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Influence of drying on the secondary structure of intrinsically disordered and globular proteins

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ABSTRACT

Circular dichroism (CD) spectroscopy of five Arabidopsis late embryogenesis abundant (LEA) proteins constituting the plant specific families LEA_5 and LEA_6 showed that they are intrinsically disordered in solution and partially fold during drying. Structural predictions were comparable to these results for hydrated LEA_6, but not for LEA_5 proteins. FTIR spectroscopy showed that verbascose, but not sucrose, strongly affected the structure of the dry proteins. The four investigated globular proteins were only mildly affected by drying in the absence, but strongly in the presence of sugars. These data highlight the larger structural flexibility of disordered compared to globular proteins and the impact of sugars on the structure of both disordered and globular proteins during drying.

1. Introduction

LEA proteins have been found in plants, invertebrates and bacteria and have been associated with cellular dehydration tolerance. Most LEA proteins have been predicted to be IDPs, i.e. to have no stable structure under physiological, fully hydrated conditions [1,2]. The genome of *Arabidopsis thaliana* contains 51 genes encoding LEA proteins [3]. Three of these proteins have been shown to be unstructured in solution, while they fold into α-helices upon drying [4,5], similar to other LEA proteins [1]. Here we report the characterization of the secondary structure of five additional Arabidopsis LEA proteins forming the two small plant specific families LEA_5 (LEA20 and LEA35, Pfam PF00477) and LEA_6 (LEA15, LEA16 and LEA17, Pfam PF10714).

The first LEA_6 gene (*PvLEA18*) was characterized as drought induced in the common bean (*Phaseolus vulgaris*) [6]. Apart from the fact that the protein does not stabilize enzymes during desiccation in vitro [7] it is functionally and structurally uncharacterized. Of the three homologous genes in Arabidopsis, *LEA15* is expressed specifically in seeds and strongly induced by the phytohormone ABA. *LEA16* is induced under salt stress in leaves, while *LEA17* is only highly expressed in flower buds, but not regulated by any stress treatments [3].

The LEA_5 group in Arabidopsis comprises only two genes. *LEA20* (*EM6*) is expressed constitutively in all investigated tissues, but is induced in leaves under salt stress and after ABA treatment, while *LEA35* (*EM1*) is seed specific [3]. LEA_5 proteins from other plant species are unstructured in solution [8–11] and protect the enzyme lactate dehydrogenase against inactivation during desiccation [12].

Due to the lack of a stable secondary structure, IDPs are generally more flexible than globular proteins [13], but the structure of globular proteins can also be influenced by drying and sugars can stabilize the structure and function of globular proteins [14,15]. The influence of sugars on the structure of dry LEA proteins has only rarely been reported. To investigate the differences in the structural responses of globular proteins and IDPs to drying in the absence or presence of sugars, we used CD and FTIR spectroscopy to compare the secondary structures of the five Arabidopsis LEA proteins of the LEA_5 and LEA_6 families with those of four globular proteins known to contain different amounts of α-helices and β-sheets.

Abbreviations: ABA, abscisic acid; BSA, bovine serum albumin; CD, circular dichroism; FTIR, Fourier-transform infrared; GRAVY, grand average of hydropathy; IDP, intrinsically disordered protein; LEA, late embryogenesis abundant; LG, β-lactoglobulin; RFO, raffinose-family oligosaccharide; RNaseA, ribonuclease A; Suc, sucrose; Thau, thaumatin; Ver, verbascose.

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Table 1
Characteristics of the Arabidopsis LEA proteins.

Protein	Pfam	pI	GRAVY	Molecular mass (kDa)
LEA15	LEA_6	4.59	−1.059	9.7
LEA16	LEA_6	4.46	−1.001	8.5
LEA17	LEA_6	5.21	−1.331	7.5
LEA20	LEA_5	6.75	−1.407	9.9
LEA35	LEA_5	5.75	−1.468	16.6

2. Materials and methods

2.1. Materials

LG from bovine milk, Thau from *Thaumatococcus daniellii*, BSA from bovine blood and Suc were obtained from Sigma (St. Louis, MO), Ver from Megazyme (Wicklow, Ireland) and RNaseA from bovine pancreas was purchased from Roche (Basel, Switzerland). D_2O (99.98%) was purchased from Deutero GmbH (Kastellaun, Germany).

