THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 15, Issue of April 11, pp. 12977–12984, 2003

Printed in U.S.A.

Transition from Natively Unfolded to Folded State Induced by Desiccation in an Anhydrobiotic Nematode Protein*

Received for publication, November 25, 2002, and in revised form, February 3, 2003
Published, JBC Papers in Press, February 4, 2003, DOI 10.1074/jbc.M212007200

Kshamata Goyal‡, Laurence Tisi‡, Amrik Basran‡§, John Browne¶, Ann Burnell¶, Jesús Zurdo‖, and Alan Tunnacliffe‡**

From the ‡Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QT, United Kingdom, the ¶Institute of Bioengineering and Agroecology, Department of Biology, National University of Ireland Maynooth, Maynooth, County Kildare, Ireland, and the ‖Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, United Kingdom

Late embryogenesis abundant (LEA) proteins are associated with desiccation tolerance in resurrection plants and in plant seeds, and the recent discovery of a dehydration-induced Group 3 LEA-like gene in the nematode *Aphelenchus avenae* suggests a similar association in anhydrobiotic animals. Despite their importance, little is known about the structure of Group 3 LEA proteins, although computer modeling and secondary structure algorithms predict a largely α-helical monomer that forms coiled coil oligomers. We have therefore investigated the structure of the nematode protein, AavLEA1, in the first such analysis of a well characterized Group 3 LEA-like protein. Immunoblotting and subunit cross-linking experiments demonstrate limited oligomerization of AavLEA1, but analytical ultracentrifugation and gel filtration show that the vast majority of the protein is monomeric. Moreover, CD, fluorescence emission, and Fourier transform-infrared spectroscopy indicate an unstructured conformation for the nematode protein. Therefore, in solution, no evidence was found to support structure predictions; instead, AavLEA1 seems to be natively unfolded with a high degree of hydration and low compactness. Such proteins can, however, be induced to fold into more rigid structures by partner molecules or by altered physiological conditions. Because AavLEA1 is associated with desiccation stress, its Fourier transform-infrared spectrum in the dehydrated state was examined. A dramatic but reversible increase in α-helix and, possibly, coiled coil formation was observed on drying, indicating that computer predictions of secondary structure may be correct for the solid state. This unusual finding offers the possibility that structural shifts in Group 3 LEA proteins occur on dehydration, perhaps consistent with their role in anhydrobiosis.

Although water is essential for life, a number of organisms can survive desiccation for extended periods by entering into a state of suspended animation. This remarkable ability, called anhydrobiosis, is found across all biological kingdoms, including bacteria, fungi, animals, and plants. Examples among higher eukaryotes include some nematode worms, such as *Aphelenchus avenae*, a soil-dwelling fungivore amenable to laboratory culture, "resurrection" plants like *Craterostigma plantagineum*, and orthodox plant seeds and pollen (1–3). The molecular mechanisms governing anhydrobiosis are not fully characterized, but several hypotheses emphasize a major role for non-reducing disaccharides. Trehalose, in animals, or sucrose, in plants, accumulates to high concentrations in many anhydrobiotic species prior to dehydration (2, 4). *In vitro* these sugars have been shown to preserve enzymes, antibodies, nucleic acids, some viruses, liposomes, and other membrane systems during and after drying (5, 6). Largely on the basis of the *in vitro* data, these sugars are proposed to act as water replacement molecules and as thermodynamic and kinetic stabilizers of biomolecules and membranes (7, 8). However, there is remarkably little evidence from living systems in support of these hypotheses (9). Furthermore, it is increasingly apparent that, if disaccharides do play an important role *in vivo*, they are insufficient to confer anhydrobiosis by themselves; other adaptations are required (9–12). In desiccation-tolerant plants, a number of genes have been identified that are induced by water stress (13, 14), but there is little information on equivalent genes in anhydrobiotic animals. We have therefore begun to characterize the genes involved in the desiccation stress response in *A. avenae* and recently described a dehydration-responsive gene, *Aavlea1*, whose cognate amino acid sequence is related to plant Group 3 late embryogenesis abundant (LEA)[1] proteins (15).

LEA proteins were first identified 20 years ago in cotton and wheat (16–19) and are produced in abundance during seed development, comprising up to 4% of cellular protein (20). Since then, up to five different groups of LEA proteins have been defined on the basis of expression pattern and sequence (21, 22). Precise functions of the LEA proteins have yet to be elucidated, but expression is linked to water stress and the acquisition of desiccation tolerance in orthodox seeds, pollen, and anhydrobiotic plants (13, 14, 22). They have been variously proposed to protect cellular structures from the effects of water loss by action as a hydration buffer, by sequestration of ions, by direct protection of other proteins or membranes, or by renaturation of unfolded proteins, although supporting evidence is limited (21, 22). The Group 3 LEA proteins, comprising sub-

* This work was funded by grants from the Leverhulme Trust, the Isaac Newton Trust, the Royal Irish Academy, and the Royal Society. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ A Science Foundation Ireland Investigator. Present address: Domantis Limited, Granta Park, Cambridge CB1 6GS, UK.

** The AWG Senior Research Fellow of Pembroke College, Cambridge. To whom correspondence should be addressed: Institute of Biotechnology, University of Cambridge, Tennis Court Rd., Cambridge CB2 1QT, UK. Tel.: 44-1223-766549; Fax: 44-1223-334162; E-mail: at10004@biotech.cam.ac.uk.

[1] The abbreviations used are: LEA, late embryogenesis abundant; CD, circular dichroism; R_s, Stokes' radius; FT-IR, Fourier transform-infrared; MOPS, 4-morpholinepropanesulfonic acid.

groups D-7 and D-29, are characterized by a repeating 11-mer amino acid motif whose consensus has been defined for plant proteins as TAE/QAAKE/QKA*X*E for the D-7 family, or more broadly as ΦΦE/Q*X*ΦKE/QKΦ*XE*/D/Q (where Φ represents a hydrophobic residue) for the D-29 family (23, 24). Recent genome sequencing projects have brought to light sequences related to Group 3 LEA proteins in the nematode *Caenorhabditis elegans* and also in the prokaryotes *Haemophilus influenzae* and *Deinococcus radiodurans* (24), indicating that this type of LEA protein at least is not restricted to plant species. The *D. radiodurans* genome also contains sequences related to other LEA proteins (25). The function of non-plant LEA proteins may also relate to water stress; mutation of two *D. radiodurans* genes encoding LEA-like sequences resulted in reduced desiccation tolerance (26), and an LEA-like protein was induced by dehydration in the entomopathogenic nematode *Steinernema feltiae* (27), as was the *Aavlea1* gene in *A. avenae* (15). The *A. avenae* protein sequence, named AavLEA1, is very similar to the plant Group 3 LEA proteins, as shown by data base comparisons, and includes several 11-mer motifs (15). These motifs differ slightly from the plant consensus, *e.g.* the first amino acid is often a positively charged lysine instead of a hydrophobic residue, but they are clearly related. It is therefore expected that AavLEA1 will adopt a conformation similar to that of plant Group 3 LEA proteins.

Structural studies on a number of LEA proteins have been performed to attempt to gain insight into function, but surprisingly little information is available for Group 3 members. No crystal structures have been reported, but secondary structure predictions can be derived from a number of algorithms available as online computer programs. Fig. 1 shows secondary structure predictions from seven such programs together with a "winner takes all" summary for the *A. avenae* Group 3 LEA protein, suggesting that it is largely α-helical throughout its length. Dure (23) used computer modeling to predict that Group 3 LEA proteins adopt amphiphilic α-helices that dimerize in an unusual right-handed coiled coil arrangement, with a periodicity defined by the 11-mer motif. Right-handed coiled coils based on an 11-mer repeat were later found in a surface layer protein from *Staphylothermus marinus* (28), demonstrating that this conformation is found in nature. Larger complexes might also arise (29), because a Group 3 LEA-like wheat protein is predicted by the MultiCoil program (30) to form trimeric coiled coils. Indeed, MultiCoil also predicts a 40% probability of coiled coils in AavLEA1, although these would be, more conventionally, left-handed. Because the structure of AavLEA1 might offer clues to its function, we decided to test the various hypotheses resulting from computer predictions to assess (*a*) whether oligomers are formed, (*b*) whether an α-helical polypeptide structure can be detected, and (*c*) whether drying has any significant effect on structure in a recombinant form of the protein.

