Temperature-Induced Extended Helix/Random Coil Transitions in a Group 1 Late Embryogenesis-Abundant Protein from Soybean[1]

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Group 1 late embryogenesis-abundant (LEA) proteins are a subset of hydrophilins that are postulated to play important roles in protecting plant macromolecules from damage during freezing, desiccation, or osmotic stress. To better understand the putative functional roles of group 1 LEA proteins, we analyzed the structure of a group 1 LEA protein from soybean (*Glycine max*). Differential scanning calorimetry of the purified, recombinant protein demonstrated that the protein assumed a largely unstructured state in solution. In the presence of trifluoroethanol (50% [w/v]), the protein acquired a 30% α-helical content, indicating that the polypeptide is highly restricted to adopt α-helical structures. In the presence of sodium dodecyl sulfate (1% [w/v]), 8% of the polypeptide chain adopted an α-helical structure. However, incubation with phospholipids showed no effect on the protein structure. Ultraviolet absorption and circular dichroism spectroscopy revealed that the protein existed in equilibrium between two conformational states. Ultraviolet absorption spectroscopy studies also showed that the protein became more hydrated upon heating. Furthermore, circular dichroism spectral measurements indicated that a minimum of 14% of amino acid residues existed in a solvent-exposed, left-handed extended helical or poly (L-proline)-type (PII) conformation at 20°C with the remainder of the protein being unstructured. The content of PII-like structure increased as temperature was lowered. We hypothesize that by favoring the adoption of PII structure, instead of the formation of α-helical or β-sheet structures, group 1 LEA proteins retain a high content of surface area available for interaction with the solvent. This feature could constitute the basis of a potential role of LEA proteins in preventing freezing, desiccation, or osmotic stress damage.

Late embryogenesis-abundant (LEA) proteins accumulate to high concentrations in plant embryos during the latter stages of seed development before desiccation (Baker et al., 1988; Dure et al., 1989; Hughes and Galau, 1989). LEA proteins also accumulate in vegetative tissues exposed to exogenous abscisic acid, as well as dehydration, osmotic, and low-temperature stress (Chandler and Robertson, 1994; Ingram and Bartels, 1996; Bray, 1997; Close, 1996, 1997; Thomashow, 1998; Nylander et al., 2001). More than seven different groups of LEA proteins have been described and categorized by virtue of similarities in their deduced amino acid sequences (Baker et al., 1988; Dure et al., 1989). The majority of LEA proteins are highly hydrophilic and display a preponderance (e.g. Ala, Gly, Glu, and Thr) or lack (e.g. Trp and Cys) of certain amino acid residues (Dure, 1993a, 1993b, 1997). Thus, LEA proteins are part of a larger, evolutionarily conserved group of hydrophilic proteins termed "hydrophilins" involved in various adaptive responses to hyperosmotic conditions (Garay-Arroyo et al., 2000).

Various functions have been proposed for different groups of LEA proteins ranging from water binding to minimize water loss and protein and membrane stabilization or protection to ion sequestration and scavenging (Dure, 1993a, 1993b; Close, 1997; Danyluk et al., 1998). LEA proteins also display diverse subcellular and tissue-specific localization patterns, suggesting that different groups or group members fulfill specific functional roles (Close, 1997; Nylander et al., 2001). LEA protein expression generally correlates well with desiccation tolerance in young seedlings (Bartels et al., 1988; Reid and Walker-Simmons, 1993; Whisitt et al., 1997) as well as salt tolerance (Moons et al., 1995) and freezing tolerance (Houde et al., 1995; Van Zee et al., 1995; Close, 1997; Danyluk et al., 1998). Furthermore, ectopic expression of a barley (*Hordeum vulgare*) group 3 LEA protein, HVA1, in transgenic rice (*Oryza sativa*) improved tolerance to

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salinity and drought stress (Xu et al., 1996). When expressed in yeast (*Saccharomyces cerevisiae*) cells, HVA1 improved growth rates under ionic (NaCl and KCl) stress as well as improving cell freezing tolerance (Zhang et al., 2000). Overexpression of a tomato (*Lycopersicon esculentum*) group 2 (*le4*) and group 4 protein (*le-25*) conferred improved yeast cell growth rates at high NaCl and KCl concentrations, respectively, with both proteins improving freezing tolerance (Imai et al., 1996; Zhang et al., 2000). Similarly, overexpression of a group 1 LEA protein from wheat (*Triticum aestivum*; *Em*) in yeast cells resulted in improved growth under high NaCl, KCl, and sorbitol conditions (Swire-Clark and Marcotte, 1999). These observations suggest that different LEA proteins may play distinct but related roles in ameliorating different stress effects. However, the exact in planta function of these different groups of LEA proteins remain unknown.

Group 1 LEA proteins are distinguished from other groups of LEA proteins by being very hydrophilic and highly conserved along the entire length of the protein (Dure, 1993, 1997). Group 1 LEA proteins are further characterized by having an internal 20-amino acid signature motif repeated up to four times depending on the species (Esperlund et al., 1992) and a high proportion of Gly, Glu, and Gln residues. These signature repeats are evident in the *Bacillus subtilis GsiB* stress protein (Stacy and Aalen, 1998), which is induced by Glc or phosphate starvation, oxygen limitation, heat, oxidation, and salinity (Völker et al., 1994). Such remarkable conservation suggests an important role in stress adaptation.