2.2. Expression and purification of recombinant LEA proteins

Full length cDNA clones for the *Arabidopsis thaliana* genes *LEA15* (At2g23110; clone RAFL 05-12-E14), *LEA16* (At2g23120; clone RAFL 04-10-D13), *LEA20* (At2g40170; clone RAFL 09-15-I02) and *LEA35* (At3g51810; clone RAFL 15-50-H15) were obtained from the RIKEN (Tokyo, Japan) RAFL collection [16,17]. *LEA17* (At2g33690) was cloned from cDNA synthesized from RNA isolated from Arabidopsis flower buds. The cDNA sequences were amplified by PCR and inserted into the Gateway pENTR.SD.D-TOPO vector (Invitrogen, Karlsruhe, Germany). The identity of the inserts was checked by sequencing. The genes were transferred into the expression vector

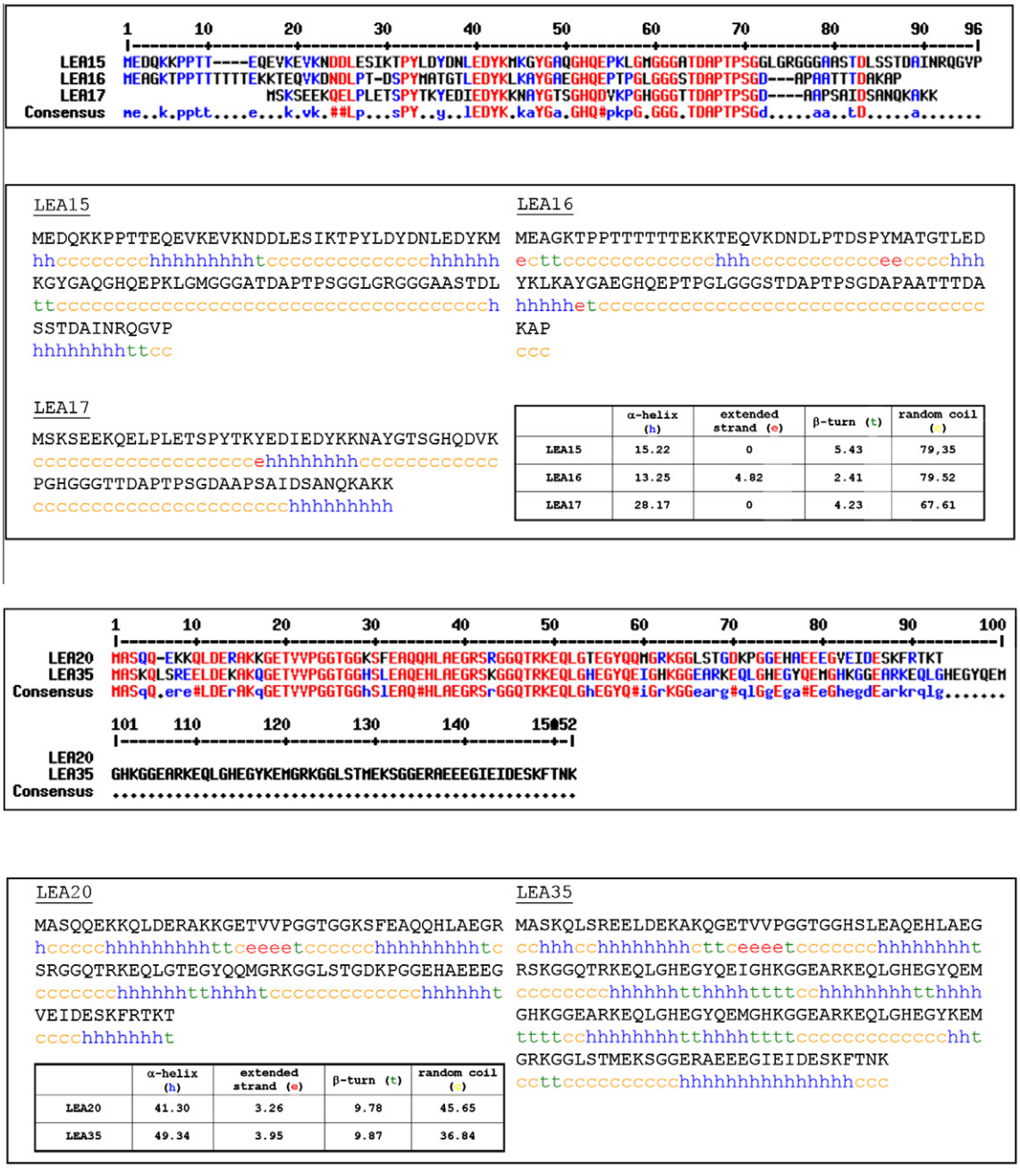

Fig. 1. Alignment of the amino acid sequences of LEA15, LEA16 and LEA17 (LEA_6) and LEA20 and LEA35 (LEA_5). The corresponding secondary structure predictions with the SOPMA tool are shown in the respective panels below the alignments.