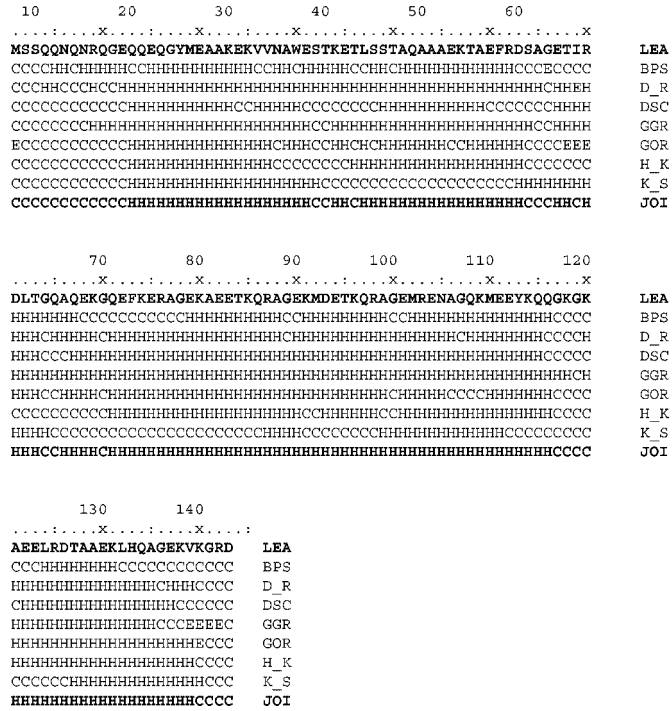

FIG. 1. **Secondary structure predictions for nematode Group 3 LEA protein.** The native LEA-like sequence from *A. avenae*, shown in *bold* at the top of each line of output and denoted LEA, was submitted to the program PELE on the SDSC Biology Workbench (workbench.sdsc.edu). Seven different structure predictions are shown, with the most likely structural feature at each residue indicated by *H* (α-helix), *E* (β-sheet), or *C* (random coil). Programs used are denoted *BPS* (77), *D_R* (78), *DSC* (79), *GGR* (80), *GOR* (81), *H_K* (82), *K_S* (83). The "winner-takes-all" joint prediction is given by JOI and shown in *bold*.

EXPERIMENTAL PROCEDURES

Production of Recombinant Nematode LEA Protein—The *A. avenae* LEA cDNA sequence reported in our earlier study (15) was reamplified by polymerase chain reaction (PCR) using oligonucleotide primers containing engineered *Nde*I (5′-GGAATTCCATATGTCCTCTCAGCAG) and *Bam*HI (5′-CGGGATCCTTAGTCGCGGCCCTT) sites (underlined) and cloned in pCR2.1-TOPO (Invitrogen). The pET15b vector (Novagen) was used to express the protein with an N-terminal His_6 tag after cloning the engineered cDNA sequence at the *Nde*I and *Bam*HI sites; the construct was verified by DNA sequence determination. The pET15b vector encodes the protein sequence MGSSHHHHHHSSGLVPRGSH at the N terminus, additional to the native AavLEA1 sequence shown in Fig. 1. The plasmid was transformed into *Escherichia coli* strain BL21(DE3), and a single bacterial colony was inoculated in 100 ml of Luria Bertani broth (LB) containing 100 μg/ml carbenicillin and grown overnight at 37 °C. 10 ml of this culture was used to inoculate 1 liter of LB plus antibiotic, and at an absorbance (A_{600}) of 0.6, gene expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 12 h at 30 °C. Cells were harvested by centrifugation at 11,000 × *g* for 10 min at 4 °C, and pellets were resuspended in 50 mM MOPS buffer (pH 6.5). The cell suspension was sonicated, and debris was removed by centrifugation at 15,000 × *g* for 10 min at 4 °C. Supernatant was applied to a 5-ml nickel chelation column pre-equilibrated with 50 mM phosphate (pH 8.0), 300 mM NaCl, 10 mM imidazole. The bound protein was eluted batchwise using increasing concentrations of imidazole, as described in the QIAexpressionist™ (Qiagen, Germany). Protein fractions were pooled and dialyzed overnight in 5 mM MOPS buffer (pH 6.5) and applied to a HiLoad Q-Sepharose column (Amersham Biosciences) equilibrated with 20 mM MOPS buffer (pH 6.5). The adsorbed proteins were eluted with a linear gradient from 0 to 0.5 M NaCl using an AKTA FPLC system (Amersham Biosciences). Fractions with AavLEA1 protein eluted as a single peak, and the purity of protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was determined spectrophotometrically using a molar extinction coefficient of 8250 M^{-1} cm^{-1}, calculated using the ProtParam program on the ExPASy server (ca.expasy.org/tools/protparam.html).

Nematode Culture and Protein Extraction—Mass cultures of *A. avenae* were grown at 20–25 °C in the dark in Duran bottles containing wheat grains that had been autoclaved and then sub-cultured with the fungus *Rhizoctonia solani* (31). To allow aeration, holes (~3 cm) had been made in bottle caps and covered with sterile filter paper. After sufficient growth (10–20 days), nematodes were isolated, washed with water, and filtered through 0.2-μm membranes (PALL Life Sciences). Filtered nematode samples were resuspended in protein lysate buffer, 50 mM Tris-HCl, pH 7.5, 75 mM NaCl, 15 mM EGTA, 1 mM dithiothreitol, 0.1% Tween 20, 60 mM β-glycerophosphate, 1 mM NaF, 0.2 mM sodium orthovanadate, 2 mM sodium pyrophosphate, plus a protease inhibitor mixture (Roche Applied Science cat. no. 1836153), and sonicated. Insoluble cellular material was removed by centrifugation for 30 min (18,000 × *g* at 4 °C), leaving a cleared lysate.

SDS-PAGE and Western Blotting—For SDS-PAGE (32), 11% SDS slab gels were run in Bio-Rad mini-Protean 3 electrophoresis cells; gels were stained with Coomassie Blue R-250. Apparent molecular weight

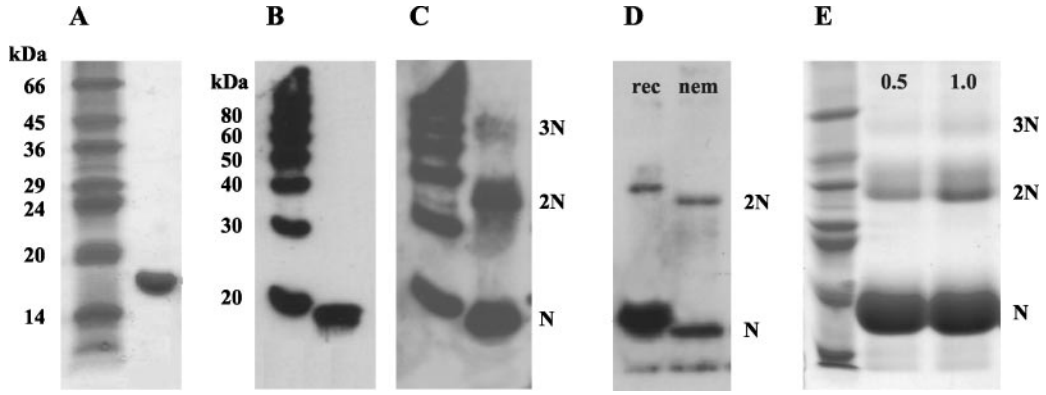

FIG. 2. **Analysis of AavLEA1 oligomerization by gel electrophoresis.** *A*, purified recombinant AavLEA1 after SDS-PAGE and Coomassie Blue staining. Molecular masses of size standards are shown in kDa. *B*, Western blot of AavLEA1 separated by SDS-PAGE and transferred to nitrocellulose. Affinity-purified rabbit polyclonal antiserum, raised against AavLEA1, was used to reveal the protein. Sizes of molecular mass standards are given in kDa. *C*, Western blot experiment, similar to *panel B* but showing monomer (*N*), dimer (*2N*), and oligomeric forms (*3N*). *D*, Western blot comparing recombinant AavLEA1 (*rec*) and native protein (*nem*) from an extract of *A. avenae* and showing monomeric (*N*) and dimeric (*2N*) forms in both. The larger size of the recombinant protein is due to the presence of an N-terminal His tag and associated sequence, which augments the monomer size by ~2 kDa. *E*, SDS-PAGE stained with Coomassie Blue of AavLEA1 after cross-linking with 0.5 or 1.0 mg/ml dimethyl suberimidate as indicated. Monomer (*N*), dimer (*2N*), and trimer (*3N*) are indicated.

