

Cryoprotective mechanism of a small intrinsically disordered dehydrin protein

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Abstract: Dehydration proteins (Dehydrins) are expressed during dehydration stress in plants and are thought to protect plant proteins and membranes from the loss of water during drought and at cold temperatures. Several different dehydrins have been shown to protect lactate dehydrogenase (LDH) from damage from being frozen and thawed. We show here that a 48 residue K₂ dehydrin from *Vitis riparia* protects LDH more effectively than bovine serum albumin, a protein with known cryoprotective function. Light scattering and 8-anilino-1-naphthalene sulfonate fluorescence experiments show that dehydrins prevent aggregation and unfolding of the enzyme. The cryoprotective effects of LDH are reduced by the addition of salt, suggesting that the positively charged K-segments are attracted to a negatively charged surface but this does not result in binding. Overall K₂ is an intrinsically disordered protein; nuclear magnetic resonance relaxation experiments indicate that the two-terminal, Lys-rich K-segments show a weak propensity for α -helicity and are flexible, and that the central, polar rich phi-segment has no secondary structure preference and is highly flexible. We propose that the phi-segments in dehydrins are important for maintaining the disordered structure so that the protein can act as a molecular shield to prevent partially denatured proteins from interacting with one another, whereas the K-segments may help to localize the dehydrin near the enzyme surface.

Keywords: cryoprotection; dehydrin; dynamics; freeze-thaw; intrinsically disordered protein; lactate dehydrogenase

Introduction

The inability of plants to flee an environmental stress has resulted in the evolution of an extensive stress response system. One example of an abiotic stress is dehydration, which can take the form of drought (lack of environmental water), high salinity (high osmolarity), or freezing (lack of liquid water). During periods of dehydration, a wide variety of plants can express dehydration proteins (dehydrins), which are also members of the plant late embryo abundant protein family.^{1–4} The accumulation of dehydrin transcripts and proteins during dehydration, and a correlation between the level of drought

tolerance and the amount of dehydrin present, strongly suggest that they are involved in protecting the plant from the negative effects of dehydration. Numerous *in vitro* functions have been described and proposed for dehydrins, including cryoprotection of lactate dehydrogenase (LDH),^{5–8} cryoprotection of purified protoplasts and chloroplasts,⁹ prevention of water loss,¹⁰ binding of excess ions,¹¹ binding of nucleic acids,¹² prevention of protein aggregation at elevated temperatures,¹³ and prevention of ice crystal growth.^{14,15} Of these functions, the most extensively studied has been the cryoprotection of LDH, where it has been shown that dehydrins are more effective than small molecules such as sucrose or other proteins such as bovine serum albumin (BSA) at protecting LDH activity from freeze-thaw damage.⁷

The cloning of many dehydrin genes from different plants has revealed a number of features, the most notable being the modular nature of their sequence, which results in a large range of sizes

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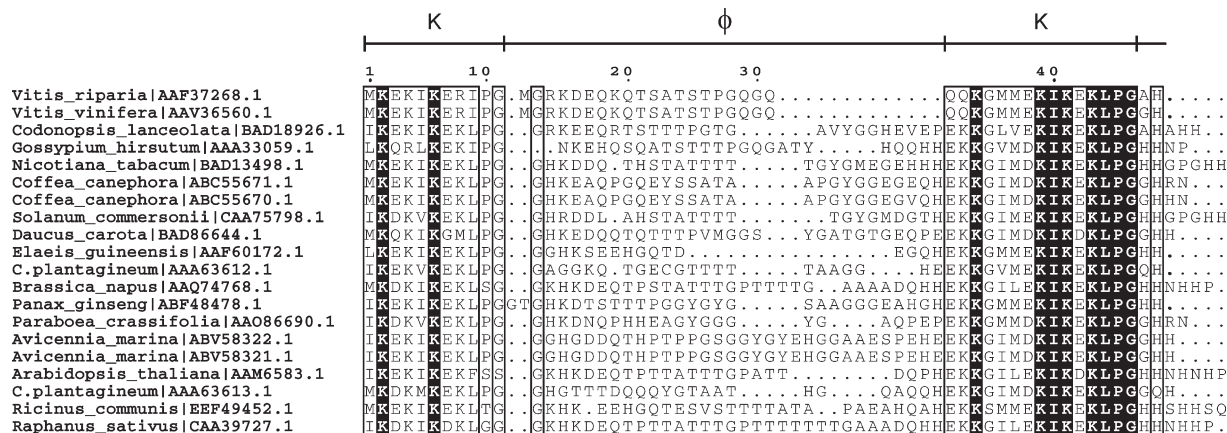


Figure 1. Alignment of K- Φ -K sequences. A ClustalW²³ generated alignment of 20 K₂ regions with the highest sequence identity to *Vitis riparia* K₂. Conserved regions are shown bound by a rectangle, where a white letter on a black background indicates a residue that is conserved in all sequences, and a black letter indicates residues that are partially conserved and/or chemically similar. The sequence numbering for *Vitis riparia* K₂ and the segment names are shown at the top of the alignment. The organism name and protein NCBI accession number are indicated on the left. The figure was generated using ESPript.²⁴