The predicted hydrophilic and high degree of random coil structure of group 1 LEA proteins have led some researchers to propose these proteins may serve as water-binding proteins that can minimize water loss (McCubbin et al., 1985; Roberts et al., 1993), act as hydration buffers to regulate water status (Dure, 1993a; Walters et al., 1997), or interact with the surface of macromolecules as a water matrix or replacement to oppose protein denaturation in dehydrating tissues (McCubbin et al., 1985; Cuming, 1999). However, few biochemical analyses using purified group 1 LEA proteins have been reported. Circular dichroism (CD) spectroscopy and hydrodynamic modeling suggested that the wheat group 1 LEA protein, Em, contains 70% random coil or nonregular, flexible secondary structure (McCubbin et al., 1985). NMR spectroscopy of a recombinant carrot (*Daucus carota*) group 1 protein, EMB-1, expressed in *Escherichia coli* revealed no defined secondary or tertiary structure of the protein in solution (Eom et al., 1996). However, understanding the detailed mechanisms by which group 1 LEA proteins may confer protection against osmotic stress will require more specific information about the biochemical and biophysical properties of these proteins. Here, we examined the structural properties of a highly purified, recombinant group 1 LEA protein from soybean (*Glycine max*; rGmD-19). UV absorption and CD spectroscopy studies performed under a range of temperatures and pH revealed that the protein is largely unstructured with 6% to 14% of the protein occupying a left-handed extended helical or poly (L-Pro)-type (PII) conformation. Furthermore, the protein was found to exist in equilibrium between two conformational states with a low degree of transitional cooperativity.

RESULTS

Overexpression and Purification of rGmD-19

To study the structural and physicochemical characteristics of group 1 LEA from soybean in detail, large (milligrams) quantities of the recombinant soybean D-19 (rGmD-19) protein were produced and purified from *E. coli*. Affinity tag sequences were removed from the pET30a expression vector to express the native protein. From total cell lysates of *E. coli* BL21 (DE3) *plysS* cells, we observed the induction of the putative GmD-19 gene product with an apparent molecular mass of 11.4 kD (Fig. 1A).

Initial purification took advantage of the unique property of rGmD-19 solubility after exposure to boiling conditions. Subsequent purification steps included preparative IEF and anion-exchange chromatography, which were needed to achieve a high degree of purification (Fig. 1B). To determine whether rGmD-19 structure was altered by heat denaturation, rGmD-19 protein was also purified without boiling. Precipitation at 60% (w/v) $(NH_4)_2SO_4$ was substituted for heat and used as the initial purification step. Preparative IEF and anion-exchange column chromatography steps followed; however, it was necessary to include an additional cation exchange column chromatography step to attain homogeneity (Fig. 1C). Both rGmD-19s purified using heat and $(NH_4)_2SO_4$ showed identical electrophoretic mobility after analysis by native or denaturing SDS-PAGE (data not shown). The identity of the proteins purified by either purification strategy was confirmed by matrix-assisted laser-desorption ionization time of flight mass spectrometry (MS). The molecular mass of rGmD-19 was 11.359 kD, which is identical to the predicted molecular mass 11.359 kD (without N-terminal f-Met). Both noninduced *E. coli* cells and cells induced for rGmD-19 overexpression showed similar growth rates, suggesting that rGmD-19 protein accumulation was not harmful to cell viability (data not shown).

Thermal Stability of rGmD-19

Differential scanning colorimetry (DSC) was used to investigate a potential cooperative structural transition of rGmD-19. The DSC scans in Figure 2A show a typical denaturation pattern of a heat-labile protein

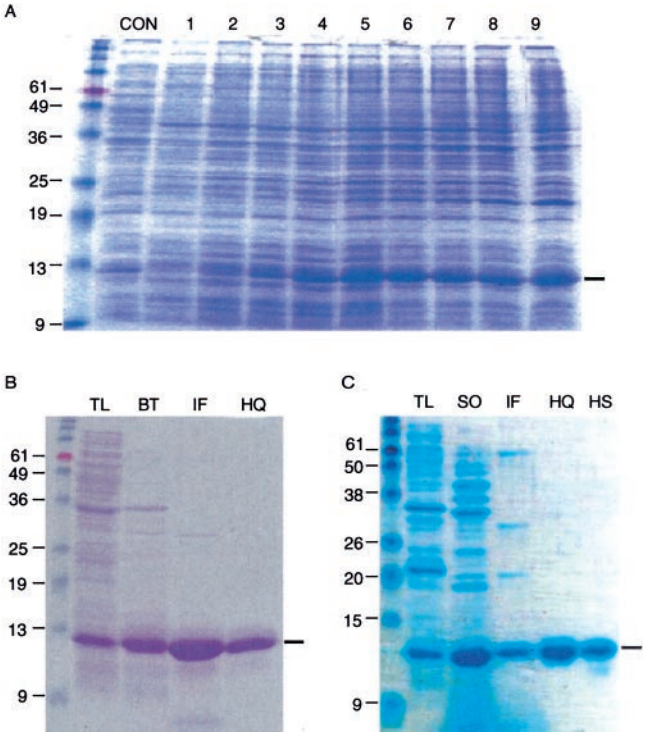

Figure 1. Expression and purification of group 1 LEA protein from *E. coli*. A, rGmD-19 accumulation at different times after isopropylthio-β-galactoside (IPTG) induction. Protein overexpression was induced with 0.1 mM IPTG when the optical density $(OD)_{600}$ was 0.8. Cells were collected at various time points and soluble proteins were extracted. Con, No IPTG; 1, induction point, 120 min; 2, 135 min; 3, 150 min; 4, 180 min; 5, 210 min; 6, 240 min; 7, 270 min; 8, 300 min; 9, 360 min. Approximately 10 μg of protein was loaded in each lane. B, Composite purification steps of rGmD-19 treated with boiling. Approximate amount of protein loaded in each lane is indicated in parentheses. TL, Total lysate (10 μg); BT, boiling treated (5 μg); IF, isoelectric focusing (IEF; 2 μg); HQ, high Q (1.5 μg). C, Composite purification steps of rGmD-19 without boiling. TL, Total lysate (10 μg); SO, 60% (w/v) $(NH_4)_2SO_4$ salting out (10 μg); IF, IEF (2 μg); HQ, high Q (2 μg); HS, high S (1.5 μg). Line designates rGmD-19. The relative mass of prestained or stainable molecular mass standards are designated in kilodaltons (kD).

(e.g. BSA). BSA undergoes thermal denaturation around 80°C. In contrast, DSC scans of rGmD-19, purified with or without boiling, show no detectable high-temperature peak (Fig. 2B). This result suggests that the protein failed to undergo any detectable irreversible denaturation. Similar results have been reported for a group 1 LEA protein isolated from pea (*Pisum sativum*) embryonic axes (Russouw et al., 1997). These DSC results are also consistent with previous studies indicating a low proportion or a lack of distinct tertiary structure in group 1 LEA proteins (McCubbin et al., 1985; Eom et al., 1996). Even though heating may alter the structure of rGmD-19 on a molecular scale, such changes may be of low cooperativity and thus not detectable by DSC. Therefore, spectroscopic methods, more sensitive at detecting molecular scale changes in structure, were tested.