was determined relative to molecular weight standards (Sigma). For blotting, proteins were first separated by SDS-PAGE on 11% slab gels and transferred to a nitrocellulose membrane (Trans-Blot transfer medium; Bio-Rad) using a Trans-Blot S.D. electrophoretic transfer cell (Bio-Rad). Immunodetection was performed using affinity-purified polyclonal rabbit antiserum raised against purified AavLEA1 by Harlan Seralab. A donkey anti-rabbit IgG peroxide-linked conjugate (Amersham Biosciences) was used for detection, and bands were visualized with enhanced chemiluminescence (ECL) detection reagent (Amersham Biosciences) and Biomax ML (Kodak) autoradiography film. Molecular weight markers used were MagicMark Western standards (Invitrogen), which comprise recombinant proteins containing an IgG binding site from protein G and are therefore revealed by the donkey anti-rabbit immunoglobulin.

Protein Cross-linking—Reactions based on Davies and Stark (33) and Coggins *et al.* (34) were carried out at room temperature in 0.2 M triethanolamine hydrochloride (pH 8.5) for 3 h with a final concentration of 0.5 mg/ml AavLEA1 and dimethyl suberimidate concentration between 0.5 and 3 mg/ml in a volume of 100 μl. At higher suberimidate concentrations, pH was adjusted with NaOH prior to addition of protein. Proteins were then denatured in SDS sample buffer for 2 h at 37 °C and appropriate amounts run on 11% SDS-slab gels, which were subsequently stained with Coomassie Blue R-250.

Analytical Ultracentrifugation—A Beckman XL-A analytical ultracentrifuge was used with the temperature of the 4-hole rotor maintained at 20 °C and an operating speed of 18,000 rpm (equilibrium runs) or 50,000 rpm (velocity runs). Optical scanning of the cells was performed at a wavelength of 280 nm and the output logged to disk for subsequent analysis. For the equilibrium runs the attainment of the equilibrium state was verified by confirming that successive scans at 1 h intervals were identical other than for random noise. Standard software was used (35): SVEDBERG for velocity analysis, NONLIN for equilibrium analysis, and SEDNTERP and BIOMOLS for estimation of partial specific volume, frictional ratios, and other routine calculations. ProFit (Quantum Soft) was used for curve fitting and graph plotting.

Size Exclusion Chromatography—A Superdex 200 column equilibrated with 50 mM Tris buffer (pH 7.0) containing 100 mM NaCl was used with an FPLC system (Amersham Biosciences) and calibrated with proteins from the LMW and HMW gel filtration calibration kits (Amersham Biosciences). Protein samples (50–500 μg) in appropriate buffers were run on the column, pre-equilibrated in the same buffer, at a flow rate of 0.25 ml/min. The apparent size and Stokes' radius ($R_{s\ gel}$) of the protein was determined relative to the standards using described methods (36).

Far UV Circular Dichroism and Fluorescence Emission Spectroscopy—CD spectra were recorded on a Jasco J-810 spectropolarimeter using a 0.1-cm path length for wavelengths between 190 and 250 nm. Temperature was controlled using a Peltier system, and data were acquired at different temperatures with 0.1 and 2 mg/ml of AavLEA1 in 50 mM phosphate buffer (pH 7.0). The buffer spectrum at each temperature set point was subtracted from the sample spectrum. Fluorescence emission spectra were recorded on a PerkinElmer LS50B luminescence spectrometer, fitted with a temperature controlling device. Data were obtained at different temperatures using an excitation wavelength of 280 nm, slit widths of 2.5 nm, and a scan rate of 60 nm/min. The concentrations of AavLEA1 used were 0.1 and 0.5 mg/ml. Fluorescence intensity and maximum emission wavelength (λ_{max}) were determined from emission spectra acquired in the 310–400 nm range.

Fourier-transform Infrared (FT-IR) Spectroscopy—Infrared spectra were recorded in a Bruker Equinox 55 FT-IR spectrometer equipped with a deuterated lanthanum triglycine sulfate (DLATGS) detector and a KBr beam-splitter and purged with a continuous flow of N_2 gas. Lyophilized protein samples were reconstituted in ^2H_2O at a concentration of 15 mg/ml and incubated at room temperature for about 10 min before measurements. Protein solutions were then passed through 0.22-μm pore syringe filters to remove any undissolved or aggregated material, and the supernatants were transferred to a clean tube. Samples were then placed between a pair of CaF_2 windows separated by a 50-μm Mylar spacer. Dehydrated samples were obtained by placing 50 μl of a 15 mg/ml solution of protein (prepared and filtered as described above) on a CaF_2 window and drying under vacuum for about 30 min. In all cases transmission spectra were collected at room temperature. For each sample 64 interferograms were collected at a spectral resolution of 2 cm^{-1}, using a scanner velocity of 10 kHz. Water vapor subtraction and baseline correction as well as additional spectra processing as indicated below were performed using GRAMS/AI (Thermo Galactic) software. Second derivatives of the Amide I band spectra were produced to determine the localization of the different spectral components. A peak fitting for each individual spectrum was performed using the spectral frequencies obtained by second derivative analysis and trough successive iterations using a variable mixture of Gaussian and Lorentzian bandshapes.

RESULTS

Gel Electrophoresis and Subunit Cross-linking Demonstrate Oligomerization of Nematode LEA Protein—A recombinant form of AavLEA1 with a predicted molecular mass of 18,175 Da was produced in *E. coli* after cloning in the pET15b expression vector. The protein was purified by nickel chelation and ion exchange chromatography and had approximately the expected mass on SDS-PAGE gels (Fig. 2*A*). MALDI-TOF mass spectrometry confirmed the mass as 18,060.1 ± 0.9 Da (data not shown), which indicated that the N-terminal methionine had been cleaved during synthesis but which otherwise agreed with predictions. To facilitate analysis of AavLEA1, a polyclonal antiserum against the purified protein was produced that recognizes it in Western blotting experiments. Intriguingly, although in some experiments AavLEA1 is visualized by the antiserum as a single band (Fig. 2*B*), oligomeric forms are also frequently observed; monomer (*N*) and dimer (*2N*) are clearly seen in Fig. 2*C*. Higher order oligomers, at least of trimer (*3N*),

are also faintly visible. A similar degree of oligomerization also occurs *in vivo* because dimers can be visualized in Western blots of nematode protein extracts; both monomer and dimer of the native form run slightly ahead of the equivalent recombinant species due to the lack of an N-terminal His tag (Fig. 2*D*). Small quantities of dimeric AavLEA1 can also be seen on SDS-PAGE gels simply stained with Coomassie Blue (data not shown). This indicates a tight association between subunits because protein samples were boiled in SDS, strongly denaturing conditions, prior to electrophoresis. To demonstrate the presence of oligomers in solution, cross-linking of purified AavLEA1 using dimethyl suberimidate was performed. Conditions were used that allow cross-links to form between the subunits of protein complexes but not between different complexes. In Fig. 2*E*, three bands are observed after incubation in dimethyl suberimidate, corresponding to monomeric, dimeric, and trimeric forms of the protein. The intensity of the bands representing oligomeric forms is lower than that of the monomer, but because cross-linking does not proceed to completion, this does not indicate the relative proportions of the oligomers observed. Larger forms were not seen, suggesting that tetramers and higher order oligomers were not present at significant levels in the protein sample used.