(6–200 kDa). The nomenclature used to describe the dehydrin sequence is the YSK notation, where each letter represents the name of a segment with a conserved sequence.¹ By definition, dehydrins contain at least one K-segment (the sequence EKKGIMKI-KEKLP), which is partly conserved among the different dehydrin members.¹ The K-segment occurs 1–12 times, with 1 or 2 repeats being the most common. The S-segment contains a tract of Ser residues and is present in one or no copies in a dehydrin. Dehydrins extracted from drought-stressed plants are phosphorylated on these serines.¹⁶ The role of phosphorylation is not clear, but may be correlated with translocation of dehydrins to the nucleus,¹⁷ or the increased negative charge could enhance the ability of the protein to bind divalent cations such as zinc.¹⁸ The Y-segment refers to the sequence (V/T)DEYGNP is similar to the nucleotide binding domain found in bacteria. Typically, 1–3 Y-segments are present at the N-terminus of a dehydrin.¹ Although not specifically included in the YSK naming system, dehydrins also contain Φ -segments, which are rich in Gly, Thr, and many other polar amino acids. This poorly conserved segment tends to be located between the Y-, S-, and K-segments. Not surprisingly, the overall high-polar residue content of dehydrins causes them to be intrinsically disordered.¹⁹ Intrinsically disordered proteins (IDPs) are molecules that, contrary to the structure-function paradigm, do not have a single, well-defined fold (for reviews see, e.g., Refs. 20 and 21). They are generally highly flexible and have minimal secondary structure, although some IDPs undergo a disorder-to-order transition after binding their target.²¹ Their many functions include cell cycle control, assembly of protein complexes, and the modification of protein activity.²⁰

Our model system for studying the biochemistry and structure of dehydrins uses the 48 residue K₂ protein from *Vitis riparia* with a segmental architecture of K- Φ -K. A previous study has shown that this dehydrin may be a splice variant of YSK₂ mRNA, which is transcribed during cold stress.²² The presence of this K₂ protein in plants has not yet been demonstrated, but the protein represents a minimal dehydrin construct that can be used to examine the role of K- and Φ -segments in its cryoprotective function. Here, we examine the structural propensity and dynamics of K₂ in solution, and measure its ability to protect LDH from freeze-thaw damage. Several mechanisms are explored, and it is shown how structural changes correlate with the amount of K₂ added.

Results

Segment alignment, structure, and dynamics

An alignment of 20 dehydrin K- Φ -K regions is shown in Figure 1, where the conserved and similar residues in the protein are highlighted. As seen with other dehydrins,⁴ the K-segments are moderately conserved. In the alignment of the first K-segment, the Lys amino acids in the 2nd and 5th positions are completely conserved, whereas the 4th and 8th positions are mostly Lys or a similarly positively charged Arg amino acid. The second K-segment contains more conserved residues, with the Lys present in all sequences in the 3rd, 8th, 10th, and 12th positions. Although the protein is highly hydrophilic, hydrophobic residues are also conserved. In the first K-segment, the 5th position is mostly Ile, whereas the 9th position is mostly Leu. Similarly, in the second K-segment, a hydrophobic Ile is completely

