Received: 14 December 2021 | Revised: 11 February 2022 | Accepted: 15 February 2022

DOI: 10.1002/pro.4288

FOR THE RECORD

THE PROTEIN SOCIETY WILEY

Desiccation-tolerance and globular proteins adsorb similar amounts of water

Julia A. Brom[1] | **Gary J. Pielak**[1,2,3,4]

[1]Department of Chemistry, University of North Carolina at Chapel Hill (UNC-CH), Chapel Hill, North Carolina, USA

[2]Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill (UNC-CH), Chapel Hill, North Carolina, USA

[3]Lineberger Cancer Center, University of North Carolina at Chapel Hill (UNC-CH), Chapel Hill, North Carolina, USA

[4]Integrative Program for Biological and Genome Sciences, University of North Carolina at Chapel Hill (UNC-CH), Chapel Hill, North Carolina, USA

Correspondence
Gary J. Pielak, Department of Chemistry, University of North Carolina at Chapel Hill (UNC-CH), Chapel Hill, NC, USA.
Email: gary_pielak@unc.edu

Funding information
National Institutes of Health, Grant/Award Number: R01GM127291

Review Editor: John Kuriyan

Abstract

When exposed to desiccation stress, extremotolerant organisms from all domains of life produce protective disordered proteins with the potential to inform the design of excipients for formulating biologics and industrial enzymes. However, the mechanism(s) of desiccation protection remain largely unknown. To investigate the role of water sorption in desiccation protection, we use thermogravimetric analysis to study water adsorption by two desiccation-tolerance proteins, cytosolic abundant heat soluble protein D from tardigrades and late embryogenesis abundant protein 4 from the anhydrobiotic midge *Polypedilum vanderplanki*, and, as a control, the globular B1 domain of staphylococcal protein G. All samples adsorb similar amounts of water, suggesting that modulated water retention is not responsible for dehydration protection by desiccation-tolerance proteins.

KEYWORDS

desiccation, disordered proteins, tardigrades, thermogravimetric analysis

1 | INTRODUCTION

Water is essential to biology,[1,2] but selected organisms from all domains of life can survive anhydrobiosis, a state in which cellular water is less than 5% w/w compared to typical values of 60–70%.[3] Although the mechanism(s) of anhydrobiosis remain largely unknown, many resistant organisms express small, disordered proteins upon desiccation.[4,5]

Drying protein-based drugs (biologics) and industrial enzymes can increase their stability and thereby avoid challenges associated with refrigerated transport and storage (the so-called cold chain) that hinder their use.[6–8] Unfortunately, many proteins do not withstand dehydration because of water's crucial role in globular protein structure and function.[9,10] Proteins can be formulated with protective molecules called excipients prior to drying,[8,11] but the process of choosing an effective excipient is empirical, and formulation often fails because we know so little about dehydration protection.[8,11,12]

Uncovering the mechanisms by which desiccation-tolerance molecules protect proteins will advance our understanding of anhydrobiosis and facilitate the logical choice and design of excipients, making enzymes and life-saving biologics more affordable and accessible.[13]

Hypotheses about the mechanism of protein dehydration protection have been proposed. In the preferential hydration hypothesis, a hydration layer is maintained by protectants that trap or crowd water at the hydrophilic surface of client proteins.[14] Using this model, one might predict that desiccation-tolerance molecules are more hygroscopic than those unrelated to desiccation

tolerance, holding on to water that then interacts with the client protein surface.

In the water replacement hypothesis, protectants provide H-bonds to client proteins that are usually made by water.[12,15] Furthermore, water can accelerate the chemical degradation of proteins such that desiccation protection might involve inhibiting water's adverse effects.[12,16–18] These ideas suggest that desiccation-tolerance molecules limit degradation by replacing water, which means they perhaps bind less water than molecules unrelated to desiccation tolerance. In summary, the amount of retained water by desiccation-tolerance proteins might be skewed in either direction compared to proteins not related to desiccation tolerance.

Here, we use thermogravimetric analysis (TGA) to quantify water sorption by two desiccation-tolerance proteins, one from a tardigrade and another from a midge. Tardigrades are microscopic animals many species of which survive desiccation and/or other extreme stresses.[19–21] Cytosolic abundant heat soluble (CAHS) proteins are unique to tardigrades, necessary for their desiccation survival, intrinsically disordered, and protect heterologously expressing cells and enzymes from desiccation damage.[22–24] They also form reversible, concentration-dependent hydrogels.[25,26] We tested CAHS D from the tardigrade *Hypsibius exemplaris*. The midge *Polypedilum vanderplanki* survives multiple cycles of complete dehydration and accumulates late embryogenesis abundant (LEA) proteins upon desiccation.[27] LEA proteins, also disordered, are involved in desiccation tolerance of many plants and animals, and inhibit protein aggregation.[27–30] We tested LEA protein 4 from *P. vanderplanki* (PvLEA4).[27] As a control we tested a globular protein not implicated in desiccation tolerance, the B1 domain of staphylococcal protein G (GB1).[31]