These experiments confirm the existence of homo-oligomers of AavLEA1 in solution and are consistent with a dimeric or trimeric coiled coil model, similar to those proposed by Dure (23) and NDong *et al.* (29). These models imply that most of the protein should adopt a oligomeric, coiled coil conformation, but the proportion of oligomeric forms is difficult to determine using denaturing gel electrophoresis because protein complexes are likely to be underestimated. Conversely, Western blotting can overemphasize weak bands in relatively long film exposures. Preliminary electrospray ionization mass spectrometry indicated that AavLEA1 was present mostly as the monomer; the presence of dimer and possibly higher order oligomers was detected but in small quantities, suggesting incomplete oligomerization (data not shown). However, although these experiments were performed under conditions designed to minimize subunit separation, it could not be ruled out that oligomeric complexes of AavLEA1 were largely disrupted by the procedure. If AavLEA1 is mainly oligomeric, though, this should be demonstrable unequivocally by analytical ultracentrifugation.

Hydrodynamic Analysis Suggests AavLEA1 Is Mostly Monomeric—Sedimentation velocity experiments were performed for a range of protein concentrations, and an apparently single boundary was observed in all cases, consistent with one predominant species being present. The plot of the inverse of sedimentation coefficient, s, against corrected concentration, c, was fitted using a linear regression routine (Fig. 3), and a value of $s^0_{20,w}$ = 1.20 S was obtained after extrapolation to infinite dilution. The relatively low value for the sedimentation coefficient of AavLEA1 is indicative of unusually high drag forces for a molecule of this molecular mass. The frictional ratio (f/f_0), derived from $s^0_{20,w}$, is given as 2.28, indicating either a highly extended structure (typical globular proteins have a frictional ratio close to 1.1 (36)) or an unstructured, swollen protein with a high degree of hydration, or some combination of both. The negative regression of s with protein concentration (Fig. 3) is most consistent with a species that is either all monomer or mostly monomer in rapid equilibrium with a proportion of dimer. Using the concentration dependence of the s value, deductions on the degree of dimerization can be made from sedimentation velocity data (37, 38). This analysis suggests, for the two structural forms postulated, that if AavLEA1 is an extended rod then very little dimer is needed to account for the data of Fig. 3, whereas for an unstructured model a significant fraction of dimer (7% dimer at a protein concentration of 2.5 mg/ml) is present. Because gel electrophoresis experiments demonstrate that monomeric and oligomeric forms co-exist, the latter model is more likely. Other possibilities, including the smallest species being a dimer, can effectively be excluded. An unequivocal value for the molecular mass of the nematode LEA protein in solution, and hence the degree of oligomerization, can be obtained from sedimentation equilibrium experiments. These were performed at a range of protein concentrations and the data fit to single-species or two-species models using the NONLIN program. Again, the best fit was observed for a single, *i.e.* monomeric species but with a small quantity of dimer present (data not shown). Higher order oligomers were not excluded by this analysis, but they must be present at a level of ≤2% total protein.

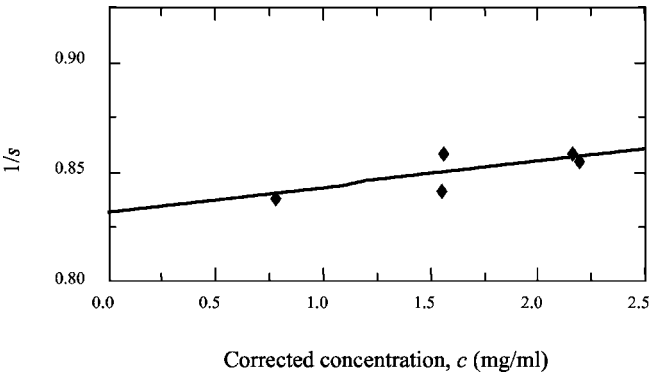

FIG. 3. **Sedimentation velocity analysis of AavLEA1 protein.** Sedimentation coefficient, $s^c_{20,w}$, was determined for five protein concentrations, c. The line fitting the inverse of s to c was extrapolated to give the sedimentation coefficient at infinite dilution, $s^0_{20,w}$; c was corrected for radial dilution.

The Stokes' radius ($R_{s\ sed}$) of AavLEA1 was calculated from sedimentation analysis to be 3.91 nm. This is larger than expected if AavLEA1 were globular in structure but is consistent with it being an extended or highly swollen protein. Gel filtration experiments gave a similar result, with the LEA-like protein running very close to bovine serum albumin (66 kDa; R_s = 3.55 nm) on a Superdex 200 column (Fig. 4); the relatively low elution volume of AavLEA1 was maintained under a variety of running conditions, at pH 6.5–9.5, in water or 0.5 M NaCl, and in 0.5 M sucrose or 1 M trehalose. The gel filtration column was calibrated using globular proteins with known R_s as standards, allowing $R_{s\ \mathrm{gel}}$ for AavLEA1 to be estimated at 3.38 nm. This is somewhat lower than the value obtained from ultracentrifugation, possibly because of matrix interaction effects, but is still far in excess of the expected R_s value for a globular protein of similar mass and supports the model of AavLEA1 having low compactness. In summary, gel electrophoresis and hydrodynamic analyses suggest that the nematode Group 3 LEA-like protein exists in solution mostly in the monomeric form but in rapid equilibrium with a small proportion of dimer. Some trimer is also observed.

Spectroscopic Analysis Shows That AavLEA1 Is Natively Unfolded—Far UV CD spectroscopy yields information on the α-helical, β-sheet, and unstructured random coil content of proteins. If AavLEA1 is largely α-helical as predicted (Fig. 1), CD spectra should have two characteristic minima near 208 and 222 nm. In contrast, a disordered structure would give a single minimum at about 200 nm and low ellipticity at 222 nm. Proteins with elements of both structural forms, and indeed of β-sheet with its typical minimum at 215 nm, would give spectra combining these features. However, significant defined secondary structure was not detected for AavLEA1; signatures of

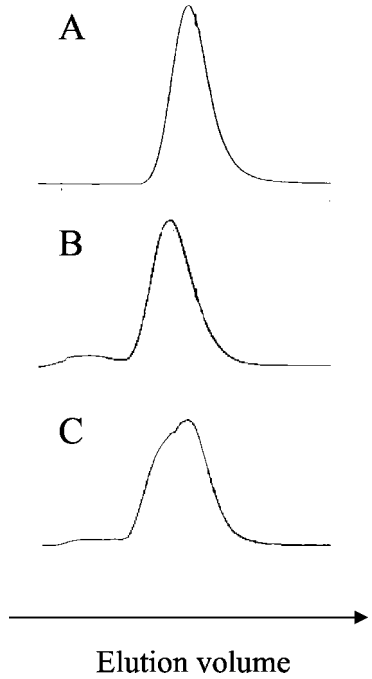

FIG. 4. **Gel filtration chromatography of AavLEA1.** *A*, purified AavLEA1 migrating on a calibrated Superdex 200 column, where protein is detected spectrophotometrically at 280 nm, has an elution volume of 14.25 ml in the experiment shown. *B*, bovine serum albumin elutes slightly ahead of AavLEA1 in a volume of 13.8 ml under the same conditions. *C*, a mixture of both proteins, giving an absorbance profile that is the sum of those of the separate proteins, is shown.

