TARDIGRADE DISORDERED PROTEINS AS POTENTIAL EXCIPIENTS FOR BIOLOGICS

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A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Chemistry in the College of Arts and Sciences.

Chapel Hill
2019

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ABSTRACT

Samantha Piszkiewicz: Tardigrade Disordered Proteins as Potential Excipients for Biologics
(Under the direction of Gary J. Pielak)

Protein-based ‘biologics’ — drugs derived from living organisms — are among the most effective therapeutic treatments on the market. However, biologics are unstable, have short half-lives and require low temperature storage, making them prohibitively expensive. Although some biologics can be stabilized by formulation with excipients, most still require low temperature storage. In our search for new, more robust excipients, we turned to the tardigrade, a microscopic animal that synthesizes cytosolic abundant heat soluble (CAHS) proteins, a family unique to the tardigrade, to protect its cellular components during desiccation. This family of proteins protects lactate dehydrogenase against vacuum-drying induced inactivation and lipoprotein lipase against freeze-thaw and freeze-drying induced inactivation. Furthermore, CAHS proteins protect both enzymes more effectively than FDA approved excipients. CAHS proteins are intrinsically disordered with some transient structure in dilute solution. However, as they are concentrated, the transient structural elements are more likely to come into contact, resulting in gelation. When water is completely removed by lyophilization, CAHS proteins inhibit hydrogen-deuterium exchange of the protein GB1 with water vapor. Collectively, these studies lay the groundwork for understanding how CAHS proteins protect tardigrades against desiccation. Furthermore, these results support CAHS proteins as potential excipients for biologic drugs.

In memory of
Annette Louise Stephens
May 5, 1935 – Dec 14, 2013

ACKNOWLEDGEMENTS

I arrived at UNC exhausted from my undergraduate experience. For almost five years, Gary Pielak has given me the space to rediscover my science mojo. Thank you for taking me seriously when I showed up in your office to pitch a totally new direction for the lab. Thank you for sticking by that project for three years as we fought for funding. Finally, thank you for valuing the mental health of your students. Unlike many graduate students, I am a healthier and happier human for my graduate experience.

This project would not exist without Professor Bob Goldstein, who first told me about the wonders of tardigrades, and Research Assistant Professor Thomas Boothby, who graciously accepted me as a collaborator on his work. Austin Smith, Rachel Cohen, and Annelise Gorensek-Benitez welcomed me into the lab and provided valuable encouragement during the early stages of this project. Alex Guseman pushed me to work smarter from the moment we arrived as graduate students. To round out the crowding half of the lab, thank you to Shannon Speer and Joey Thole for your encouragement and positive energy.

Over the last four years I have had the privilege of training undergraduate students Aakash Mehta, Kenny Nguyen, and Owen Warmuth, as well as high school student Ashlee Propst. Thank you for your dedication. I am so proud to count you as co-authors.

In addition to Gary and Thomas, I leave the tardigrade project in the very capable hands of Candice Crilly, Claire Stewart, Jack Eicher, Octavio Origel, my former

undergraduate trainees, Owen Warmuth and Kenny Nguyen, and my Caltech sister, Adriana Hertel-Wulff. I'm so excited to see what discoveries you make.

I stuck with this project in part because of support from numerous scientists outside of the lab including Brooke Christian, Kathryn Gunn, Karl Koshlap, Chris Luft, Saskia Neher, Marc ter Horst, Bonnie Wallace, Ellen Young, and Greg Young. I also need to thank Kelly Parsons for believing in our work and guiding us through the provisional patent application process. I have learned so much about patents and companies from you that I did not expect to learn in graduate school.

Halfway through my 4th year of graduate school, I was hit by a car while crossing the street. Gary was beyond understanding when I showed up to lab with a broken pipetting hand. My trainee, Owen, and Thomas's undergraduate trainee, Cody Weyhrich, stepped up to assist me in lab until my cast was removed. At one point or another, practically every member of the Pielak, Li, Brustad, and Weeks labs helped me get around and function in lab until I finished physical therapy. It would have been very easy to lose my sanity without all of your support.