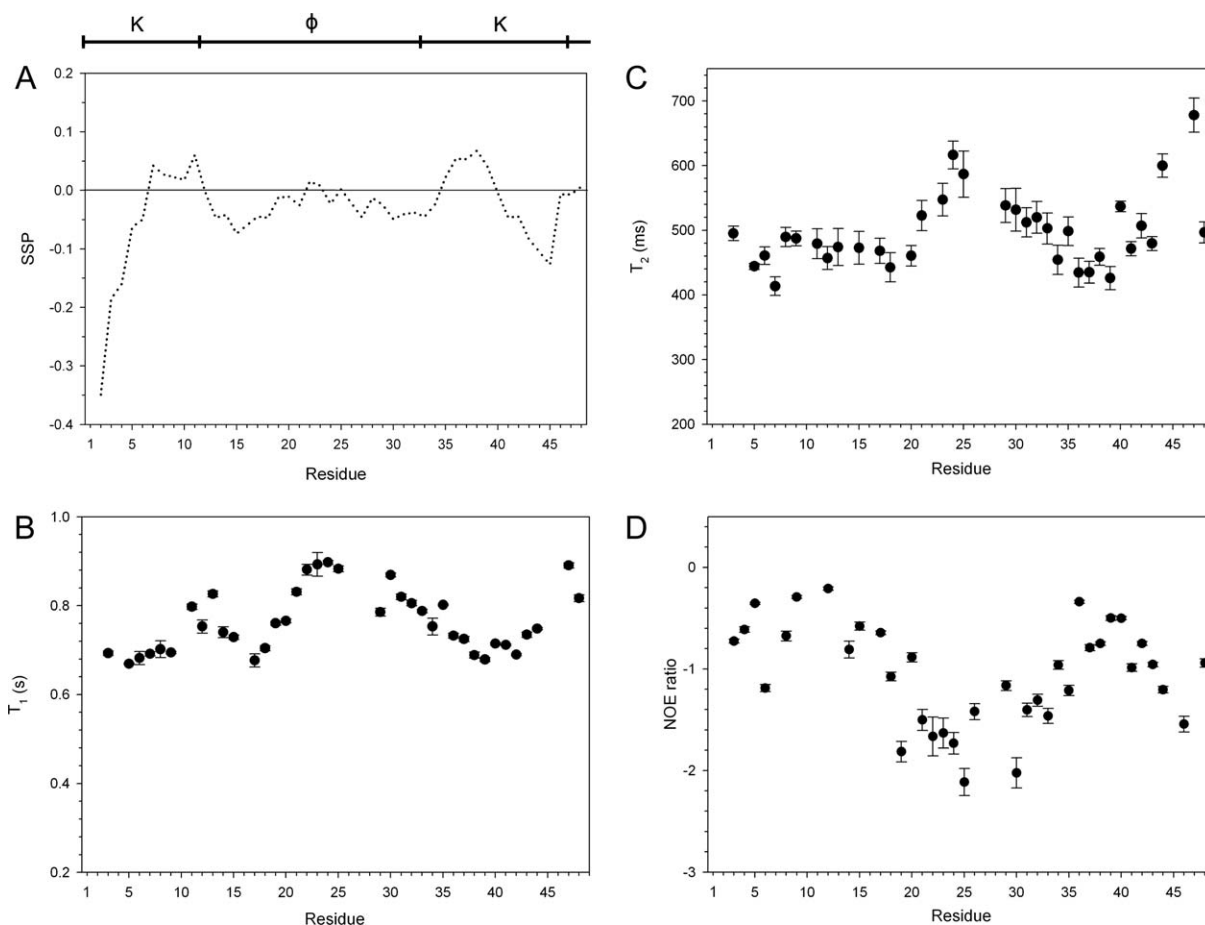


Figure 2. Structure and dynamics of K_2 in solution. The arrangement of the K- and ϕ -segments is shown at the top of the figure. A: The SSP scores of K_2 were calculated using H_{α} , C_{α} , and C_{β} chemical shifts and are plotted on a per residue basis. B: T_1 , (C) T_2 , and (D) ^{15}N -NOE relaxation data of K_2 . Error bars represent the error in fitting the relaxation decay curves as described in CCPNMR.

conserved in the ninth position, whereas a hydrophobic Leu is completely conserved in the 13th position. In the majority of the alignments, both K-segments end with (hydrophobic)-Pro-Gly. The regions following the second K-segment are 2–8 residues in length, and are rich in His and Gly residues. The Φ -segment in the middle of the alignment is highly variable in sequence and length, being rich in Gly, Thr, Ser, and Ala residues (i.e., amino acids with small side chains), with Gln being the most common amino acid with a longer side chain.

The segmental architecture is also reflected in the structural propensity of *V. riparia* K_2 in solution. The secondary structure propensity (SSP) program can be used to describe the presence of transient secondary structures in IDPs, where positive scores are equated with propensity of the disordered ensemble to be in an α -helical conformation, and negative scores indicate the propensity to be in a β -strand conformation.²⁵ For the K_2 studied here, the overall SSP pattern is symmetric about the middle of the sequence except for the first 4 and the last two residues [Fig. 2(A)]. The core of the K-segments has a

tendency to form a helix-like structure, whereas the N-terminal end of the first segment and the C-terminal end of the second segment tend to be more strand-like. The final two residues have no secondary structure preference. For the Φ -segment, the beginning and end of this region show a propensity toward being extended, while the middle has very little consistent preference for either secondary structure.

Like the SSP scores, the heteronuclear ^{15}N relaxation data show that the protein is not highly structured [Fig. 2(B–D)]. For folded, globular proteins, heteronuclear nuclear Overhauser effect (NOE) values are positive and near 1. As can be seen in Figure 2(D), none of the residues have NOEs that are positive, which shows that the K_2 protein has high flexibility and is undergoing fast motions. The T_1 [Fig. 2(B)] and T_2 [Fig. 2(C)] measurements also indicate that the protein is very flexible. All of the relaxation data show the same pattern between the different regions as indicated by the SSP scores, where residues in the K-segments are flexible, and the residues in the Φ -segments are even more flexible, with the highest amount of motion seen in the

