability to stop the growth of ice crystals. We can exclude the possibility that the lack of activity is due to the lack of a posttranslational modification for two reasons. The first is that the only known modification of dehydrin, phosphorylation of the S-segment, cannot occur in PCA60 because it has no such Ser tract. The second is that Dhn5 purified from barley (Hordeum vulgare) does not have thermal hysteresis (L. Bravo, personal communication). Therefore, our results show that the previous reports of AFP-like activity in dehydrins are likely due to contamination and that dehydrins do not protect enzymes by inhibiting ice growth. This also agrees with our observation that AFPs are very rigid proteins (Graether et al., 2003; Graether and Sykes, 2004) and not disordered, a property possibly required for them to form ice-like waters on their surface (Garnham et al., 2011).

**Materials and Methods**

**Dehydrin Protein Production**

The K2 protein was expressed and purified as described previously (Livernois et al., 2009; Hughes and Graether, 2011). The K-peptide (containing one K-segment) and KK-peptides (containing two K-segments without the δ-segment) were synthesized by CanPeptide. These constructs were made more than 95% pure by reverse-phase HPLC using a Biobasic C18 column (Fisher Scientific) with buffer A (0.1% trifluoroacetic acid [w/v] in water) and buffer B (0.1% trifluoroacetic acid [w/v] in acetonitrile) as the mobile phases. Separation was performed using a linear gradient of 1% buffer B to 100% buffer B over 60 min at a flow rate of 1 mL min⁻¹. Fractions containing the peptides were subsequently pooled and lyophilized. For the KN constructs, a gene insert encoding a-K-K-K protein sequence flanked by a 5' S-segment restriction site and a 3' Xhol restriction site (to encode compatible overhangs) was synthetically made. The pET22b-K2 plasmid (Livernois et al., 2009) was modified to contain an Xhol restriction site. The first construct, K2N, was made by ligating the gene insert at the 3' end of the K2 encoded gene. Ligation of the KN gene insert into the KN plasmid causes the S-segment restriction site to be lost but maintains the 3' Xhol site. Because of this, the insertion process could be repeated to create the KN KN and KN KN protein constructs. The expression and purification of the KN proteins was performed identically to that described for K2 (Livernois et al., 2009).

The natural dehydrin plasmids were generous gifts (peach *Prunus persica* dehydrin [PC60]) from M.E. Wisniewski, Appalachian Fruit Research Station, U.S. Department of Agriculture, Kearneysville, WV [Wisniewski et al., 1999]; *Thalangella saluinea* dehydrin (TsDHN-2) from G. Harauz, University of Guelph [Rahman et al., 2010]; barley (*Hordeum vulgare*) dehydrin (Dhn5) from T.J. Close, University of California, Riverside [Bravo et al., 1999]; and *Opuntia streptacantha* dehydrin (OpsDHN-1) from J.-F. Jiménez Bremont, Instituto Potosino de Investigación Científica y Tecnológica, San Luis Potosi, Mexico [Ochoa-Alfaro et al., 2012]) and were expressed and purified as described for K2. The expression and purification of *Vitis riparia* YSK2 has been described previously (Livernois et al., 2009).
CD

CD data of the dehydrin proteins were collected using a Jasco-815 CD spectropolarimeter. All protein samples were dissolved in 10 mM sodium phosphate, pH 7.4, at a concentration of 0.16 mg mL$^{-1}$. A quartz cuvette with a 2-mm path length (Hellma) containing the protein sample was scanned from 250 to 190 nm. The spectra were averaged over eight accumulations, and all experiments were performed at 25°C using a thermally controlled water bath.

LDH Cryoprotection Assay and Analysis

The cryoprotection assay was performed as described previously (Hughes and Graether, 2011). Briefly, LDH was prepared by overnight dialysis against 10 mM sodium phosphate buffer, pH 7.4. The dehydrins and reagents were dissolved in 10 mM sodium phosphate, pH 7.4, buffer. LDH samples (7.5 μL of 50 μg mL$^{-1}$) were mixed with the dehydrins (7.5 μL of varying concentrations of protein) or buffer only (7.5 μL) in a 1.5-mL microcentrifuge tube. The concentration of LDH in all of the freeze-thaw assays was 0.18 μL. The samples were immersed in liquid nitrogen for 30 s and thawed by immersion in a circulating water bath at 4°C for 5 min. This treatment was performed a total of five times. LDH activity was measured by diluting the enzyme to 0.5 μg mL$^{-1}$ into 750 μL of 10 mM sodium phosphate, pH 7.4, 2 mM NADH, and 10 μM pyruvic acid. Enzyme activity was followed on a Cary 100 spectrophotometer (Varian) by measuring $A_{340}$ to follow the oxidation of NADH. Data from individual assays are plotted as percentage recovery of LDH activity versus additive concentration. The results were fitted to:

$$\% \text{LDH recovery} = \frac{x}{1 + e^{-ax}}$$

where $e$ is Euler’s number, $x$ is the additive concentration, $x_i$ is the percentage recovery in the absence of the additive, and $a$ and $b$ are fitted variables. The $PD_{50}$ value was determined by solving for $x$ at the predicted value of 50% recovery of enzyme activity. The PEGs used in the assay are listed in Supplemental Table S4, including their range of $M_t$ values and average hydrodynamic radii. Note that PEGs are polydisperse polymers. Assuming that the sizes of PEGs are evenly distributed around the average $M_t$, one would expect that the lower protective activity of polymers smaller than average is compensated by the higher activity of polymers that are larger than average.

Ice Recrystallization Inhibition

Ice recrystallization inhibition measurements were performed as outlined by Livernois et al. (2009). Briefly, the proteins were dissolved at a concentration of 1 mg mL$^{-1}$ in a 30% (w/v) Suc solution. The solution (5 μL) was applied between a glass coverslip and the glass slide and placed on a freezing microscope stage. The sample was cooled to −40°C at a rate of 30°C min$^{-1}$, after which the temperature was raised to −0°C at a rate of 30°C min$^{-1}$. At this point, the temperature was held constant and a photograph was taken to represent 0 min. After 1 h at −6°C, a second photograph of the sample was taken to represent 60 min. The presence of ice recrystallization inhibition was verified by visual comparison of samples between 0 and 60 min. Type I AFP was used as a positive control, while a solution containing only 30% (w/v) Suc was used as a negative control. The brightness of the images was increased digitally to enhance the contrast of the ice crystal edges.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Summary of experimental data from the dehydrin constructs.

Supplemental Table S2. Summary of experimental data from the natural dehydrins.

Supplemental Table S3. Summary of experimental data from the structured proteins.

Supplemental Table S4. Summary of experimental data from various PEGs.

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LITERATURE CITED


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