Additionally, I have been a more resilient person throughout grad school thanks to the support of many friends. Thank you to the Women in Science group and STEM Pride of the Triangle as well as my fellow co-founders and volunteers in the WinSPIRE Summer High School Research Program for working with me to make tangible change towards gender and LGBTQ equality. To the Tuesday Night Knitters, thank you for providing me with a weekly sanity check. To my Caltech family, thank you for always

having my back. To my parents, thank you for understanding the stress of my career and always supporting me through it. And finally, to Alyssa Vollaro, Earl Ritchie, and Kristen Biernat, I would not have finished this journey without your friendship.

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LIST OF ABBREVIATIONS AND SYMBOLS

°C	degrees Celsius
%	percent
®	registered trademark
AEBSF	4-benzenesulfonyl fluoride hydrochloride
A. thaliana	*Arabidopsis thaliana*
atm	atmosphere
BSA	bovine serum albumin
C. elegans	*Caenorhabditis elegans*
$CaCl_2$	calcium chloride
CaF_2	calcium fluoride
CAHS	cytosolic abundant heat soluble protein
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate
cm	centimeter(s)
cm^{-1}	inverse centimeters
d	day(s)
deHase	dehydrogenase
dextran 20	20 kDa dextran
DGGR	1,2-di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresofurin ester)
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
DM-β-cyclodextrin	2,6-Di-O-methyl-β-cyclodextrin
DNA	deoxyribonucleic acid

E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
Ficoll 70	70 kDa Ficoll®
g	gram(s)
g	g-force, gravitational acceleration
G’	storage modulus
G’’	loss modulus
GB1	B1 domain of streptococcal protein G
GE	General Electric
h	hour(s)
H^+	hydrogen ion
H. dujardini	*Hypsibius dujardini,* recently renamed *Hypsibius exemplaris*
H. exemplaris	*Hypsibius exemplaris*
HCl	hydrochloric acid
HDX	hydrogen-deuterium exchange
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hphob	Kyte and Doolittle hydrophobicity
HP-β-cyclodextrin	hydroxypropyl-β-cyclodextrin
HSQC	heteronuclear single-quantum coherence
HT	high tension voltage
Hz	hertz
IDP	intrinsically disordered protein
K	kelvin

kcal	kilocalories
KCl	potassium chloride
kDa	kilodalton
KH_2PO_4	potassium phosphate monobasic
L	liter(s)
LB	Lennox broth
LDH	lactate dehydrogenase
LEA	late embryogenesis abundant
LPL	lipoprotein lipase
M	molar
M^{-1}	inverse molar
mg	milligram(s)
$MgSO_4$	magnesium sulfate
MHz	megahertz
min	minute(s)
mL	milliliter(s)
mM	millimolar
mol	mole(s)
MW	molecular weight
MWCO	molecular weight cut off
$M\Omega cm^{-1}$	resistivity in molar ohms per centimeter
n/a	not applicable
n/d	not determined

NaCl	sodium chloride
NAD^+	oxidized nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
Na_2HPO_4	sodium phosphate dibasic
ng	nanogram(s)
nm	nanometer(s)
NMR	nuclear magnetic resonance spectroscopy
PAGE	polyacrylamide gel
PBS	phosphate buffered saline
PDB	Protein Data Bank
PDB ID	PDB identification code
PEG 3350	3.35 kDa polyethylene glycol
PEG	polyethylene glycol
pH	negative log (base 10) of the molar concentration of hydronium ions
pI	isoelectric point
ppm	parts per million
rad	radian
rpm	rounds per minute
s	second(s)
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
SH3	the T22G variant of the N-terminal SH3 domain of *Drosophila* signal transduction protein drk
SRCD	synchrotron circular dichroism spectropolarimetery

TMAO	trimethylamine-N-oxide
Tris	tris(hydroxymethyl)aminomethane
Ubq	ubiquitin
vol/vol	volume per volume
y	year(s)
α	alpha
β	beta
γ	gamma
δ	delta
Δε	molar circular dichroism
ε	epsilon
ε	extinction coefficient
μg	microgram(s)
μm	micrometer(s)
μM	micromolar
μL	microliter(s)