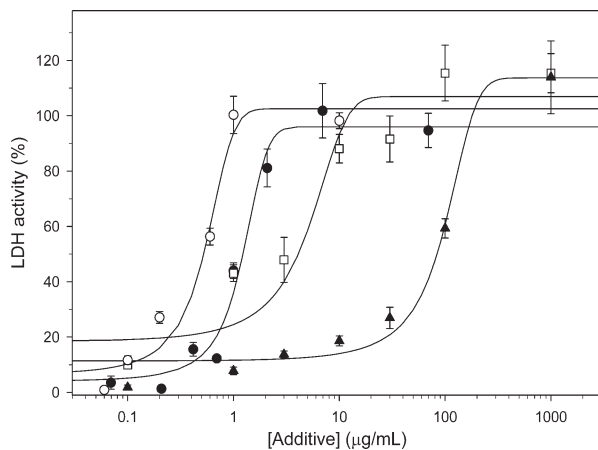


Figure 3. Cryoprotection of LDH. The protection of LDH activity after freezing and thawing of the enzyme was examined in the presence and absence of additives (K_2 , YSK_2 , BSA, or lysozyme) over a range of concentrations. The activity of unfrozen LDH is defined as 100% activity. K_2 , solid circle; YSK_2 , open circle; BSA, open square; lysozyme, solid triangle. Error bars represent the standard deviation of $n = 6$ (K_2 , BSA, lysozyme) or $n = 3$ (YSK_2). The lines represent fits to the sigmoidal equation: % LDH activity = $\frac{a}{1 + e^{-\frac{(x-x_0)}{b}}}$, where x is the concentration of the additive.

middle of the protein. The last two C-terminal residues show an increase in the amount of flexibility compared with the preceding residues in the K-segment. The lower flexibility in the K-segments and the higher flexibility in the Φ -segments support the SSP data, that is, the presence of transient structure in the K-regions and the lack of any structure in the Φ -region.

Denaturation and cryoprotection of LDH

One *in vitro* protective mechanism that has been shown for a number of dehydrins is their ability to protect LDH from losing activity due to being frozen and thawed.^{5–8} For controls, the assay was also performed in the presence of BSA (a known cryoprotective agent) and lysozyme (a poor cryoprotective agent). As shown in Figure 3, the K_2 and YSK_2 proteins have cryoprotective abilities that are better than BSA, an observation that has also been made with other dehydrins.⁷ The fitted curves have a sigmoidal shape when plotted as log concentration versus percent recovery, indicating that below a threshold concentration of ~ 0.5 $\mu\text{g/mL}$ K_2 there is no ability to protect LDH and, at high concentrations (>4 $\mu\text{g/mL}$), there is a saturating effect on enzyme protection. YSK_2 was able to provide 50% recovery of LDH activity at approximately twofold lower protein concentration, showing that the larger protein provides more efficient protection. LDH protection by lysozyme was weak, with the curve plotted in the figure representing the most effective protection that we measured. On occasion, we observed no pro-

tection under identical experimental conditions, but this inconsistency in protection was never observed with BSA, YSK_2 or K_2 . Other researchers have used lysozyme as a negative control in the LDH assay, with one group reporting essentially no protection,⁸ and the other group reporting weak protection similar to what was observed here.²⁶

Several structural properties of LDH before and after freezing were examined to determine how the treatment has damaged the protein. These experiments were repeated in the presence and the absence of K_2 at three concentrations, one of which resulted in no recovery of activity, one with $\sim 50\%$ recovery of activity, and one which recovered 100% of the LDH activity. The use of several concentration of K_2 with differing effects on the recovery of LDH allows one to distinguish between structural changes in the enzyme that are correlated with the cryoprotective effects of K_2 and those that are not.

The first property examined was the oligomeric state of LDH before and after freezing. Native and fully active LDH is a tetramer, whereas dimers and monomers have no activity.²⁷ Unfrozen and freeze-thawed samples of LDH were loaded onto a Superdex 200 gel filtration column. As shown in Figure 4(A), both treated and untreated proteins eluted at exactly the same position and gave the same peak shape. This shows that the freeze-thaw process used here did not cause the enzyme to irreversibly dissociate.

During gel filtration, a decrease in the amount of eluted LDH was observed after the freeze-thaw cycle, which suggested that the protein was aggregating. To examine the role of aggregation, the scatter at A_{340} of several enzyme samples before and after freezing were measured [Fig. 4(B)]. The absorbance readings were corrected for scatter caused by buffer and/or K_2 . For the LDH alone sample, the scatter increased ~ 100 -fold after the freeze-thaw treatment, indicating that aggregation contributes to the loss of LDH activity. Surprisingly, the addition of K_2 at a concentration that produced 50% protection reduced the scatter to background levels, and the addition of K_2 that would result in 0% activity increased scatter only 50-fold compared with the 100-fold increase seen for frozen LDH alone [Fig. 4(B)]. This suggests that the prevention of aggregation is not the only factor in the dehydrin cryoprotective mechanism, because it does not account for the loss of activity seen in the presence of lower concentrations of K_2 .