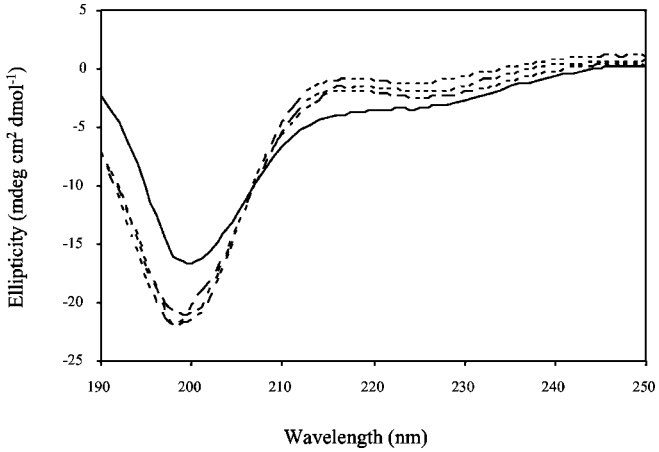

FIG. 5. **Far UV CD spectroscopy of AavLEA1.** The *solid line* represents the CD spectrum of AavLEA1 at 75 °C. The three *dotted lines* are very similar spectra at 4 and 25 °C and at 4 °C again after cooling from 75 °C.

α-helix and β-sheet were absent, with the spectrum showing instead that the protein is most likely unstructured through a temperature range of 4 to 75 °C (Fig. 5). A slight increase in ellipticity at 200 nm on shifting to higher temperature, together with a decrease at 220 nm, could be interpreted as an increase in α-helical content. On cooling to 4 °C, the spectrum reverts to that seen previously at this temperature, showing that any structural changes induced by heating are fully reversible. This is the opposite to what is observed in globular proteins, where heating causes unfolding, but the effect has been noted previously with unstructured proteins and could reflect increased strength of hydrophobic interactions at elevated temperature (39). CD spectra did not change appreciably throughout a concentration range of 0.1 to 2 mg/ml, suggesting

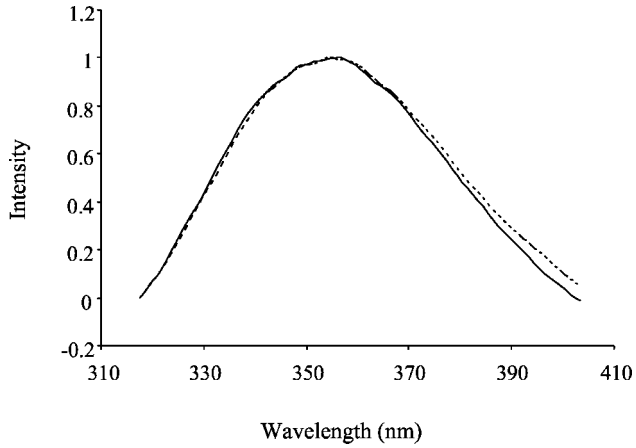

FIG. 6. **Emission fluorescence spectroscopy of AavLEA1.** The *dotted line* represents the fluorescence emission spectrum of the single tryptophan residue in AavLEA1 at 4 °C. The *solid line* shows fluorescence at 65 °C. Both curves have a λ_{max} at 355 nm, indicative of a fully solvated tryptophan side chain.

that any secondary structure of AavLEA1 is not influenced by protein concentration (data not shown).

To confirm the largely unstructured nature of AavLEA1, fluorescence emission spectroscopy was performed. Tryptophan residues in proteins exhibit different fluorescence maxima (λ_{max}) dependent on the hydrophobicity of their environment, which commonly reflects the degree of solvent exposure of the tryptophan side chain. For fully solvated tryptophans, a λ_{max} at 355 nm is observed; tryptophans that are buried within the protein have a λ_{max} as low as 310 nm. The single tryptophan residue in AavLEA1, at position 49 of 162 (allowing for the His tag, position 30 of 143 in the native nematode sequence; Fig. 1), gives rise to a λ_{max} at 4 °C of 355 nm, which is indicative of a solvent-exposed side chain (Fig. 6). The emission profile does not change appreciably on heating the sample to 65 °C; on cooling, the spectrum is indistinguishable from the original (data not shown). This implies that any increased folding with temperature, as suggested by CD analysis, does not mask the tryptophan at position 49. Therefore, both far UV CD and fluorescence emission experiments are consistent with a natively unfolded structure for the nematode Group 3 LEA protein. Preliminary proton nuclear magnetic resonance studies (data not shown) also suggested a lack of stable structural elements within AavLEA1.

A large proportion of the proteome of many species is predicted to include partially or wholly unfolded proteins; in the yeast *Saccharomyces cerevisiae*, for example, 30% of proteins are predicted to be partially, and 6% to be wholly, disordered (40). A simple predictor of whether a given protein falls into this group is obtained from calculation of mean normalized hydrophobicity ($<H>$) and mean net charge at neutral pH ($<R>$). When these parameters were plotted by Uversky and colleagues (41) for a set of 275 folded and 91 unfolded proteins, it was discovered that the large majority of natively folded and unfolded proteins fall either side of a boundary line empirically defined by the equation $<H>_b = (<R> + 1.151)/2.785$. Thus, for a given value of $<R>$, if $<H>$ is less than $<H>_b$, the protein is predicted to be unfolded. For AavLEA1, $<R> = 0.014$, giving $<H>_b = 0.418$. Mean hydrophobicity was calculated to be 0.340, locating the nematode protein within natively unfolded space on the Uversky plot. Uversky (39) further distinguished between natively unfolded proteins containing some degree of secondary structure, so-called pre-molten globules, and fully unfolded random coils; these two categories of unfolded protein fall on different lines of a plot of $\log(R_s)$ *versus*

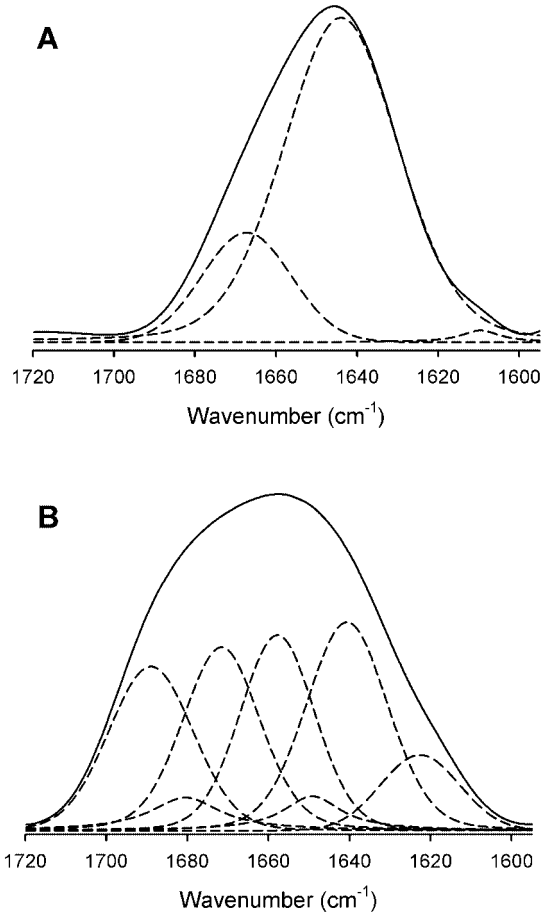

FIG. 7. **FT-IR spectroscopy of AavLEA1.** Decomposition of the amide I band spectra corresponding to soluble (*A*) and dried (*B*) protein. Second derivatives of the respective spectra were used to obtain the different band positions used in the curve fitting. The spectrum of rehydrated AavLEA1 overlaps completely with that of the protein in solution, as shown in *panel A* (data not shown).

log(M_r). AavLEA1 has values of log(R_s) = 1.58 (for R_s in Ångstroms, $R_{s\ sed}$ = 39.1 Å) and log(M_r) = 4.26 and locates on the line occupied by coil-like proteins rather than pre-molten globules, agreeing with experimental data.