UV Absorption Spectroscopy

rGmD-19 contains only two aromatic residues, one Tyr at position 57 and one Phe at position 89. Therefore, the near-UV absorption spectrum should be dominated by the absorption characteristics of the Tyr residue. The UV absorption spectrum of rGmD-19 showed two absorption maxima located at 228 and 275 nm (Fig. 3). The maximum at 275 nm is characteristic of the absorption by Tyr and confirms the absence of Trp. The maximum at 228 nm is dominated by the absorption of Tyr, but probably also has a contribution from the absorption of Phe and His residues (Demchenko, 1986).

Secondary Structure of rGmD-19

The secondary structure of rGmD-19 in solution was estimated from the far-UV CD spectrum at different temperatures and pHs. The CD spectra of rGmD-19 at pH 5, 8, and 9 remained unchanged (data

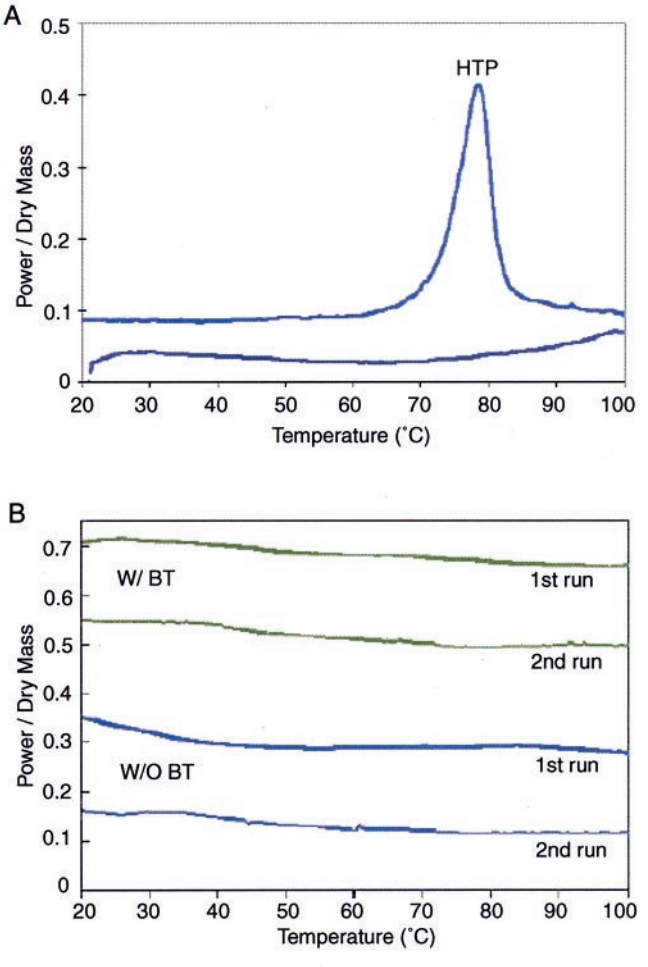

Figure 2. Heating thermograms of bovine serum albumin (BSA) and rGmD-19 using DSC. DSC heat scans of BSA (A) or rGmD-19 (B) with and without boiling treatment (BT) were performed at 10°C min^{-1} from 0°C to 100°C. Plots of each scans were offset slightly for clarity. HTP, High temperature peak.

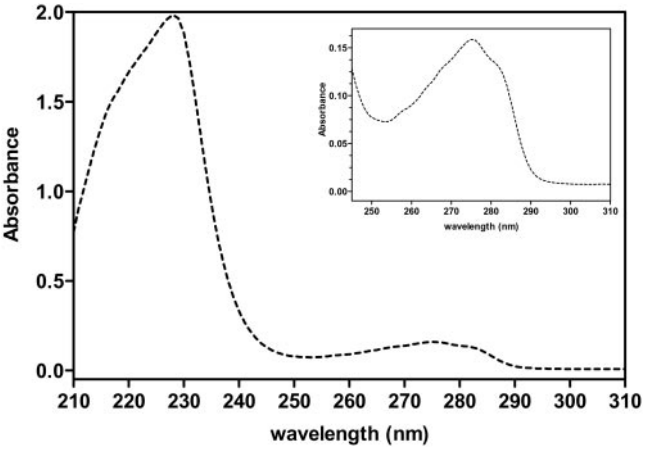

Figure 3. UV absorption spectrum of rGmD-19 (77 μM) in 50 mM phosphate buffer, pH = 7, 24°C. The inset shows the expanded near-UV region of the spectrum.

not shown), indicating that in this pH range the structure of the protein was not modified. A simple inspection of the CD spectra of rGmD-19 shows a strong negative band at 200 nm, which is generally found in highly unfolded proteins (Woody, 1992). The structural composition of the protein was estimated by the method of Sreerama et al. (1999), using a reference data set of 37 proteins that included the CD spectra of five unfolded proteins. The results obtained at 20°C (Table I) indicated that this protein has a very low content of the typical secondary structural elements of α-helices or β-strands and a high content of "unordered" structure.