Therefore, we used the fluorescent dye 8-anilino-1-naphthalene sulfonate (ANS) to explore what further changes may be occurring to the structure of LDH. ANS is used to examine various unfolded states since its fluorescence intensity changes dramatically when the probe binds to solvent exposed hydrophobic patches.^{28–30} The emission scans of

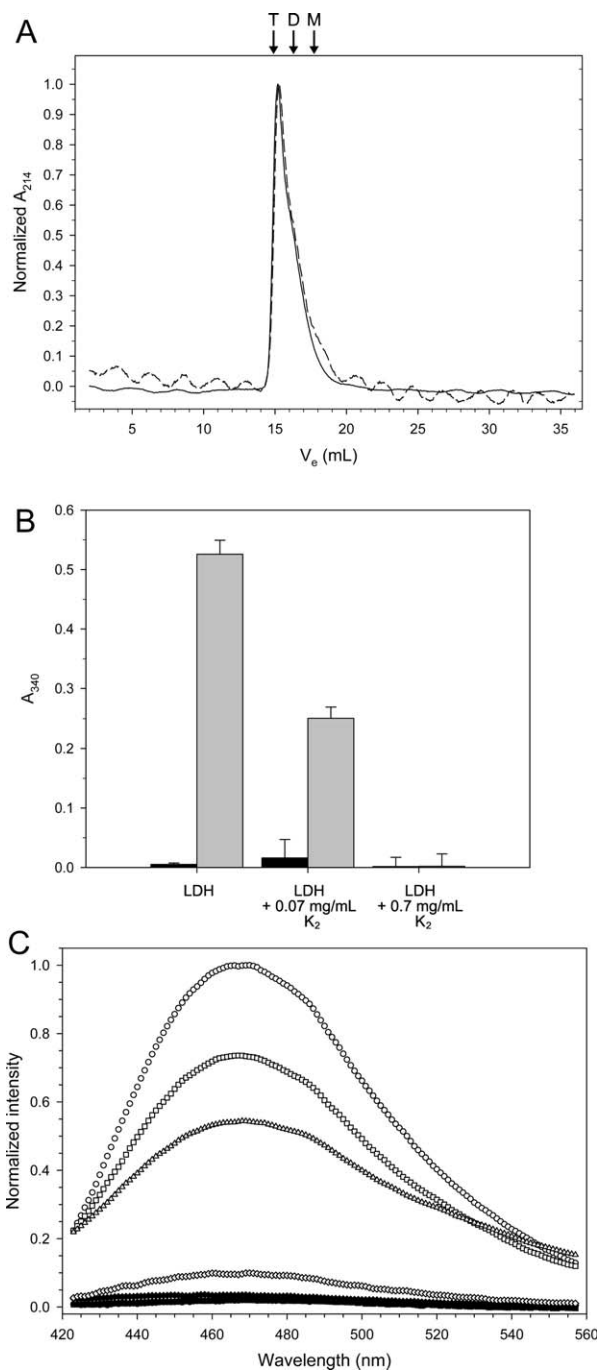


Figure 4. Effect of freezing and freeze-protection on the structure of LDH. **A:** Gel filtration of 200 μ g of LDH before (solid line) and after (dashed line) being frozen and thawed. The arrows and labels indicate the predicted elution volume of the various oligomeric states: T, tetramer; D, dimer; M, monomer. V_e represents the elution volume of the sample. **B:** Light scatter measured at 340 nm. The bar graph represents data from $n = 6$ measurements, and the error bars show their standard deviation. The sample conditions are listed below each entry, where black represents unfrozen samples and gray represents frozen and thawed samples. **C:** Emission scans of ANS fluorescence of 1 μ M LDH in the presence and the absence of K_2 . Samples were scanned before (solid symbols) and after (open symbols) freeze-thaw treatment. Symbols: LDH alone, circle; LDH with 0.028 mg/mL K_2 , square; 0.28 mg/mL K_2 , triangle; 0.65 mg/mL K_2 , diamond.

frozen and thawed LDH alone showed that the intensity at 470 nm increased 47-fold compared with the untreated control, demonstrating that hydrophobic residues had been exposed [Fig. 4(C)]. Increasing concentrations of K_2 showed decreasing ANS fluorescence to the point where at maximal protection by K_2 the fluorescence returned to near background levels. Therefore, the cryoprotective effects of K_2 are a combination of preventing aggregation and partial denaturation of LDH.

Cryoprotection by K_2

How K_2 and LDH may be interacting was subsequently examined using nuclear magnetic resonance (NMR). 15 N-heteronuclear single quantum coherence (HSQC) spectra have been extensively used to map interaction sites between a protein and its ligand, because changes in the 1 H and 15 N chemical shifts can be correlated with the binding of a ligand.³¹ 15 N-HSQC spectra of K_2 in the presence and the absence of a three molar excess of LDH were overlapped. As can be seen in Figure 5(A), there was absolutely no shift in any of the resonances, demonstrating that there is no direct binding interaction between K_2 and LDH (i.e., the interaction has a K_d weaker than the millimolar range).