pDEST17 (Invitrogen) to express the proteins with an N-terminal 6xHis-tag under the control of the T7 expression system.

The pDEST17 constructs were transformed into several *Escherichia coli* strains. Optimal protein expression levels were obtained in Rosetta pLysSRARE (*LEA15, LEA35*), BL21 (Novagen, Darmstadt, Germany) (*LEA16*) and BL21 Star (Invitrogen) (*LEA17, LEA20*). Recombinant proteins were purified as described in detail previously [5,18]. After dialysis, the proteins were >95% pure as estimated from SDS–PAGE and Coomassie blue staining. Purified proteins were lyophilized and stored at −20 °C.

2.3. CD spectroscopy

CD spectra were obtained with a Jasco-715 spectropolarimeter (Jasco Instruments), as described in detail recently [5,18]. Spectra were analyzed with the CDPro software [19] using three different algorithms: CONTINLL, CDSSTR and SELCON3. Sets of reference spectra containing denatured proteins were chosen for the analysis. Since the results were similar in each case for the three parallel samples and all algorithms, averages are shown.

2.4. FTIR spectroscopy

All proteins were dissolved in D_2O at a concentration of 2 mg/ml. Where indicated proteins in D_2O were mixed with Suc or Ver dissolved in D_2O to reach final concentrations of 10 μM protein and 100 mM sugar. Dry samples were prepared by spreading 50 μl of protein or protein/sugar solutions on CaF_2 windows. The samples were dried in desiccators over silica gel at 28 °C for 24 h and measured in triplicate in a vacuum cuvette placed in the infrared beam as described previously [20–22]. Samples were kept under vacuum for at least 15 min before measurements to remove any residual water, yielding essentially anhydrous samples [4,23]. Hydrated samples were placed between CaF_2 windows separated by a 0.01 mm Teflon spacer and were measured in the same cuvette at ambient pressure [4]. All measurements were performed with a Perkin–Elmer GX2000 FTIR spectrometer by co-adding 216 spectra for each measurement. Spectra were analyzed using Perkin–Elmer Spectrum 5.0.1 software. Differences in protein secondary structure were estimated by analyzing the Amide I peak (1700–1600 cm^{-1}).

2.5. Secondary structure prediction

The secondary structure elements in the LEA proteins were predicted with the online tool SOPMA (http://www.npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) [24].

3. Results and discussion

3.1. Primary and secondary structure of the LEA_5 and LEA_6 proteins

These LEA proteins show very similar characteristics (Table 1). They are slightly acidic to neutral, highly hydrophilic and, with the exception of LEA35 (16.6 kDa), very small proteins (under 10 kDa). They show a high degree of sequence similarity within the two groups, but none between the groups (Fig. 1), as noted on the basis of a phylogenetic tree analysis before [3]. The three LEA_6 proteins were predicted by the SOPMA tool to be about 70–80% random coil, with the rest mainly α-helical (Fig. 1, top panel). The central, highly conserved part of all three proteins was predicted to be disordered with only six to eight amino acids in an α-helical configuration.

The sequences of the N-terminal parts of the two LEA_5 proteins are 65% identical over the first 81 amino acids, but LEA35 contains an additional C-terminal extension of 67 amino acids (Fig. 1, bottom panel). Both proteins were predicted to carry a N-terminal α-helix of 12 (LEA20) or 16 (LEA35) amino acids. The complete proteins were predicted to be 40–50% α-helical, with the remainder mainly in a random coil configuration.

To experimentally test these predictions, recombinant proteins were investigated by CD spectroscopy. In solution the spectrum of LEA15 (Fig. 2A) showed typical features of a largely unstructured protein with a well defined minimal ellipticity at around 200 nm. After drying the spectrum was massively changed, indicating a gain of structure. However, the CD spectrum of LEA15 did not exhibit the double minimum at 208 and 222 nm characteristic of predominantly α-helical proteins. The CD spectra of the other investigated LEA proteins, both in the hydrated and dry state, were similar to those presented in Fig. 2A and are therefore not shown.