CHAPTER 1: PROTECTING ENZYMES FROM STRESS-INDUCED INACTIVATION

Overview

In this review, we summarize the ability of a variety of additive molecules to protect enzymes and other proteins against common stresses of manufacture, transport, and storage: vacuum-drying, freeze-thawing, and freeze-drying. Although dozens of studies have investigated how to protect enzymes against these stresses, little can be concluded from considering these reports with respect to one another. Some molecules have no protective ability in one report and full protective ability in another. Many reports of no protection likely result from insufficient quantity of additive used. Reports of synergism test few combinations of molecules and are rarely if ever reproduced in the literature. Researchers are further divided on the mechanisms behind protection. Additional work is needed to understand the protective nature of many of these additives and the mechanisms behind protection.

Introduction

Biologics, protein-based drugs, are the most effective therapeutic treatments on the market.[1] However, these medications are inherently unstable and can easily degrade with changes to their environment.[2] The World Health Organization even varies its stability guidelines for biologic drugs to accommodate the temperature and humidity ranges of different regions around the world.[2] Furthermore, biologics usually require transport and storage in refrigerated conditions, also called the cold chain, which adds additional challenges and expense to maintaining a drug's integrity.[3] Enzymes are

similarly poised to revolutionize the industry of chemical synthesis; however, optimizing these reactions require stabilizing enzymes in non-physiological, and often non-aqueous, environments.[4]

In this review, we set out to summarize the ability of a variety of additive molecules to protect enzymes and other proteins against common stresses of biologic and industrial enzyme manufacture, transport, and storage: vacuum-drying, freeze-thawing, and freeze-drying. Many of these additives are already used as or have potential to be excipients, non-active drug ingredients that are formulated with biologics to stabilize and protect the active ingredient.

Vacuum-drying Stress

Removing water from biologic drugs and industrial enzymes drastically reduces the weight of these products, decreasing shipping costs. Water can be removed from liquid formulations in a vacuum chamber, which is often combined with a centrifuge (e.g a Speedvac®).

We have compiled reports of enzyme protection during vacuum-drying (Table 1.1). The highest degree of protection for each additive is further summarized in Figure 1.1. Focusing on the highest protection may amplify findings of false-positives but minimizes the detection of false-negatives, which are likely given that many of these studies tested a single concentration or mole ratio rather than producing a dose curve.

The stress of vacuum-drying resembles anhydrobiosis, suggesting interrogation of desiccation-tolerant organisms could result in the discovery of new excipients. Many desiccation tolerant organisms, including *Caenorhabditis elegans*, *Saccharomyces cerevisiae,* and *Anopheles gambiae,* use the non-reducing disaccharide trehalose.[5-7]

Heterologous expression of a trehalose transporter improves the desiccation tolerance of Chinese hamster ovary cells,[8] and trehalose biosynthetic genes improve the drought resistance of rice.[9] *In vitro* studies show that high concentrations of trehalose protect lactate dehydrogenase (deHase) against vacuum drying-induced inactivation.[10-14] Trehalose also protects activity of the restriction enzymes *Bam*HI, *Eco*RI, and *Hind*III.[15,16]

Most sugars tested have some ability to protect enzymes against desiccation. The sucrose polymer Ficoll™ 70 fully protects lactate deHase, although a higher g/L concentration is required than for trehalose.[14] The glucose polymer dextran 20, disaccharide sucrose, and sugar alcohol mannitol partially inhibit lactate deHase inactivation in a concentration dependent manner.[14] Additionally, Ficoll™ and dextran reduce desiccation-induced aggregation of the water-soluble T-REx293 proteome.[17] Maltodextrin and raffinose protect the restriction enzyme *Eco*RI against desiccation-induced inactivation, although the authors were unable to quantify this protection.[16] The disaccharide maltose partially protects lactate deHase activity at intermediate concentrations, but protection diminishes at both low and high concentrations.[14] Of sugars tested in the compiled publications, only glucose has no protective ability.[14] The protective ability of sugars may depend on the absence of reactive carbonyl groups.[14]

The sugars trehalose, raffinose, mannitol, and glucose are also osmolytes, a class of molecules that restore homeostasis in organisms under osmotic stress. However, only sugar-based osmolytes protect enzymes against desiccation. The osmolyte and sugar alcohol mannitol has some ability to protect lactate deHase, but even at high concentrations it protects only a fraction of the enzyme.[14] The amino acid

osmolyte glycine, and its methylated form, betaine, have no ability to protect lactate deHase.[14]