The lack of a direct interaction suggests that the cryoprotective function of dehydrins may be to act as a shield that prevents denatured LDH from associating with other LDH molecules. The cryoprotection by YSK₂ is slightly better than that provided by K_2 (see Fig. 3). This is most likely a reflection of the increased size of YSK₂ (13.9 kDa vs. 5.4 kDa for K_2), making it a larger shield. Further support comes from the cryoprotection studies using the 80 kDa P-80 dehydrin from barley, which is even more effective than the two grape proteins at protecting LDH from freeze-thaw denaturation.³²

K_2 molecules would need to stay close to an LDH molecule to prevent this aggregation. The conserved K-segments in dehydrins are highly positively charged due to the presence of several Lys residues. To examine whether long-range electrostatics have any role in dehydrin cryoprotection, NaCl was added to the cryoprotective assay, with K_2 present at a concentration which should provide $\sim 100\%$ protection. Figure 5(B) shows the bar graph of changes in LDH activity in the presence and the absence of the salt. All of the measurements are expressed as percent LDH activity, where unfrozen LDH in the absence of salt is defined as 100%. The addition of NaCl reduced the activity of unfrozen LDH in the presence and absence of K_2 . The presence of salt has reduced the percent activity recovered to 54% for both unfrozen LDH controls. However, the LDH sample with K_2 and salt recovered only 41% of its unfrozen activity, suggesting that electrostatic interactions between the positively charged K_2 and the

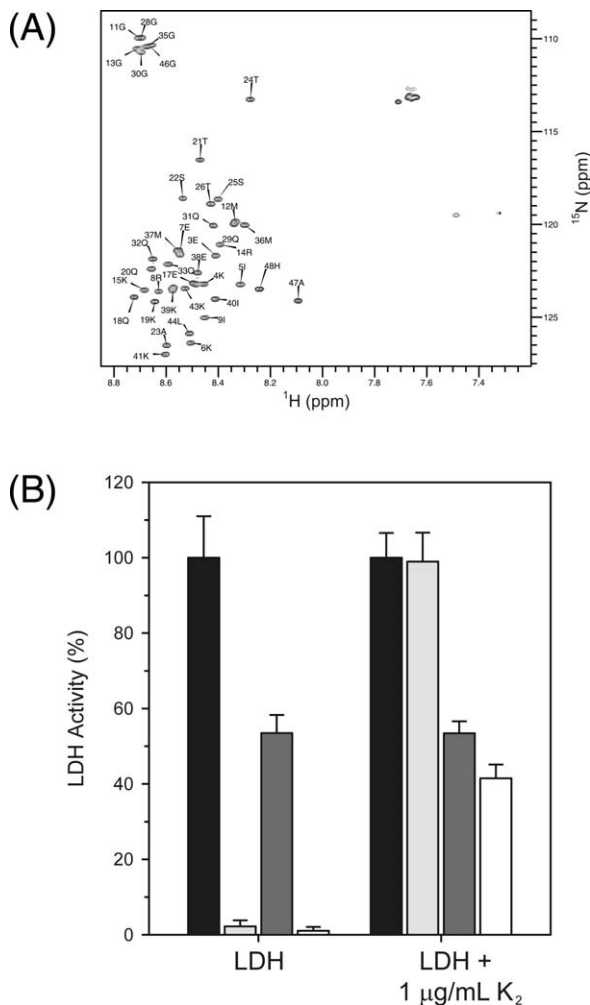


Figure 5. K_2 interaction with LDH. (A) ^{15}N -HSQC of 0.1 mM of K_2 collected in the absence (black contours) or the presence (gray contours) of 0.3 mM LDH. The peak labels show the residue number and single letter amino acid code of K_2 . B: Cryoprotection of LDH by K_2 is mediated by weak electrostatic interactions. The bar graph shows the percent LDH activity of samples assayed in the presence and the absence of salt with and without 1 $\mu\text{g/mL}$ K_2 . The data represent $n = 6$ measurements with the error bar corresponding to the standard deviation. Unfrozen samples with no NaCl, black bars; frozen samples with no NaCl, light gray; unfrozen samples with 1M NaCl, dark gray; frozen samples with 1M NaCl, white.

negatively charged LDH surface help keep the two proteins in close proximity without binding.

Discussion

Alignment and dynamics of K_2

The alignment of the K_2 sequences shows the conservation of Lys residues and two hydrophobic residues in the K-segments, and the lack of conservation in the Φ -segment (Fig. 1). The conservation patterns are also reflected in the differences in the structural propensity and dynamics data between these two segments. As an IDP, all of K_2 is flexible but the K-

segments show a weak tendency for forming an α -helix, whereas the Φ -segment has no clear secondary structure preference and is even more flexible than the K-regions (Fig. 2). It has been previously proposed that the K-segments form amphipathic helices, but here we show that in solution the protein is very weakly helical. It is only after a dehydrin binds a ligand, such as a micelle, that a notable amount of helical structure is induced.³³ We suggest that the high number of small, polar residues in the Φ -segment ensures that the protein remains highly flexible and is unable to form even transient structures. The presence of several hydrophobic amino acids are disfavored because they may cause the structure to partly collapse, whereas the presence of only like charged amino acids may cause extended structures to form because of charge–charge repulsion. Therefore, the nature of the amino acids in the Φ -region ensures that no structure can form, whereas the K-segments are able to form only transient α -helices.