Secondary structure estimates derived from these CD spectra indicated that the hydrated LEA proteins were between 61%

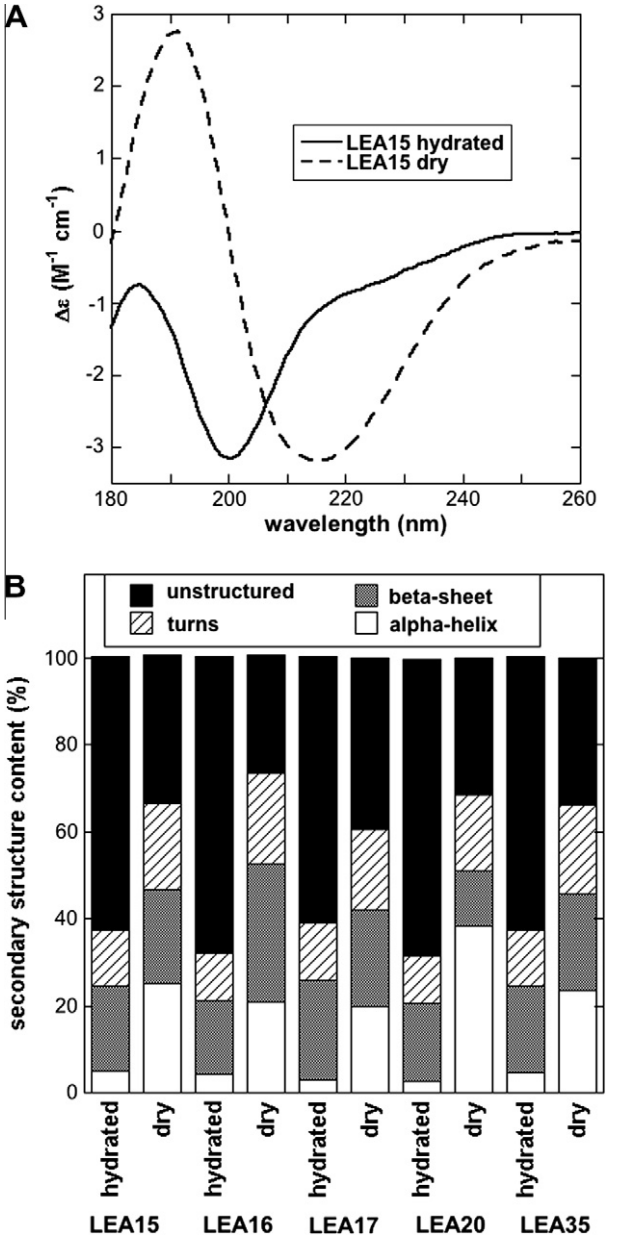

Fig. 2. CD spectra of hydrated and dry LEA15 (A) and the structural composition of LEA15, 16, 17, 20 and 35 in the hydrated and dry state as calculated from the respective CD spectra (B).