Temperature-Induced Structural Changes Monitored by UV Absorption

Temperature-induced changes in the hydration of the Tyr residue of rGmD-19 were studied by second derivative UV absorption spectroscopy. This method is a reliable, quantitative, and sensitive tool to study the hydration of aromatic residues (Ragone et al., 1984; Demchenko, 1986; Soulages and Bendavid, 1998). Using Tyr fluorescence spectroscopy, Russouw et al. (1997) reported no difference in the Tyr environment of a group 1 LEA protein from pea before and after heat treatment (80°C). In contrast to this earlier work, Figure 4A shows the spectral changes that occur when the temperature was increased from 10°C to 80°C. In the spectral region displayed, the temperature-induced changes observed in the second derivative spectrum of rGmD-19 reflect the changes in the hydration of the Tyr residue. Even though the Tyr residue was highly hydrated at 10°C, the spectral changes observed indicate that the protein becomes more hydrated as the temperature is increased. These changes suggest that heating promotes a structural transition involving an unfolding process. In addition to the changes in the intensity of the second derivative spectra, another important feature observed in this study is the presence of several isobestic points (Fig. 4A). The presence of an isobestic point indicates a transformation involving two components. The fact that these isobestic points remain unchanged in the entire range of temperatures studied suggests that, in the 12°C to 80°C temperature range, there is an equilibrium between two conformational states. Because second derivative spectra obey Beer's law, as well as zero order spectra (Demchenko, 1986), we can use the difference between the second derivative values at any two given wavelengths as a measure of the relative change in the population of the conformational states. We have done this using the values of the derivative at 279 and 283 nm. Figure 4B shows that between 12°C and 80°C there is a continuous change in $\Delta(279–283)$ $[\delta^2\epsilon/\delta\lambda^2]$. Consistent with the DSC results, the slope of the plot suggests that the cooperativity of this conformational transition is quite low.

Effect of SDS Micelles, Phospholipid Liposomes, and Trifluoroethanol (TFE) on the Secondary Structure of rGmD-19

Even though rGmD-19 exhibited a very low degree of structural organization in aqueous medium, we studied its tendency to adopt helical structure by determining the CD spectrum in a solution containing 50% (v/v) of TFE, a helix-promoting solvent. In the presence of 50% (v/v) TFE, rGmD-19 acquired a significant degree of α-helical structure (Fig. 5). The difference spectrum has the typical features of an α-helical polypeptide (Fig. 5, inset). Using the difference spectrum it is estimated that, at 50% (v/v) TFE, the α-helical content of the protein is approximately 30%. This estimate indicated that the primary structure of the protein imposes significant restrictions to the adoption of α-helical structures. Using a similar approach, the Em protein from wheat was previously shown to increase in α-helical content from 13% to 29% in the presence of 45% (v/v) TFE (McCubbin et al., 1985).

Ismail et al. (1999) have observed that a group 2 LEA (dehydrin) protein from cowpea (*Vigna unguiculata*) acquired α-helical structure in the presence of SDS, suggesting that this protein may contain α-helical structures in a lipid-bound state in vivo. To determine if the structural properties of rGmD-19 might be influenced by lipid interactions, we tested the effect of SDS and unilamellar liposomes of dimyristoyl phosphatidylcholine on rGmD-19 structure. The CD spectra of rGmD-19 in the presence of SDS

Table I. *Secondary structure fractions of rGmD-19*

The fractions were determined by the method of Sreerama et al. (1999). Data were acquired at pH = 7 and 20°C.

	α-Helix	β-Strand	Turns	Unordered
Fraction %	7.9	5.4	3.5	82.5

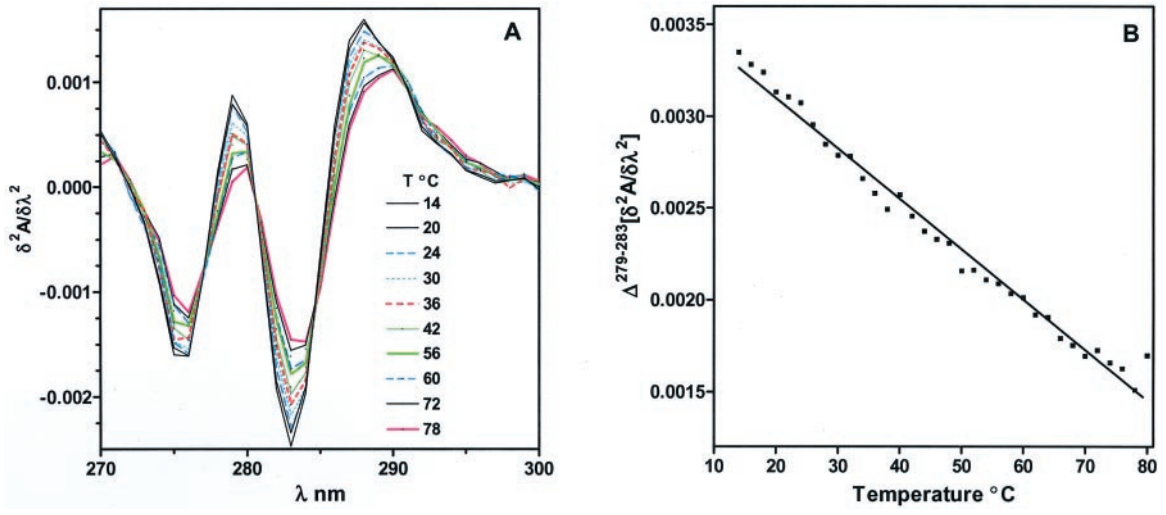

Figure 4. Effect of temperature on the second derivative spectrum of rGmD-19. A, The spectra shown, reading downward at 283 nm, represent increasing temperatures from 14°C to 78°C. The temperatures are indicated in the figure. B, The temperature-induced changes are represented using the difference between the derivative values at 279 and 283 nm: $\Delta(279–283)$ $[\delta^2\epsilon/\delta\lambda^2]$.

(Fig. 5), and the difference spectrum (spectrum in the presence of SDS minus spectrum in the absence of SDS), indicated that the protein interacts with SDS micelles and gains some α-helical structure. In the presence of detergent, the protein contains 8% of α-helical structure. Because SDS micelles have a very high charge density (negative), their interaction with the protein may not be physiologically relevant. To investigate whether the protein could interact with other lipid surfaces, we also incubated rGmD-19 with unilamellar liposomes of dimyristoyl phosphatidylcholine at a 20:1 lipid:protein molar ratio. This incubation produced no changes in the CD spectrum of rGmD-19 (data not shown), suggesting that the protein is unable to interact with zwitterionic phospholipid membranes. This result is in agreement with previous studies showing the lack of a specific phospholipid-protein interaction of other cold-induced proteins (Uemura et al., 1996; Webb et al., 1996). Although these results cannot discount the possibility that this group 1 LEA protein may interact with and be influenced by interaction with membrane lipids in vivo, we suggest that such interactions are unlikely given the extremely hydrophilic nature of this protein.