Denaturation and cryoprotection of LDH

The denaturation of the model enzyme LDH has been studied using a number of chemical and physical stresses. The loss of activity after various treatments has been attributed a loss of its tetrameric state,³⁴ aggregation,⁵ and/or changes in its three-dimensional structure.^{35,36} We examined each of these possibilities to see what happens to LDH in our freeze-thaw protocol.

A sample of LDH was applied to a gel filtration column before and after being frozen and thawed. The results show that the oligomeric state was completely unaffected [Fig. 4(A)]. Low temperatures cause LDH to dissociate into monomers,³⁷ but the reversible nature of tetramerization appears to be unaffected by the freeze-thaw cycles. A dramatic loss in absorbance intensity was observed after LDH was treated, suggesting that the protein could be aggregating and thus losing activity. The addition of K_2 reduces the scatter of the thawed samples. However, there is a considerable reduction in the amount of scatter observed even at a K_2 concentration where all LDH activity is lost, and the addition of K_2 that results in a 50% reduction of LDH activity brought the scatter down to background levels [Fig. 4(B)]. This does not suggest that preventing aggregation is unimportant in the cryoprotective function of K_2 , but instead highlights a difference between *in vitro* and *in vivo* dehydrin assays. Performing these experiments with soluble protein extracts from plant cells would result in considerably more aggregation due to the much higher concentration of proteins and other macromolecules compared with *in vitro* conditions. Such results were seen in the work by Tunnacliffe and coworkers,³⁸ where a disordered protein from a desiccant-tolerant nematode reduced

the amount of aggregation of the water soluble proteome.

To explore what other structural changes may be occurring to LDH, the denaturation of the enzyme was followed using ANS as a probe to see if hydrophobic residues were being exposed. The results here show that the freezing and thawing of LDH alone causes a dramatic increase in ANS fluorescence [Fig. 4(C)], indicating that LDH has exposed its hydrophobic residues at some point during the freeze-thaw process. Similar results were observed with a *Rhododendron* dehydrin.³⁹ Increasing amounts of K₂ reduced the ANS fluorescence intensity. These results support the idea that dehydrins protect LDH activity by preventing both the aggregation and partial denaturation of the enzyme.

Cryoprotective mechanism

To determine whether there is a direct interaction between K₂ and LDH, the ¹⁵N-HSQC spectrum of ¹⁵N-K₂ alone was compared with that of the dehydrin in the presence of unlabeled LDH [Fig. 5(A)]. The resulting spectra completely overlap, indicating that the two proteins do not bind to each other. The data suggest that dehydrins do not appear to prevent LDH from denaturing by covering the surface of the protein before the freeze process.

We also examined whether there is an extremely weak association between the K-segment of K₂ and LDH that was mediated by long-range electrostatic forces. In this model, the dehydrin would not bind LDH, but would stay preferentially localized near the enzyme. The freezing and thawing of LDH in the presence of sodium chloride and K₂ at a concentration that should recover all of the pre-treatment LDH activity was only ~75% active relative to the unfrozen sample. This suggests that the K-segments of dehydrins could be important in maintaining a local concentration of K₂ around its target enzyme for cryoprotection. Because the “typical” cytoplasmic protein has a negatively charged surface at physiological pH,⁴⁰ it would make sense that a dehydrin would be positively charged from the Lys residues in the K-segments. It has been shown previously that the removal of the K-segments from several dehydrins lowered the recovery of LDH activity after freezing and thawing the enzyme, whereas the deletion of a Φ-like segment had no such effect.³⁹ Interestingly, the same study stated that a K-segment peptide did not show any cryoprotective effect. This would indicate that a flexible Φ-region and the presence of two K-segments are required to ensure that the dehydrin is large enough to prevent enzyme denaturation.

Our observations here are similar to those observed for the polyelectrostatic interaction between the intrinsically disordered Sic1 and Cdc4, where Sic1 interacts with its target only after a

number of residues have been phosphorylated (i.e., several negative charges are introduced).⁴¹ Like K₂, Sic1 does not appear to undergo a disorder-to-order transition in the presence of its ligand.

We have demonstrated that a small dehydrin is able to protect LDH from damage due to freezing and thawing. This comes about when the intrinsically disordered K₂ acts as a molecular shield to prevent partially denatured LDH from aggregating.

Materials and Methods

Protein purification and preparation

Unlabeled and ¹⁵N-labeled K₂ were purified as previously described.⁴² The proteins were desalted by reversed-phase high pressure liquid chromatography (HPLC) using a Biobasic C18 column (Fisher Scientific, Mississauga, ON, Canada) 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. Samples containing K₂ were pooled and lyophilized. Rabbit type II LDH protein (cat. no. L2500, Sigma-Aldrich, Oakville, ON, Canada) was prepared by overnight dialysis against 10 mM sodium phosphate buffer, pH 7.4.