AavLEA1 Acquires Secondary Structure on Drying—FT-IR spectra collected from soluble AavLEA1 samples in ^2H_2O show an amide I pattern indicative of a mainly disordered polypeptide (Fig. 7*A*), in agreement with CD data. Second derivative analysis of the amide I band showed two main peaks at 1644 cm^{-1} and 1667 cm^{-1}, characteristic of random coil structures (42). A minor component at 1610 cm^{-1} could be due to side chains of amino acids being highly represented in the sequence of the protein, such as glutamine (43) which accounts for almost 10% of the total amino acids of AavLEA1. Natively unfolded proteins are known to become more structured when complexed with partner molecules or exposed to altered physiological conditions (39–41); therefore we examined the effect of dehydration, the natural stress vector experienced by anhydrobiotes, on AavLEA1 structure.

Interestingly, when the protein is dried under vacuum, the amide I spectrum of AavLEA1 experiences major changes in both its overall shape and in the distribution of spectral components, suggesting that important conformational changes have occurred. The overall amide I spectrum of this form of the protein exhibits a maximum at 1657 cm^{-1}, which together with the existence of an important component at 1658 cm^{-1} indicates the acquisition of α-helical structure by the protein when dehydrated (Fig. 7*B*; Table I). Intriguingly, components detected at 1641, 1672, and 1689 cm^{-1} (and perhaps also that at 1623 cm^{-1}) are consistent with the arrangement of α−helical structures as coiled coils (44). According to the existing literature, the absence of components around 1630 cm^{-1} together with the location of the spectral maximum of the overall amide I band (1657 cm^{-1}) and its spectral weight (1659 cm^{-1}) suggest, however, the absence of a "superhelical pitch," *i.e.* any superhelical assembly is expected to originate from the lateral association of helices in a straight manner without any twisting, as suggested previously for coiled coil models (44). As mentioned, the band at 1623 cm^{-1} could be one of those attributed to the superhelical arrangement of dehydrated AavLEA1. Alternatively, together with the component at 1680 cm^{-1}, it might also account for a residual amount of inter-molecular β-sheet structure, perhaps because of aggregation of a small fraction of the protein during dehydration. Finally, the bands at 1649 cm^{-1} and 1680 cm^{-1} could account for residual random conformations in the sample. The FT-IR spectrum of rehydrated AavLEA1 is completely superimposable with that of the original protein in solution (Fig. 7*A*), indicating that the structural rearrangements imposed by desiccation are fully reversible.

TABLE I
FT-IR amide I components of AavLEA1 samples and their corresponding areas

Solution[a]		Dehydrated[at]	
Wavenumber	Area	Wavenumber	Area
cm^{-1}	%	cm^{-1}	%
1667	19.9	1689	19.1
1644	78.9	1680	4.2
1610	1.2	1672	20.3
		1658	20.5
		1649	3.7
		1641	24.1
		1623	8.1

[a] Peak fitting was performed as indicated under "Experimental Procedures."

DISCUSSION

The gene *Aavlea1* encodes a protein in the anhydrobiotic nematode *A. avenae* with marked similarity to the Group 3 LEA proteins found in many maturing plant seeds. The precise function of LEA proteins in plants has not been defined, although their expression is associated closely with acquisition of desiccation tolerance (45). Tomato, wheat, and barley LEA proteins have been shown to confer increased resistance to osmotic and freeze stress when introduced into yeast (46–49), and a barley LEA protein improved tolerance to water deficit in transgenic rice (50) and wheat (51). *In vitro*, a Group 3 LEA protein from the alga *Chlorella* decreased freeze damage of the enzyme lactate dehydrogenase (52), and our unpublished results suggest that the nematode protein behaves similarly. Therefore, LEA proteins seem able to offer partial protection to biological structures at the molecular and cellular level against the effects of water loss, but how this function is linked, if at all, to structure is unclear. This report provides the first structural information, in both hydrated and dry states, on a fully characterized Group 3 LEA-like protein from any species, including plants.

The literature (23, 29) and several computer programs predict that Group 3 LEA proteins adopt a largely α-helical structure, possibly as a coiled coil homodimer or higher order species. However, gel electrophoresis and hydrodynamic experiments on AavLEA1 indicate that although limited oligomerization occurs the majority species is the monomer. The protein is apparently wholly unfolded in solution with little evidence obtained by far UV CD, fluorescence emission, and

FT-IR spectroscopy for any defined conformation. Failure to achieve crystallization of AavLEA1[2] is consistent with this. Calculations made from the hydrodynamic data about the degree of hydration of the protein show that an ideal, compact protein of M_r 18,060 would have a value of R_s = 1.72 nm, but the observed value for AavLEA1 ($R_{s\ sed}$) is 3.91 nm. The ratio of the molecular volumes calculated from these radii gives $V_s/\bar{v}$, the ratio of partial specific hydrated volume of the protein to partial specific volume of the protein alone. For AavLEA1, $V_s/\bar{v}$ = 11.8 and, because $\bar{v}$ is estimated to be 0.705 ml/g using the SEDNTERP application, this gives V_s = 8.32 ml/g. If we assume that volume not occupied by protein is occupied by water with the same density as bulk phase, then the degree of hydration of AavLEA1 is V_s minus $\bar{v}$ = 7.6 ml/g, and the ratio of volume of water per unit volume of protein is 7.6/0.705 or 10.8. This indicates a high level of associated water, because a typical globular protein would have a $V_s/\bar{v}$ value of around 1.5, giving a degree of hydration of 0.365 ml/g. Therefore, in this model AavLEA1 has ~20-fold more associated water than a typical globular protein of equivalent size, which is consistent with the recognized hydrophilicity of LEA proteins (21, 22). On the basis of these observations, it appears that structural predictions are incorrect, at least for the purified, solution state of AavLEA1, and that instead it adopts a conformation termed natively unfolded (39, 41) or intrinsically disordered (40). One concern with the use of a recombinant molecule is the possible interference of the N-terminal His tag in protein folding. However, we have obtained almost identical gel electrophoresis and hydrodynamic data with a version of AavLEA1 where the N-terminal His tag has been removed by thrombin cleavage or by partial proteolysis; in addition, a different recombinant form of the protein, where a His tag is positioned at the C terminus, also exhibits an unfolded conformation (data not shown). Although their sequence is unrelated to that of Group 3 LEA proteins, the other main categories of LEA protein (*i.e.* Group 1 and Group 2, the latter frequently referred to as dehydrins) also seem to be wholly or partially natively unfolded (53–60), suggesting that this is a general characteristic of LEA proteins.

An intriguing feature of natively unfolded proteins and one that might throw light on the function of LEA proteins is that although such proteins lack structure they do not lack function. The vast majority of proteins or protein domains in this category have recognized activities, and almost all are known to bind specific ligands or target molecules (40, 41). For example, the yeast nucleoporin Nup2p, which forms part of the nuclear pore complex, interacts specifically with importin $\alpha\beta$ (Kap60p/Kap95p) and is required for nuclear import of importin-$\alpha\beta$-dependent cargoes, is natively unfolded (61). Substrates for disordered proteins include other proteins but also DNA or RNA, nucleotides, and cations. Crucially, binding to partner molecules can induce a switch between unfolded and folded states, as with heme-induced folding of apocytochrome *c* (62) and the CFP-10 major T-cell antigen of *Mycobacterium tuberculosis*, which is disordered in solution but becomes structured on binding to its cognate molecule ESAT-6 (63). It is therefore possible that LEA proteins have specific binding targets within desiccating cells that confer more ordered secondary structure. However, the high concentrations of LEA proteins found within cells, estimated to be >200 μM in cotton seeds prior to desiccation (20), would necessitate an equally abundant protein binding partner, and we might expect such proteins to have been identified already. LEA proteins of different groups where they are present in the same cell might form hetero-oligomers, although this seems unlikely given that both partners would be unstructured. LEA proteins might also bind small molecules, and Walters *et al.* (64) have reported tight, stoichiometric binding of sugars to a mixture of wheat LEA proteins. The effect of this binding on LEA protein structure was not assessed by the authors, but we did not detect any difference in mobility of AavLEA1 on gel filtration in the presence of 0.5 M sucrose or 1 M trehalose, which might be expected if sugar binding has a dramatic effect on folding.