2 | RESULTS

To determine if the mechanism of desiccation protection is related to water adsorption, we performed TGA on lyophilized samples of GB1, CAHS D, and PvLEA4. Immediately after 24 hr of lyophilization, samples possess 10–11% water by mass (Figure 1), which is less than one surface layer of water for GB1 (Table S1). Upon exposure to 75% relative humidity, the water content sharply increases before plateauing by 24 hr. PvLEA4 samples plateau with a water content ~19%, CAHS D ~16%, and GB1 ~18%, which amounts to slightly more than one layer on the surface of GB1, similar to other results (Table S1).[32,33] From 2 hr on, the pattern from highest to lowest water content is PvLEA4, GB1, CAHS D (Figure 1). However, the water content of PvLEA4 and CAHS D samples is within the uncertainty for GB1 at each timepoint except 4 hr, where the water content of PvLEA4 is higher than that of GB1 (Figure 1). After exposure to 75% relative humidity for 72 h, all samples adsorb similar amounts of water, showing a total water content of ~17% (Figure 1). These results are comparable to estimations of water sorption based on the hydrophilic groups in a dry protein; the procedure of Leeder and Watt[34,35] predicts equilibrium water contents for our proteins of ~19–21%, not far from our experimental results (Table S2, Figure 1).

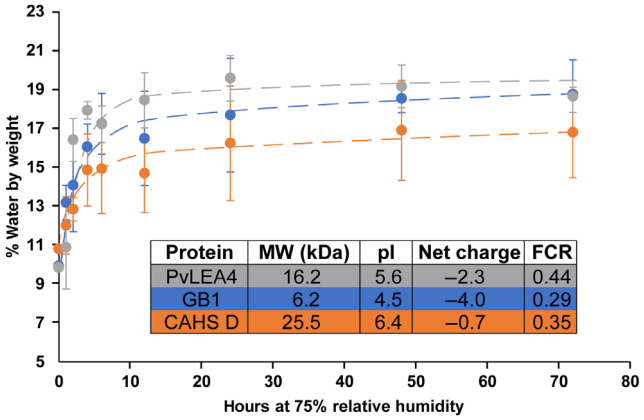

FIGURE 1 Water content, in percent weight of solid protein sample, as determined by TGA while heating at 4°C/min. Samples comprising 2 mg of GB1, CAHS D, or PvLEA4 in 650 µl 1.5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 6.5 were lyophilized for 24 hr, then exposed to 75% relative humidity at room temperature. Error bars represent the *SD* from three independent experiments. Molecular weight (MW), isoelectric point (pI), net charge calculated at pH 6.5, and FCRs are shown in the inset. CAHS D, cytosolic abundant heat-soluble protein D; FCRs, fraction of charged residues; GB1, B1 domain of staphylococcal protein G; PvLEA4, LEA protein 4 from *P. vanderplanki*; TGA, thermogravimetric analysis

3 | DISCUSSION

The observation that PvLEA4 and CAHS D adsorb neither more nor less water than GB1 (Figure 1) and other globular proteins at 75% relative humidity (e.g., lysozyme, ribonuclease, chymotrypsinogen, bovine somatotropin)[34,36] shows that neither elevated nor depressed sorption is a distinguishing feature of desiccation-tolerance proteins. In the dry state, a globular protein comprises an ensemble of partially folded conformations,[32,37] which suggests that the vapor-accessible surface of a dry globular protein is more comparable to that of a dry disordered protein than it is to the surface of a globular protein in solution.

Although water coordination to client proteins may be an aspect of their mechanism, simple accumulation is

not sufficient to explain the function of desiccation-tolerance proteins. Similarly, although desiccation-tolerance proteins may replace water H-bonds to client proteins while combating plasticizing and degradative effects, they do not adsorb less (or more) water than other proteins. We have also examined gelatin, a disordered protein unrelated to desiccation tolerance. A gelatin-GB1 mixture adsorbs a similar amount of water as mixtures of CAHS D and GB1 or PvLEA4 and GB1.[33] In the same residue-level study of dehydration protection, we show that water content of client protein/protectant protein mixtures does not correlate with dehydration protection.[33]

These data also suggest that the ability of CAHS D to form hydrogels[25,26] is not explained by hygroscopicity, because CAHS D adsorbs no more water than nongelling proteins (GB1, PvLEA4, etc.).

Neither charge, fraction of charged residues, amino acid composition, nor hydrophobicity completely explain the amounts of retained water (Figure 1, Tables S3 and S4). However, PvLEA4, the protein with the highest fraction of charged residues, on average retains more water than GB1 or CAHS D. This observation suggests that more charged proteins adsorb more water, in agreement with studies showing that hydrophilic groups in a dry protein correlate with sorption capacity.[34,35,38,39]

In summary, desiccation-tolerance proteins adsorb water similarly to globular proteins, suggesting that modulated water retention does not explain desiccation protection. In vivo, other molecules may modulate hydration near desiccation-tolerance proteins, but desiccation-tolerance proteins themselves do not necessarily bind water differently than other proteins. Investigating what properties are particular to desiccation-tolerance proteins will reveal their protective mechanism(s), allowing rational design of excipients to make protein products more affordable and accessible.