LDH cryoprotection assay and analysis

A modified technique of Lin and Thomashow⁷ was used to measure LDH activity and examine the cryoprotective effects of the K₂ dehydrin and controls. All proteins and reagents were resuspended in 10 mM sodium phosphate, pH 7.4. LDH samples (8 μL of 50 μg/mL) were mixed with protective protein (8 μL of varying concentrations of K₂, BSA, or lysozyme) or buffer (8 μL) in a 1.5 mL microcentrifuge tube. Five cycles of freezing (immersion in liquid nitrogen for 30 s) and thawing (immersion in a circulating water bath at 4°C for 5 min) were performed. LDH activity was measured by diluting the enzyme to 0.5 μg/mL into 750 μL of reaction mix (10 mM sodium phosphate, pH 7.4, 2 mM NADH, 10 mM pyruvic acid). Oxidation of NADH was followed by monitoring A₃₄₀ on a Cary 100 (Varian, Mississauga, ON, Canada) over 2.5 min, during which time the reaction rate was linear. For the cryoprotection assays performed in the presence of salt, the same procedure was followed but 1M NaCl was added to the sample before freeze-thaw treatment.

Gel filtration

LDH (200 μg) was injected onto a Superdex G200 column before and after freeze-thaw treatment as described for the cryoprotection assay. The sample was eluted at a flow rate of 0.5 mL/min using 50 mM sodium phosphate, pH 7.4, 150 mM NaCl as the buffer. The column was calibrated with molecular

weight standards using the same buffer and flow rates.

Structure of thawed LDH

For the scatter experiments, 0.15 mg/mL of LDH in 10 mM sodium phosphate, pH 7.4 was frozen and thawed as described in the cryoprotective assay protocol in the presence and the absence of K_2 at concentrations of 0.07 and 0.7 $\mu\text{g/mL}$. The scatter was measured at A_{340} on a Cary 100 at a temperature of 25°C before and after the samples were frozen and thawed. Absorbance readings were corrected for contribution from buffer and K_2 .

For ANS fluorescence, 1 μM of LDH and 10 μM of ANS were dissolved in 10 mM sodium phosphate, pH 7.4, in the presence and the absence of K_2 at three concentrations (0.028, 0.28, and 0.65 mg/mL). The samples were frozen and thawed as described in the cryoprotection assay protocol. Fluorescence data were acquired on an Alphascan-2 spectrofluorometer (Photon Technology, South Brunswick, NJ), where emission scans were obtained by exciting the sample with $\lambda_{\text{ex}} = 350$ nm and scanning from 420 to 560 nm. Scans were corrected for contributions from buffer and K_2 alone.

NMR data collection and analysis

Experiments were collected on a Bruker Avance DRX600 spectrometer equipped with a cryogenic triple resonance probe at a temperature of 300 K, and ^1H and ^{15}N referencing was performed relative to 2,2'-dimethyl-2-silapentane-5-sulfonate (DSS) as described.⁴³ The T_1 , T_2 , and $\{^1\text{H}\}$ - ^{15}N -NOE relaxation experiments⁴⁴ were collected using 0.5 mM ^{15}N - K_2 in 600 μL of NMR buffer (20 mM sodium phosphate, pH 6.0, 10 mM NaCl, 0.01% sodium azide, 0.1 mM DSS and 5% D_2O (v/v)). For all three relaxation experiments, 1024 (^1HN) and 128 (^{15}N) complex data points were acquired with a total of eight transients per t_1 increment using spectral widths of 8370.5 (^1HN) and 1338.0 (^{15}N) Hz. For the T_1 experiments, relaxation delays of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 s were applied. For the T_2 experiments, relaxation delays of 16.064, 32.128, 48.192, 64.256, 80.320, 112.450, 144.580, 176.700, 208.830, and 240.960 ms were applied. The $\{^1\text{H}\}$ - ^{15}N steady-state NOE values represent the intensity ratio from the two HSQC spectra with and without ^1H saturation applied prior to the ^{15}N excitation pulse. For the acquisition of ^{15}N -HSQC spectra of 0.1 mM K_2 in the presence and the absence of 0.3 mM LDH, 1024 (^1HN) and 128 (^{15}N) complex data points were acquired with a total of four transients per t_1 increment using spectra widths of 7211.5 (^1HN) and 2128.6 (^{15}N) Hz.

Spectra were processed using NMRPipe⁴⁵ and peak assignments were made using the CCPNMR analysis software version 2.1.⁴⁶ Chemical shifts of K_2 were obtained from Biological Magnetic Reso-

nance Bank (BMRB) accession number 16445.⁴⁷ The T_1 and T_2 relaxation times were analyzed by fitting the cross-peak intensity decay to a two parameter, single exponential decay function $I(t) = I_0 e^{-t/T_{1,3}}$, where I_0 is the intensity at time $t = 0$ and $I(t)$ is the intensity after a delay time t for the T_1 or T_2 experiment. The errors were estimated from the uncertainty of the nonlinear fits. For the calculation of the SSP, the program SSP²⁵ was run with the RefDB data set for random coil and secondary structure chemical shift values.⁴⁸

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