Shifts in temperature, pH, and concentration of counter ions can also increase folding of disordered proteins (39), and a limited temperature effect was noted for AavLEA1 and some other LEA proteins. However, desiccation induced a more dramatic effect on AavLEA1 structure; FT-IR spectroscopic analysis showed that the protein became more folded, developing a significant α-helical component, when dried. Furthermore, spectral components were present that were consistent with the formation of superhelical, and possibly coiled coil-like, structures. This is a highly unusual observation because protein dehydration is most often associated with a loss of structure and aggregation (65) rather than with an increase in structure, folding, or subunit assembly and offers the exciting possibility that structural shifts in Group 3 and possibly other categories of LEA proteins depend on the availability of water. Other workers have observed that apparently unfolded LEA (or similar) proteins become more structured when water activity is decreased by *e.g.* trifluoroethanol (56, 58, 66), high salt concentration (54), or drying in the presence of sucrose (67). This has obvious physiological relevance in desiccation-tolerant systems, including the anhydrobiotic nematode *A. avenae*, but leads to the further question of what the functions of the folded, presumably partially dehydrated, LEA proteins might be. *In vitro*, a pollen protein increases the glass transition temperature (T_g) of a sucrose glass into which it is incorporated (67), prompting the consideration that LEA proteins might stabilize the cytoplasmic sugar glasses implicated in the protection of biomolecules during anhydrobiosis (7, 68, 69). However, this stabilizing function is also exhibited by poly(L)lysine (70) and bovine serum albumin.[3] Indeed, other polymers with high intrinsic T_g, such as hydroxyethyl starch (71), also increase the T_g of a two-component polymer-sugar glass; the phenomenon is therefore not specific to LEA proteins. The high concentration of protein in the cytoplasm (80–300 mg/ml) (72,73) also means that there is no shortage of available protein for glass formation with or without the presence of LEA proteins.

Dehydration apparently induces α-helix formation in AavLEA1 and potentially coiled coil oligomerization, suggesting that computer predictions of its structure may be fulfilled in the dry, if not the hydrated, state. Increased folding and oligomerization could be driven by a combination of decreased protein hydration and molecular crowding effects, which exert thermodynamic pressure on proteins to adopt a compact structure. Crowding would be augmented by loss of water from the cytoplasm, where a 10% reduction in cellular water could result in an increase in thermodynamic activity of volume-excluding protein species of up to 10-fold (74). We might speculate that AavLEA1 coiled coils would be able to form more complex structures, reminiscent of intermediate filaments (IFs). IFs are cytoskeletal components based on coiled coil dimers of, for example, the various keratins or lamins that extend throughout the cytoplasm or nucleoplasm and provide intracellular support and physical strength to the cell (75). Additional LEA protein-derived filaments might increase resistance to the physical stresses imposed during desiccation that can lead to cell deformation and collapse (76). Moreover, LEA protein fil-

[2] D. Leys, personal communication.

[3] S. Ring, personal communication.

aments could work together with sugar glasses in a manner analogous to steel-reinforced concrete, where the filaments might increase the tensile strength of the amorphous carbohydrate matrix. Such hypotheses, in particular the dehydration-dependent oligomerization of LEA proteins, are directly testable and will form the basis of future experiments.

Acknowledgments—We thank Prof. Arthur Rowe and colleagues of the National Centre for Macromolecular Hydrodynamics Business Centre, University of Nottingham, for performing analytical ultracentrifugation experiments and for helpful advice on sedimentation analysis. Several colleagues at the University of Cambridge provided assistance during the project: Drs. Len Packman and Richard Turner, Department of Biochemistry, performed mass spectrometry; Prof. Chris Dobson, Department of Chemistry, allowed access to the spectropolarimeter and FT-IR apparatus; and Dr. Michael Wise, Department of Genetics, made valuable comments on the manuscript.