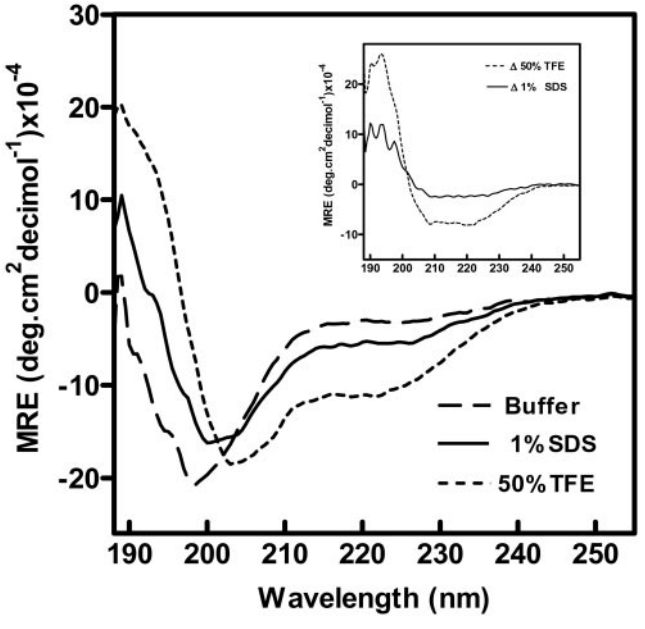

Figure 5. Effect of SDS and TFE on the secondary structure of rGmD-19. The CD spectra of rGmD-19 were obtained in 5 mM sodium phosphate buffer, pH 6.5, at room temperature. The samples of rGmD-19 containing SDS or TFE were prepared 1 h before the acquisition of the spectra. The inset shows the difference spectra that were obtained after subtracting the spectrum of rGmD-19 in buffer from the spectra obtained with either SDS or TFE.

Temperature-Induced Structural Changes Monitored by CD

Earlier investigations of a pea group 1 LEA protein using CD spectroscopy showed that heat treatment had no effect on the CD spectrum of the protein (Russouw et al., 1997). To more thoroughly investigate possible temperature- induced structural changes in rGmD-19, we obtained CD spectra at several temperatures (Fig. 6A). Contrary to the effect of pH, raising the temperature promoted significant changes in the CD spectrum of rGmD-19. Deconvolution of the spectra obtained at different temperatures by the method of Sreerama et al. (1999) renders no significant changes in the fractions of different secondary structural elements. Despite this, and although there are no changes in the shape of the

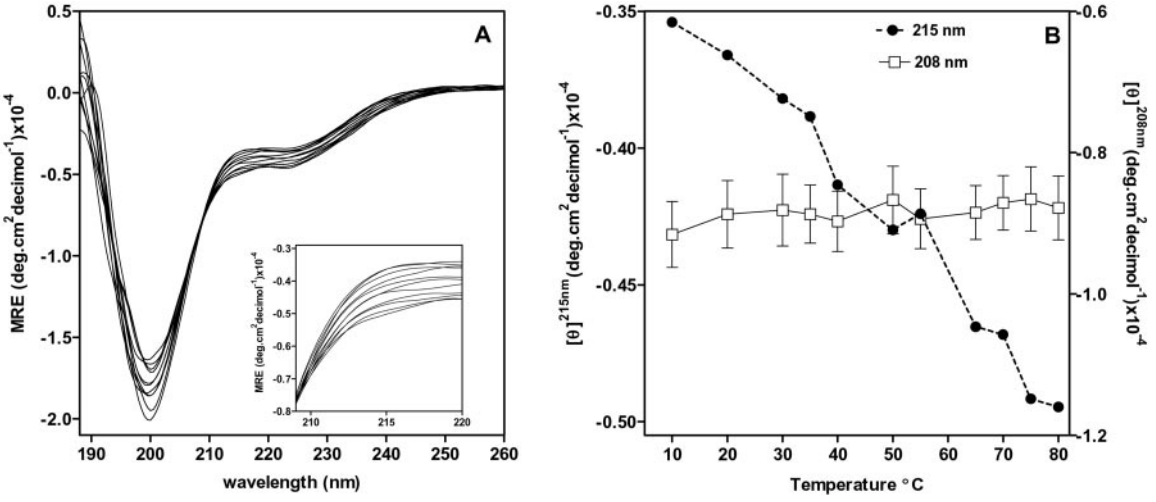

Figure 6. Effect of increasing temperature on secondary structure of rGmD-19. A, CD Spectrum of rGmD-19. The CD spectrum was acquired in a 7.7 μM protein solution in 50 mM buffer Na-phosphate. The spectra shown, reading downward at 220 nm, represent increasing temperatures: 10°C, 20°C, 30°C, 35°C, 40°C, 50°C, 55°C, 60°C, 65°C, 70°C, 75°C, and 80°C. The inset shows the convergence of the ellipticities at 208 nm. B, The temperature-induced changes in the CD spectrum are represented using the ellipticity values obtained at 208 and 215 nm. Symbols are indicated in the figure.

spectrum, increasing the temperature produces a gradual and significant increase in the negative band centered between 210 and 230 nm as well as a concomitant decrease in the intensity of the negative band centered at 200 nm. Besides the changes in these two negative bands, it is also evident that at approximately 208 nm and −7,900 degree cm^2 dmol^{-1}, there is an isodichroic point. This point is maintained in the temperature range studied, 10°C to 80°C, suggesting the presence of an equilibrium between two conformational states (Fig. 6B). This observation is consistent with the isobestic points observed by UV absorption and provides further support to the existence of a two-state equilibrium.

The decrease in intensity of the 200-nm band, and the increase in intensity of the band near 220 nm (Figs. 6A and B), which are characteristic of an increase in α-helical content, led us to consider the possibility that increased temperature-induced folding of the protein rather than unfolding. However, the fact that the increase in temperature was accompanied by an increase in the hydration of the protein, as inferred from the UV absorption study, indicated that this possibility was not likely. In fact, the changes observed by UV absorption spectroscopy are indicative of an unfolding process. As indicated below these changes were due, in fact, to an unfolding process.