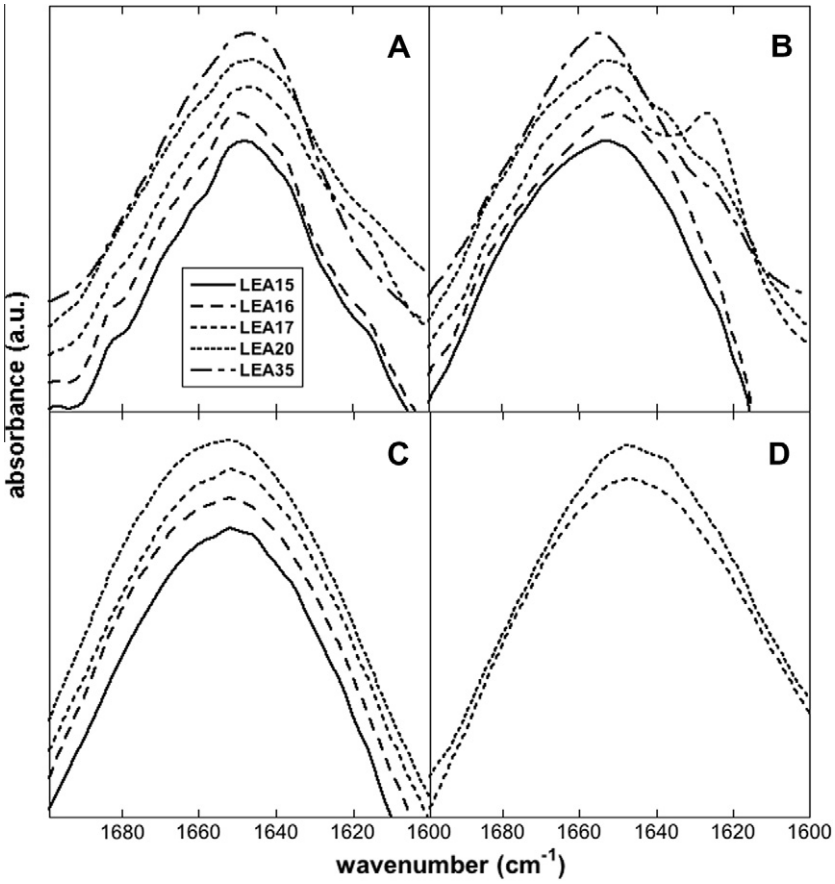

Fig. 3. Normalized Amide I peaks of the hydrated (A) and dry (B) LEA proteins. LEA15, LEA16 and LEA17 comprise the LEA_6 group, while LEA20 and LEA35 represent the LEA_5 family. Panels (C) and (D) show the Amide I peaks of the same proteins in the dry state in the presence of Suc and Ver, respectively. Proteins and sugars were dissolved in D_2O, to final concentrations of 10 μM and 100 mM, respectively. The different peaks in each panel were off-set from each other for better visibility.

(LEA17) and 68% (LEA20) unstructured (Fig. 2B). In addition, they contained around 20% β-sheet and only a negligible fraction of α-helices. After drying the proportion of β-sheet remained similar, with the exception of LEA16, where it doubled and LEA20, where it was strongly reduced. The α-helix content of the proteins increased during drying to 20–25%. Only LEA20 showed the typical structure of a dry LEA protein with an α-helix content of 51%. In addition, all proteins were between 27% and 40% unstructured in the dry state.

A comparison of the secondary structure predictions (Fig. 1) and the structural content determined by CD spectroscopy (Fig. 2) reveals that for the LEA_6 proteins the predicted structure (70–80% random coil) corresponds most closely to the structure in solution (61–65% unstructured). This correspondence became even better (51–60% unstructured) when we repeated the prediction with the full sequence of the recombinant proteins, including the tag and linker sequences (Supplementary Fig. 1). This is in contrast to reports that secondary structure prediction programs such as SOPMA predict rather the structure of dry LEA proteins [4,5,25], which was only true for LEA20 with about 40% α-helix. In this case, the predictions for the native sequence and for the sequence including the tag and linker were essentially identical (Supplementary Fig. 1). The predictions, either for the native or the tagged sequence of LEA35, on the other hand, showed no clear correspondence to the structure determined by CD spectroscopy under either condition. It is unclear why the correspondence between predicted and measured structure content varies so drastically between these proteins, since their physical properties (charge, hydrophobicity) are very similar (Table 1).

3.2. Influence of drying and sugars on the secondary structure of IDPs

We used FTIR spectroscopy to further investigate the secondary structure of the recombinant LEA proteins. Sugars such as Suc and the RFO Ver are frequently accumulated in plant seeds when they become desiccation tolerant [26,27]. The ability of sugars to form glasses and to establish H-bonds with macromolecules and membranes during drying are the main reasons for their stabilizing effects on biological structures during desiccation [28,29].

Information about the secondary structure of proteins from FTIR spectra can be found in the Amide I peak (1700–1600 cm^{-1}) which is composed of several overlapping component peaks representing α-helices (1660–1650 cm^{-1}), β-sheets (1640–1620 cm^{-1}), turns (1670–1660 cm^{-1}) and unordered regions (1640 and 1650 cm^{-1}) [30,31]. A peak at around 1620 cm^{-1} is associated with intermolecular β-sheet aggregates [32,33]. H_2O shows an absorbance peak at about 1645 cm^{-1}, overlapping strongly with the protein Amide I peak. We therefore dissolved all proteins and sugars in D_2O for the measurement of hydrated samples, as the D_2O peak is shifted to around 1200 cm^{-1} and therefore does not interfere with the Amide I peak [30,31].