REFERENCES

1. Crowe, J. H., Hoekstra, F. A., and Crowe, L. M. (1992) *Ann. Rev. Physiol.* **54,** 579–599
2. Oliver, M. J., Tuba, Z., and Mishler, B. D. (2000) *Plant Ecol.* **15,** 85–100
3. Clegg, J. S. (2001) *Comp. Biochem. Physiol.* **128B,** 613–624
4. Perry, R. N. (1999) *Parasitology* **119,** S19-S30
5. Crowe, J. H., Crowe, L. M., Carpenter, J. F., and Wistrom, C. A. (1987) *Biochem. J.* **242,** 1–10
6. Colaco, C., Sen, S., Thangavelu, M., Pinder, S., and Roser, B. (1992) *Bio-Technol.* **10,** 1007–1011
7. Crowe, J. H., Carpenter, J. F., and Crowe, L. M. (1998) *Ann. Rev. Physiol.* **60,** 73–103
8. Bolen, D. W., and Baskakov, I. V. (2001) *J. Mol. Biol.* **310,** 955–963
9. Tunnacliffe, A., and Lapinski, J. (2003) *Philos. Trans. R. Soc. Lond. (B Biol. Sci.*), in press
10. Higa, L. M., and Womersley, C. Z. (1993) *J. Exp. Zool.* **267,** 120–129
11. Womersley, C. Z., and Higa, L. M. (1998) *Nematologica* **44,** 269–291
12. Hoekstra, F. A., Golovina, E. A., and Buitink, J. (2001) *Trends Plant Sci.* **6,** 431–438
13. Ingram, J., and Bartels, D. (1996) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47,** 377–403
14. Bartels, D., and Salamini, F. (2001) *Plant Physiol.* **127,** 1346–1353
15. Browne, J., Tunnacliffe, A., and Burnell, A. (2002) *Nature* **416,** 38
16. Dure, L., III, Greenway, S. C., and Galau, G. A. (1981) *Biochemistry* **20,** 4162–4168
17. Grzelczak, Z. F., Sattolo, M. H., Hanley-Bowdoin, L. K., Kennedy, T. D., and Lane, B. G. (1982) *Can. J. Biochem.* **60,** 389–397
18. Galau, G. A., Hughes, D. W., and Dure, L., III (1986) *Plant Mol. Biol.* **7,** 155–170
19. Baker, J. C., Steele, C., and Dure, L., III (1988) *Plant Mol. Biol.* **11,** 277–291
20. Roberts, J. K., DeSimone, N. A., Lingle, W. L., and Dure, L., III (1993) *Plant Cell* **5,** 769–780
21. Bray, E. A. (1993) *Plant Physiol.* **103,** 1035–1040
22. Cuming, A. (1999) in *Seed Proteins* (Shewry, P. R., and Casey, R., eds) pp. 753–780, Kluwer Academic Publishers, Dordrecht, Netherlands
23. Dure, L., III (1993) *Plant J.* **3,** 363–369
24. Dure, L., III (2001) *Protein Peptide Lett.* **8,** 115–122
25. Makarova, K. S., Aravind, L., Wolf, Y. I., Tatusov, R. L., Minton, K. W., Koonin, E. V., and Daly, M. J. (2001) *Microbiol. Mol. Biol. Rev.* **65,** 44–79
26. Battista, J. R., Park, M.-J., and McLemore, A. E. (2001) *Cryobiology* **43,** 133–139
27. Solomon, A., Salomon, R., Paperna, I., and Glazer, I. (2000) *Parasitology* **121,** 409–416
28. Stetefeld, J., Jenny, M., Schulthess, T., Landwehr, R., Engel, J., and Kammerer, R. A. (2000) *Nat. Struct. Biol.* **7,** 772–776
29. NDong, C., Danyluk, J., Wilson, K. E., Pocock, T., Huner, N. P. A., and Sarhan, F. (2002) *Plant Physiol.* **129,** 1368–1381
30. Wolf, E., Kim, P. S., and Berger, B. (1997) *Protein Sci.* **6,** 1179–1189
31. Evans, A. A. F. (1970) *J. Nematol.* **2,** 99–100
32. Laemmli, U. K. (1970) *Nature* **227,** 680–685
33. Davies, G. E., and Stark, G. R. (1970) *Proc. Natl. Acad. Sci. U. S. A.* **66,** 651–656
34. Coggins, J. R., Lumsden, J., and Malcolm, A. D. B. (1977) *Biochemistry* **16,** 1111–1116
35. Cole, J. L., and Hansen, J. C. (1999) *J. Biomol. Tech.* **10,** 163–176
36. Siegal, L. M., and Monty, K. J. (1966) *Biochim. Biophys. Acta* **112,** 346–362
37. Rowe, A. J. (1977) *Biopolymers* **16,** 2595–2611
38. Rowe, A. J. (1992), in *Analytical Ultracentrifugation in Biochemistry and Polymer Science* (Harding, S. E., Rowe, A. J., and Horton, J. C, eds) pp. 394–406, Royal Society of Chemistry, London
39. Uversky, V. N. (2002) *Eur. J. Biochem.* **269,** 2–12
40. Dunker, A. K., Lawson, J. D., Brown, C. J., Williams, R. M., Romero, P., Oh, J. S., Oldfield, C. J., Campen, A. M., Ratliff, C. M., Hipps, K. W., Ausio, J., Nissen, M. S., Reeves, R., Kang, C., Kissinger, C. R., Bailey, R. W., Griswold, M. D., Chiu, W., Garner, E. C., and Obradovic, Z. (2001) *J. Mol. Graph. Model.* **19,** 26–59
41. Uversky, V. N., Gillespie, J. R., and Fink, A. L. (2000) *Proteins Struct. Funct. Genet.* **41,** 415–427
42. Byler, D. M., and Susi, H. (1986) *Biopolymers* **25,** 469–487
43. Krimm, S., and Bandekar, J. (1986) *Adv. Protein Chem.* **38,** 181–364
44. Heimburg, T., Schunemann, J., Weber, K., and Geisler, N. (1999) *Biochemistry* **38,** 12727–12734
45. Blackman, S. A., Obendorf, R. L., and Leopold, A. C. (1995) *Physiol. Plantarum* **93,** 630–638
46. Honjoh, K.-I., Oda, Y., Takata, R., Miyamoto, T., and Hatano, S. (1996) *J. Plant Physiol.* **155,** 509–512
47. Imai, R., Chang, L., Ohta, A., Bray, E. A., and Takagi, M. (1996) *Gene* **170,** 243–248
48. Swire-Clark, G. A., and Marcotte, W. R., Jr. (1999) *Plant Mol. Biol.* **39,** 117–128
49. Zhang, L., Ohta, A., Takagi, M., and Imai, R. (2000) *J. Biochem.* **127,** 611–616
50. Xu, D., Duan, X., Wang, B., Hong, B., Ho, T.-H. D., and Wu, R. (1996) *Plant Physiol.* **110,** 249–257
51. Sivamani, E., Bahieldin, A., Wraith, J. M., Al-Niemi, T., Dyer, W. E., Ho, T.-H. D., and Qu, R. (2000) *Plant Sci.* **155,** 1–9
52. Honjoh, K., Matsumoto, H., Shimizu, H., Ooyama, K., Tanaka, K., Oda, Y., Takata, R., Joh, T., Suga, K., Miyamoto, T., Iio, M., and Hatano, S. (2000) *Biosci. Biotechnol. Biochem.* **64,** 1656–1663
53. McCubbin, W. D., Kay, C. M., and Lane, B. G. (1985) *Can. J. Biochem. Cell Biol.* **63,** 803–811
54. Russouw, P. S., Farrant, J., Brandt, W., Maeder, D., and Lindsey, G. G. (1995) *Seed Sci. Res.* **5,** 137–144
55. Russouw, P. S., Farrant, J., Brandt, W., and Lindsey, G. G. (1997) *Seed Sci. Res.* **7,** 117–123
56. Soulages, J. L., Kim, K., Walters, C., and Cushman, J. C. (2002) *Plant Physiol.* **128,** 822–832
57. Ceccardi, T. L., Meyer, N. C., and Close, T. J. (1994) *Protein Expr. Purif.* **5,** 266–269
58. Lisse, T., Bartels, D., Kalbitzer, H. R., and Jaenicke, R. (1996) *Biol. Chem.* **377,** 555–561
59. Ismail, A. M., Hall, A. E., and Close, T. J. (1999) *Plant Physiol.* **120,** 237–244
60. Hara, M., Terashima, S., and Kuboi, T. (2001) *J. Plant Physiol.* **158,** 1333–1339
61. Denning, D. P., Uversky, V., Patel, S. S., Fink, A. L., and Rexach, M. (2002) *J. Biol. Chem.* **277,** 33447–33455
62. Stellwagen, E., Rysary, R., and Babul, G. (1972) *J. Biol. Chem.* **247,** 8074–8077
63. Renshaw, P. S., Panagiotidou, P., Whelan, A., Gordon, S. V., Hewinson, R. G., Williamson, R. A., and Carr, M. D. (2002) *J. Biol. Chem.* **277,** 21598–21603
64. Walters, C., Ried, J. L., and Walker-Simmons, M. K. (1997) *Seed Sci. Res.* **7,** 125–134
65. Dong, A., Prestrelski, S. J., Allison, S. D., and Carpenter, J. F. (1995) *J. Pharm. Sci.* **84,** 415–424
66. Boothe, J. G., Sönnichsen, F. D., de Beus, M. D., and Johnson-Flanagan, A. M. (1997) *Plant Physiol.* **113,** 367–376
67. Wolkers, W. F., McReady, S., Brandt, W. F., Lindsey, G. G., and Hoekstra, F. A. (2001) *Biochim. Biophys. Acta* **1544,** 196–206
68. Burke, M. J. (1986) in *Membranes, Metabolism, and Dry Organisms* (Leopold, A. C., ed) pp. 358–364, Cornell University Press, New York
69. Sun, W. Q., and Leopold, A. C. (1997) *Comp. Biochem. Physiol.* **117B,** 327–333
70. Wolkers, W. F., van Kilsdonk, M. G., and Hoekstra, F. A. (1998) *Biochim. Biophys. Acta* **1425,** 127–136
71. Crowe, J. H., Oliver, A. E., Hoekstra, F. A., and Crowe, L. M. (1997) *Cryobiology* **3,** 20–30
72. Zimmerman, S. B., and Trach, S. O. (1991) *J. Mol. Biol.* **222,** 599–620
73. Swaminathan, R., Hwang, C. P., and Verkman, A. S. (1997) *Biophys. J.* **72,** 1900–1907
74. Minton, A. P. (2000) *Curr. Opin. Struct. Biol.* **10,** 34–39
75. Fuchs, E., and Cleveland, D. W. (1998) *Science* **279,** 514–519
76. Wolfe, J., Dowgert, M. F., Maier, B., and Steponkus, P. L. (1986), in *Membranes, Metabolism, and Dry Organisms*, (Leopold, A. C., ed) pp. 286–305, Cornell University Press, New York
77. Burgess, A. W., Ponnuswamy, P. K., and Sheraga, H. A. (1974) *Isr. J. Chem.* **12,** 239–286
78. Deléage, G., and Roux, B. (1987) *Protein Eng.* **4,** 289–294
79. King, R. D., and Sternberg, M. J. E. (1996) *Protein Sci.* **5,** 2298–2310
80. Garnier, J., Gibrat, J. F., and Robson, B. (1996) *Methods Enzymol.* **266,** 97–120
81. Garnier, J., Osguthorpe, D. J., and Robson, B. (1978) *J. Mol. Biol.* **120,** 97–120
82. Holley, L. H., and Karplus, M. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 152–156
83. King, R. D., and Sternberg, M. J. E. (1990) *J. Mol. Biol.* **216,** 441–457