Evidence of an Extended Helix Conformation

The difference spectrum (Δ10°C–75°C) of rGmD-19 is shown in Figure 7. This difference spectrum is characterized by an intense negative CD band centered at 200 nm, and a positive CD band above 200 nm. The positive band indicates that the contribution of α-helical structures to the CD spectrum is very low. Otherwise, we should expect a decrease in α-helical content as the temperature increases, and a negative CD band above 200 nm in the difference spectrum. It is interesting that these two features observed in the difference spectrum are present in the CD spectra of peptides rich in poly(Pro) II-like (PII) structures (Park et al., 1997). This similarity suggested that the spectral changes observed in rGmD-19 could be because of a temperature-induced extended helix/random coil transition. The difference spectrum shows a maximum at 215 nm, which is coincident with the maximum observed in peptides adopting PII-like conformations. Additional support

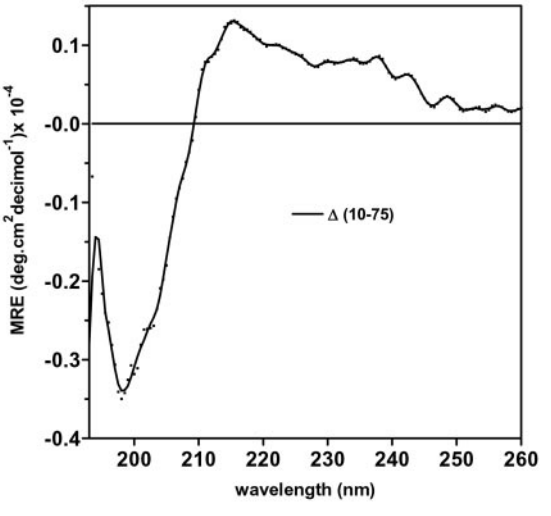

Figure 7. CD difference spectrum. The CD component that disappears upon heating is illustrated in the graph by the CD difference spectrum between (CD at 10°C − CD at 75°C).

for this possibility arises from the coordinates of the isodichroic point observed in Figure 6A (208 nm and -7, 900 deg cm^2 dmol^{-1}). These coordinates are coincident with the coordinates observed in model peptides that undergo PII/random coil transitions (Woody, 1992).

Park et al. (1997) have investigated a series of host/guest peptides and suggested limiting values of ellipticity for the PII and the unordered structures of $+9{,}580$ and $-5{,}560$ deg cm^2 dmol^{-1} at 222 nm. Using these suggested values, we can estimate the fractions of unfolded and PII conformations in rGmD-19 at different temperatures. To perform this calculation, we also assumed that the α-helical content of the protein was nearly zero at high and low temperatures. This assumption appears to be well supported by the maintenance of the isodichroic point along the entire temperature range studied. If this transition would involve three states, unordered, α-helical, and PII, one should not expect the maintenance of the isodichroic point. Using these limiting values, and the ellipticity of rGmD-19 at pH 7.0 and 10°C, we inferred that rGmD-19 contains a minimum of 14% of its residues in an extended-helix conformation. This fraction would be reduced to 6% at 80°C. This estimation represents a minimum estimate because the presence of a small amount α-helical structure would decrease significantly the estimation of the PII-like structure.

DISCUSSION

Previous in vivo functional studies of a group 1 LEA protein from wheat have shown that this protein can ameliorate the detrimental effects of ionic or osmotic stress in yeast cells (Swire-Clark and Marcotte, 1999). Despite these observations, the mechanisms by which this protein acts to afford such protection remain largely unknown. Predicted structural features of group 1 LEA proteins and their highly hydrophilic nature have led researchers to suggest that this group of highly conserved proteins protect cells or tissues from damage by maintaining or replacing water at the interaction interface with other macromolecules (McCubbin et al., 1985; Dure et al., 1989; Cuming, 1999). However, detailed in vitro biochemical, physicochemical, and structural analyses are needed to gain a clearer picture of how group 1 LEA proteins function. Toward this end, we have purified large amounts of soybean group 1 LEA protein from *E. coli* to initiate detailed structural analyses. We first used DSC scans to determine if rGmD-19 showed a cooperative unfolding transition characterized by a heat absorption band. In contrast to BSA, rGmD-19 showed no heat absorption peaks in DSC scans from 10°C to 100°C. This result was independent of whether the protein was purified by heat denaturation or $(NH_4)_2SO_4$ precipitation (Fig. 2B). These DSC results of rGmD-19 are entirely consistent with the secondary structure analysis of CD data, which showed that the protein assumes a largely unordered structure in solution. This observation is also consistent with previous hydrodynamic and CD or NMR spectroscopy studies, which reported that group 1 LEA proteins possess little or no apparent defined secondary structures (McCubbin et al., 1985; Eom et al., 1996). One-dimensional NMR analysis of a group 1 protein (EMB-1) from carrot indicated that its polypeptide backbone was extremely flexible on a sub-nanosecond time scale (Eom et al., 1996). Attempts to induce structure in the EMB-1 protein by approximating dehydration conditions with TFE and ethanol failed to demonstrate any identifiable structure.

The major finding of this study is that we demonstrated that rGmD-19 actually undergoes a temperature-induced structural transition. Moreover, we show that low temperatures favor the adoption of a PII structure. PII helices are secondary structural elements important for many structural proteins, in unfolded proteins, and in protein-protein interaction domains (Creamer, 1998; Stapley and Creamer, 1999). Most PII helices are only five to 12 amino acids in length, and their formation is favored by Pro residues; however, Gln and positively charges amino acids, which compose 30% of GmD-19 amino acids, also favor their formation. Our study also shows that the temperature-induced spectroscopic changes observed in rGmD-19 are because of the equilibrium between an extended-helix conformation, PII, and a random coil or unordered conformation. Similarly, Lisse et al. (1996) reported that a dehydrin-like LEA protein (Dsp16) underwent temperature- and denaturant-dependent order-disorder structural transitions. However, PII structures were not identified in this report. Furthermore, one-dimensional NMR studies suggested the presence of interactions within the polypeptide chain that might stabilize or slow the rapid equilibrium between conformational states, but no PII structures were elucidated (Lisse et al., 1996). Our preliminary results suggest that a related group 2 LEA protein from soybean (DHN1) undergoes structural transitions similar to both rGmD-19 and Dsp16 (J. Soulages, unpublished data). Thus, the adoption of PII structures might be a common property of group 1 and 2 LEA proteins. To our knowledge, experimental evidence supporting the presence of PII structures in any LEA proteins has not been previously reported.