Fig. 3A and B shows the Amide I peaks of the LEA proteins in the hydrated and dry state, respectively. The Amide I peaks from all hydrated proteins were situated between 1646 and 1650 cm^{-1} in agreement with the CD spectra indicating them to be largely unstructured. After drying, the Amide I peaks were shifted to positions between 1650 and 1654 cm^{-1}, reflecting the transition to a more α-helical configuration. The Amide I contour of LEA17 showed an additional peak at 1620 cm^{-1}, typical for a partly aggregated

protein. Both Group_5 proteins showed evidence of a smaller degree of aggregation from a shoulder at this wavenumber. Due to their highly hydrophilic character, aggregation of LEA proteins during drying has only rarely been observed [4,34].

In vivo LEA proteins never occur in isolation, but, in addition to other proteins, low molecular weight solutes such as amino acids and sugars are present. Especially sugar concentrations often increase concomitantly to the induction of LEA proteins under stress conditions [1,2]. In solution, 100 mM Suc or Ver had no measurable effect on the structure of the LEA proteins (data not shown). In the dry state, however, both Suc (Fig. 3C) and Ver (Fig. 3D) completely prevented LEA protein aggregation. This may, at least in part, be related to the well-known property of sugars such as Suc and Ver to form a glass during drying, thereby severely restricting molecular diffusion [35]. The Amide I peak maxima were located at 1652 cm^{-1} in the presence of Suc and at 1646 cm^{-1} for LEA17 and 1648 cm^{-1} for LEA20 in the presence of Ver. The peak positions in the presence of Suc were similar to those of the pure dry proteins, in agreement with recent data on three LEA_1 proteins from soybean [36,37]. Ver shifted the peaks to a position corresponding to the structure of the hydrated proteins, emphasizing the structural flexibility of these IDPs.

3.3. Influence of drying and sugars on the secondary structure of globular proteins

To study the effects of drying on globular proteins, we used the proteins BSA (69.3 kDa), LG (18.4 kDa), RNaseA (13.7 kDa) and Thau (22 kDa) with well defined, but different secondary structures. In addition, we chose LG, RNaseA and Thau because their molecular masses are comparable to those of the studied LEA proteins and therefore allow for more direct comparison. In the hydrated state, the Amide I peak of BSA was located at 1650 cm^{-1} (Fig. 4A), in agreement with the 66% α-helix content [38]. The other proteins had their Amide I contours centered at 1636 cm^{-1} (LG), 1640 cm^{-1} (RNaseA) and 1644 cm^{-1} (Thau), in agreement with their higher β-sheet content. LG is composed of an eight-stranded antiparallel β-barrel and one major α-helix [39]. For RNaseA earlier FTIR measurements estimated its β-sheet content at 45–50% [40,41], while Thau consists primarily of two β-sheets forming a flattened β-barrel [42].

The proteins were only mildly affected by drying (Fig. 4B), in agreement with a FTIR study of the effects of lyophilization on four different globular proteins [15]. The small shift (about 4 cm^{-1}) to lower wavenumbers for LG and Thau suggests a higher β-sheet content of these proteins after drying, in agreement with an FTIR study on the effects of freeze-drying on the secondary structure of several globular proteins [41]. These authors observed on average an increase in β-sheet content of about 20%. For BSA and RNaseA the small shift of the Amide I peak to higher wavenumbers suggests a small increase in α-helicity, while for lyophilized RNaseA an increase in β-sheet content has been reported [41]. The mild effects on the globular proteins are in strong contrast to the massive changes in secondary structure observed by both CD (Fig. 2) and FTIR (Fig. 3) spectroscopy for the LEA proteins, in agreement with the presumed higher degree of structural flexibility in IDPs [13].