4 | MATERIALS AND METHODS

4.1 | Materials

Ampicillin, kanamycin sulfate, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Thermo Fisher) were used without further purification. H_2O with a resistivity >17 MΩ cm^{-1} was used to prepare buffers. A constant relative humidity of 75 ± 5% as measured by a digital hygrometer (Fisherbrand TraceableGO™ Bluetooth datalogging digital hygrometer) was created by sealing a 0.5-L chamber containing 200 ml of H_2O saturated with sodium chloride (Thermo Fisher).[40]

4.2 | Protein expression and purification

The pET11a plasmid (Novagen) containing the gene for the T2Q variant of GB1 was provided by Leonard D. Spicer's laboratory at Duke University (Durham, North Carolina). This variant, which we call GB1, was chosen because the mutation prevents N-terminal deamidation.[41] The pET28b plasmid containing the gene for CAHS D was engineered as described.[22] The pET28b plasmid containing the gene for PvLEA4 fused to an N-terminal hexahistadine (His-) tag and a TEV protease cleavage site was ordered from Gene Universal Inc. Vectors were transformed into Agilent BL21 Gold (DE3) *Escherichia coli* as described.[22]

A single colony was used to inoculate 100 ml of Luria-Bertani broth (Fisher, 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) supplemented with the antibiotic ampicillin (GB1) or kanamycin (CAHS D and PvLEA4) to a final concentration of 60 μg/ml. The culture was shaken at 37°C overnight (New Brunswick Scientific I26 incubator, 225 rpm). Ten milliliters of the overnight culture were used to inoculate 1 l of antibiotic-supplemented LB. One-liter cultures were shaken at 37°C until they reached an optical density at 600 nm of 0.6–0.8, at which point protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (1 mM final concentration). Three hours after induction, cells were harvested via centrifugation at 4,000*g*. The cell pellet from each culture was resuspended in 10 ml of 20 mM Tris, pH 7.5, and stored at −20°C.

Cell pellets from GB1 expression were lysed by sonication (500-W dismembrator 1/8-in. tip, 15% amplitude [Fisher Scientific]) for 8 min using a 2 s on/1 s off duty cycle, and then GB1 was purified as described.[42] CAHS D was purified as described.[23] PvLEA4 was purified as described.[33] Purified proteins were exchanged into H_2O by dialysis (ThermoScientific Snakeskin™ dialysis tubing, 3,500 Da molecular weight cutoff), and divided into 2 mg aliquots. Aliquots were flash-frozen, lyophilized, and stored at −20°C. Purity was confirmed by observation of a single band on sodium dodecylsufate polyacrylamide gel electrophoresis and by quadrupole time-of-flight mass spectrometry (ThermoScientific, Q Exactive HF-X) in the UNC Mass Spectrometry Chemical Research and Teaching Core Laboratory.

4.3 | Thermogravimetric analysis

Aliquots of purified, lyophilized protein were resuspended in 650 μl of 1.5 mM HEPES buffer, pH 6.5, flash-frozen, and lyophilized (LABCONCO FreeZone 1 Liter Benchtop Freeze Dry System) for 24 hr. Samples

were then placed, without caps, in a chamber with a controlled relative humidity of 75 ± 5%, created as described above. Individual tubes were removed after 0, 1, 2, 4, 6, 12, 24, 48, and 72 hr, and protein samples were loaded into a TA Instruments model 550 thermogravimetric analyzer on an open Pt pan and heated from 25 to 175°C at a rate of 4°C/min under a N_2(g) sample purge of 60 ml/min and a balance purge of 40 ml/min. The well-defined mass loss ending around 125°C was used to quantify H_2O content.[43,44] Thermograms were analyzed using Trios V5.1.0.56403 software.

ACKNOWLEDGMENTS

This research was supported by NIH grant R01GM127291 to Gary J. Pielak. The authors thank Samantha Stadmiller for GB1 preparation. The authors thank Brandie Ehrmann from the UNC Chemistry Mass Spectrometry Core Laboratory for equipment maintenance and advice, Elizabeth Pielak for comments on the manuscript, and the Pielak Laboratory for helpful discussion.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS

Julia A. Brom: Conceptualization (lead); data curation (lead); formal analysis (lead); investigation (lead); methodology (lead); validation (lead); visualization (lead); writing—review and editing (lead). **Gary J. Pielak:** Conceptualization (lead); project administration (lead); writing—review and editing (equal).

ORCID

Julia A. Brom https://orcid.org/0000-0001-5058-8680
Gary J. Pielak https://orcid.org/0000-0001-6307-542X

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SUPPORTING INFORMATION

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How to cite this article: Brom JA, Pielak GJ. Desiccation-tolerance and globular proteins adsorb similar amounts of water. Protein Science. 2022;31(5):e4288. https://doi.org/10.1002/pro.4288