Large regions of random coil conformation are considered to promote the efficient interaction of group 1 LEA proteins with water and aid in their ability to prevent cellular water loss (McCubbin et al., 1985; Eom et al., 1996; Cuming, 1999). The high degree of random coil formation in rGmD-19 structure suggests it can fulfill such a role. In addition, PII helices are highly solvent exposed and are expected to contribute to solvent interactions. Because of the distri-

bution of highly polar amino acid residues, rGmD-19 may also be able to serve as a partial replacement for water in desiccated cell (Crowe et al., 1992; Cuming, 1999). Even though the biochemical roles of PII structures have not been firmly established, the fact that decreasing the temperature induces an increase in the structural organization of the protein without compromising the interaction of the protein with the solvent constitutes an indication about a potential role of the PII structure and of GmD-19 in protection against freezing, desiccation, or osmotic stress damage. Thus, although the content of PII structure increases significantly as the temperature decreases, there is no increase in the apparent content of α-helical or β-structures, which would decrease the number of potential interactions between the solvent and the protein backbone. Compared with a random coil structure, the α-helical or β-sheet structures represent a dramatic decrease in the number of H-bond interactions between the solvent and the polypeptide backbone as well as a decrease in the exposed surface area of hydrophobic and hydrophilic residues. However, the extended helical conformation of the PII structure retains a high degree of exposed surface area. It has been estimated that residues within PII helices expose between 50% and 60% more surface area than do average residues (Adzhubei and Sternberg, 1993).

PII structures are also found in certain glycosylated polar fish antifreeze proteins (Bush et al., 1984; Lane et al., 1998, 2000). Antifreeze glycoproteins (AFGP) are responsible for non-colligative freezing point depression, inhibition of ice nucleation and crystal growth in aqueous solutions, and differentially effect membrane stability during freezing (Yeh and Feeney, 1996; Davies and Sykes, 1997; Tomczak et al., 2001). AFGP8 has a slight cryoprotective effect against freezing-induced membrane leakage. Similar to group 1 LEA proteins, AFGP8 has no long-range ordered structure and has significant flexibility (Lane et al., 1998, 2000). One possible mechanism for AFGP action is to promote cooperative hydrogen bonding over its length sufficient for disruption of ice crystal growth (Yeh and Feeney, 1996). Unlike fish AFGPs, our mass spectrometry measurements of rGmD-19 demonstrated that it lacks glycosylation. Furthermore, computer-assisted motif searches for post-translational modification motifs confirmed that rGmD-19 lacks consensus glycosylation motifs. This is important because the antifreeze property of the glycoproteins from arctic fish is most often attributed to the water-carbohydrate interactions via hydrogen bonding, rather than to water-peptide interactions. However, despite the absence of glycosylation, we suggest that in a hydrophilic protein that is rich in PII structure at low temperatures, the peptide backbone and side chains could act as an efficient protectant against dehydration, osmotic, and/or freezing damage. Deeper insights into the mechanism of action of group 1 LEA proteins should come from two-dimensional or multidimensional NMR studies leading to a solution structure. Such information would be useful in the development of modeling algorithms for predicting PII structures (Creamer, 1998; Siermala et al., 2000). This information could also be used to develop a molecular model for the interaction between the amino acid side chains and backbone with water in the future.

Many unordered proteins or domains can adopt a well-defined structure upon binding to target molecules. Recent observations by Ismail et al. (1999) showed that a group 2 LEA (dehydrin) protein from cowpea can adopt increased α-helical content in the presence of SDS, suggesting a possible role in protein or membrane stabilization for this protein. Our study showed that this is also the case with rGmD19. However, the physiological relevance of the limited folding promoted by SDS is unclear. We failed to detect any interaction between GmD19 and unilamellar liposomes made of the most abundant phospholipid found in any cell. This observation raises additional doubts regarding the potential for group 1 LEA proteins to engage in membrane protection via direct interaction with membrane lipids. Other researchers have proposed that the cryoprotective role of LEA-related COR proteins (e.g. COR15a) arises from its interaction with membrane lipids (Steponkus et al., 1998). This interaction, which alters the intrinsic curvature of the lipid monolayer of the inner chloroplast envelope, is mediated by the COR15am polypeptide, which is composed largely of amphipathic α-helical regions. In contrast, our results showed that the group 1 LEA protein assumes a largely random coil in solution and is, therefore, much less likely than group 2 LEA or COR15a proteins to interact with membranes. Although additional studies should be conducted to determine if group 1 LEA proteins can adopt certain structures upon interaction with other macromolecules, the evidence provided here is inconsistent with such interactions being important for their in vivo function.

In conclusion, we suggest that rGmD-19 can interact efficiently with water because of its hydrophilicity and the adoption of an extended helical conformation, at very low temperatures, in combination with unordered random coil structures. Future in vitro and in vivo biochemical and physicochemical analyses, including examination of the hydration properties of purified, recombinant rGmD-19, are under way and should provide important clues about the functional roles that group 1 LEA proteins play in combating macromolecular destabilization because of dehydration-related stresses.