However, drying the globular proteins in the presence of sugars had stronger effects on their secondary structure (Fig. 4C and D), while the presence of Suc or Ver had no influence in the fully hydrated state (data not shown). The presence of Suc promoted

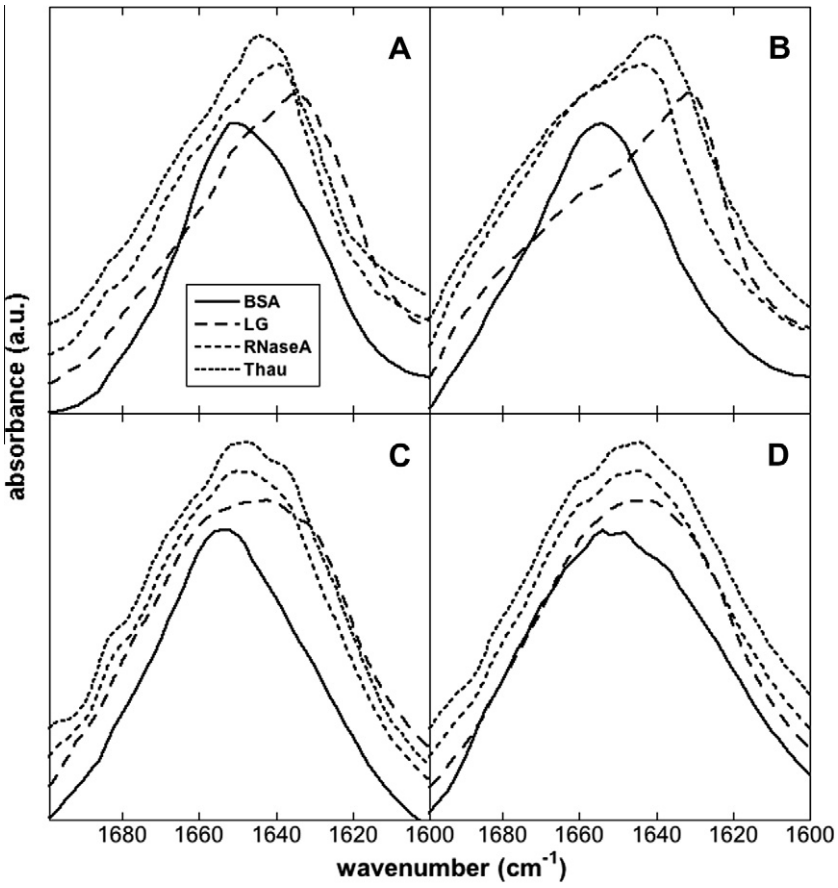

Fig. 4. Normalized Amide I peaks of the hydrated (A) and dry (B) globular proteins BSA, LG, RNaseA and Thau. Panels (C) and (D) show the Amide I peaks of the same proteins in the dry state in the presence of Suc and Ver, respectively. All experimental conditions were identical to those in Fig. 3. The different peaks in each panel were off-set from each other for better visibility.

α-helix formation in the proteins that contained a significant amount of β-sheets in the hydrated state. Their peak positions were shifted to higher wavenumbers by 14 cm^{-1} (LG), 10 cm^{-1} (RNaseA) and 4 cm^{-1} (Thau) by drying. The presence of Ver induced smaller shifts, probably indicating that the tetrasaccharide interacted less with the proteins. Functionally, it has been shown for a variety of enzymes that their activities can be preserved by sugars both during air-drying and freeze-drying [43,44].

The strong differences between the proteins dried in the presence or absence of sugars are in contrast to an FTIR study that examined the influence of lyophilization on the structure of six globular proteins, detecting only minor differences [45]. Other studies that compared FTIR spectra between proteins in solution and lyophilized in the absence and presence of sugars suggested that protein secondary structure was most similar in hydrated proteins and in proteins dried with sugar [14,15].

The major difference between the present and previous studies is the drying method. While we used slow air drying, all other studies used lyophilization, where samples are first frozen in liquid nitrogen and then dried under vacuum. Air-drying is more meaningful in the context of the biological phenomenon of anhydrobiosis, while lyophilization is the method of choice in a technical, e.g. pharmaceutical, context. The present data suggest that sugar–protein interactions and structural transitions in proteins during drying may be more pronounced when water is removed slowly than in samples that are rapidly frozen to −196 °C prior to drying.

Collectively, our data confirm the larger structural flexibility of IDPs compared to globular proteins. The secondary structure estimates indicate more structural diversity in LEA proteins after drying than was previously assumed based on data showing dry LEA proteins to be predominantly α-helical. In addition, we found that secondary structure prediction programs do not always predict the structure of dry LEA proteins. Aggregation of LEA proteins during drying was prevented by sugars, suggesting that it may not occur in vivo. The secondary structure of globular proteins was only little affected by drying, but the effects increased in the presence of sugars. Finally, it should be emphasized that the globular proteins investigated here are monomeric. The stability of oligomeric enzymes during drying entails an additional level of complexity [46].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.11.067.

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