MATERIALS AND METHODS

Cloning and Expression of Soybean (*Glycine max*) Group 1 LEA

The soybean group 1 (GmD-19) LEA cDNA coding region (321 bp; accession nos. U66317 and AAB68027; Burns

et al., 1997) was amplified using ULTma DNA polymerase (Promega, Madison, WI) and gene-specific primers containing *Nco*I (5′-CATGCC*ATG*GCATCTCGTCAAAAC-3′) or *Eco*RI (5′-CGGATTCCTCACTTATCC TGGTCTTC-3′) restriction sites. The amplified fragment was digested with *Nco*I/*Eco*RI and ligated into the *Nco*I/*Eco*RI sites of the pET30a *Escherichia coli* expression vector (Novagen, Madison, WI). His and S-tag sequences (157 bp) were then removed from the cloning vector by inverse PCR using *Pfu* polymerase (Life Technologies, Rockville, MD) and outward-facing primers (5′-GGCGCGCCCTCCTTCTTAAAGTTAA-3′ and 5′-GGCGCGCCATGGCATCTCGTCAAA-3′) containing AscII restriction sites. The amplification product was digested with AscII and religated with T4 ligase. The integrity of the cloned insert was confirmed by automated DNA sequencing. The resulting pET30a::D-19 was introduced into *E. coli* BL21 (DE3) plysS cells and grown in 2× yeast tryptone medium under kanamycin selection (50 μg mL^{-1}) at 37°C with vigorous agitation (300 rpm). Recombinant GmD-19 protein expression was induced by adding IPTG to a final concentration of 0.1 mM when cells reached an OD_{600} of 0.8. Cells were harvested when the culture reached an OD_{600} of 1.5. Expression of the recombinant protein (rGmD-19) was confirmed by 15% (w/v) SDS-PAGE.

Purification of rGmD-19 from *E. coli*

Bacterial cells were harvested by centrifugation at 5,000*g* and resuspended in B-PER Bacterial Extraction buffer (Pierce, Rockford, IL) in the presence of Complete Mini protease inhibitor cocktail (Boehringer Mannheim, Indianapolis). The soluble cell lysate extract was sonicated to reduce viscosity, heat denatured in boiling water for 10 min., and clarified by centrifugation at 26,500*g* for 20 min. The clarified supernatant was concentrated using a Centricon Plus-80, MWCO 5000 polycarbonate centrifugal filter (Amicon, Beverly, MA) and dialyzed overnight against 10 mM Tris-HCl, pH 7.5, using dialysis membrane tubing (SnakeSkin MWCO 3000, Pierce). The dialyzed extracts were subjected to preparative IEF in the presence of ampholytes (2% [v/v], pH range 5–7; Bio-Rad Laboratories, Hercules, CA) using a Rotofor Cell (Bio-Rad Laboratories) at 15-W constant power for 4 h. Fractions were surveyed by 12% (w/v) SDS-PAGE and fractions containing rGmD-19 protein were pooled and stored at −20°C.

Further purification of the pooled IEF fractions was conducted using anionic exchange column chromatography on a High-Q column (Bio-Rad Laboratories) in 10 mM Tris-HCl, pH 7.5, eluted by a 0 to 250 mM NaCl gradient at 1 mL min^{-1} flow rate. Collected fractions (2 mL) were analyzed by SDS-PAGE, pooled, and stored as the final purified proteins. Alternatively, rGmD-19 was purified without heat denaturation by 60% (w/v) ammonium sulfate precipitation. Clarified supernatant was recovered and desalted using a Centricon Plus-80, MWCO 5000 polycarbonate centrifugal filter (Amicon) and dialyzed before preparative IEF and anion-exchange column chromatography. Cation exchange column chromatography using a High-S column (Bio-Rad Laboratories) equilibrated with 10 mM Na-acetate, pH 4.8, was used as a final purification step. rGmD-19 was eluted using a 0 to 500 mM NaCl gradient at 1 mL min^{-1} flow rate and fractions containing the purified protein were pooled and desalted by dialysis as described above.

Mass Spectrometry

Molecular mass determination of rGmD-19 was performed by matrix-assisted laser-desorption ionization time of flight MS (Proflex, Bruker, Karlsruhe, Germany) using sinapinic acid as a matrix. Samples were prepared using a $ZipTip_{C18}$ reverse phase column (Millipore, Bedford, MA) following manufacturer's instructions. After desalting, samples were dissolved in 50% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid and then dried by gentle heating before MS analysis. Cytochrome C (molecular mass 12,384 D) was used as a calibration standard.

Differential Scanning Calorimetry (DSC) and Thermal Analysis

Three milligrams of lyophilized protein powder was weighed on a DSC volatile samples pan and hydrated under 80% (v/v) relative humidity controlled by saturated KNO_3 at room temperature. Thermal events were measured from 0°C to 100°C at a rate of 10°C min^{-1} using a Differential Scanning Calorimeter DSC-4 and DSC-7 (Perkin-Elmer, Norwalk, CT) calibrated for temperature using methylene chloride (−95°C) and indium (156°C) standards and for energy with indium (28.54 Jg^{-1}) as previously described (Leprince and Vertucci, 1995). Helium gas was used for purging at a rate of 20 mL min^{-1}. To standardize the dry mass of each sample, heat flow in every DSC scan was divided by the sample dry weight.

UV Absorption Spectroscopy

UV absorption spectra were recorded with an HP 8453 diode array spectrophotometer (Hewlett-Packard, St. Paul). The concentration of rGmD-19 was calculated from the absorbance of the samples at 280 nm in the presence of 6 M guanidinium-HCl (ϵ_{Tyr} = 1, 285 cm^{-1} M^{-1}; Pace et al., 1995). Second derivatives were calculated by the Savitzky-Golay differentiation technique using a filtering length of 9. The temperature dependence of the difference between the second derivatives at 283 and 279 nm was determined in 50 m[scap]m phosphate buffer solutions. The sample temperature was modified and controlled by a Peltier temperature-controlled cell holder.

CD Analysis

CD spectra were acquired with a CD spectropolarimeter (model J715, Jasco, Easton, MD) using a 0.1-cm path-length cell over the 184- to 260-nm range. The temperature was controlled by a circulating water bath (model RTE 111, Neslab, Newington, NH) and determined directly into the cell using a thermocouple. CD spectra were acquired every

1 nm with 2 s averaging time per point and a 1-nm bandpass. Quadruplicate average spectra were corrected for the blank and smoothed. CD data were expressed as mean residue ellipticity values and analyzed by the method of Sreerama et al. (1999).

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