Although the sugar polymers dextran and Ficoll™ are protective, the synthetic polymer polyethylene glycol has no ability to protect lactate deHase against vacuum-drying regardless of the concentration used.[14] Polyethyleneimine provides partial protection to LDH and polyvinylpyrrolidone protects *Eco*RI during vacuum-drying, although protection of the latter was not quantified.[16,18] These mixed result suggest that macromolecular crowding does not confer desiccation tolerance.

Organisms across all kingdoms of life also synthesize intrinsically-disordered proteins in response to stress.[19] These proteins are often classified as hydrophilins, defined by Garay-Arroyo *et al.*[20] as having high glycine content (>6%) and a high hydrophilicity index[21] (>1.0). Late embryogenesis abundant (LEA) proteins are likely best known of this family. Their heterologous expression increases the desiccation tolerance of yeast and the hyperosmotic tolerance of human cells.[22,23] During vacuum drying, LEA proteins reduce desiccation-induced aggregation of the water-soluble T-REx293 and *Aphelenchus avenae* proteomes and partially protect ADP-glucose-pyrophosphorylase and glucose-6-phosphodeHase activity in the soluble leaf proteome of *Arabidopsis thaliana*.[17,24,25] *In vitro,* LEA proteins protect lactate deHase, fumarase, and citrate synthase activity and inhibit the latter's aggregation.[12,13,17,22,24,26] Additionally, formulation with LEA proteins reduces the loss of fluorescence of the protein mCherry during vacuum-drying and rehydration.[17]

Another family of intrinsically disordered proteins, cytosolic abundant heat soluble (CAHS) proteins, is required by tardigrades to survive desiccation.[10]

Heterologous expression of CAHS proteins increases the desiccation tolerance of both *Escherichia coli* and *Saccharomyces cerev*isiae, and, *in vitro,* CAHS proteins protect lactate deHase against desiccation.[10,14] Mitochondrial abundant heat soluble proteins from tardigrades increase the hyperosmotic tolerance of human cells, but these proteins have not yet been studied as protectants *in vitro*.[23]

Despite no link to desiccation tolerance, many other proteins protect enzymes against vacuum-drying *in vitro*. The most common globular protein control for vacuum-drying experiments, bovine serum albumin (BSA), protects activity of lactate deHase, and citrate synthase and prevents aggregation of the latter, although it does not protect activity of fumarase or fluorescence of the red fluorescent protein mCherry.[10,12-14,17,22,26] The disordered signaling protein flgM protects lactate deHase more effectively than CAHS proteins against vacuum-drying induced inactivation.[14] Ubiquitin and an SH3 domain are similarly effective protectants.[14] α-Crystallin and β-lactoglobulin have some protective ability.[13,24] Ribonuclease A, thaumatin from the pathogenesis-related protein family 5,[27] and the chaperone HSP70 have no protective ability,[17,22,24] but this could result from an insufficient amount of additive.

That so many proteins with well-studied functions and no known link to desiccation tolerance protect enzymes against vacuum-drying induced inactivation suggests that protection is not conferred by a particular amino acid sequence.[14] Rather, protection is a general property of proteins that are themselves resilient to desiccation.[14]

Table 1.1. Ability of additives to protect proteins against vacuum-drying.

Test Protein	Assay	Output	Additives tested	Protection
ADP-glucose-pyrophosphorylase	activity	absorbance	β-lactoglobulin LEA protein ribonuclease A	none[24] partial[24] none[24]
Aphelenchus avenae proteome	aggregation	light scattering	LEA protein	full[25]
*Bam*HI	activity	DNA digestion	trehalose	partial[15]
citrate synthase	activity aggregation	absorbance light scattering	BSA LEA protein BSA HSP70 LEA protein	partial[12] full[12] none[12] none[17] full[12,17]
*Eco*RI	activity	DNA digestion	maltodextrin polyvinylpyrrolidone raffinose sucrose trehalose	partial[16] partial[16] partial[16] partial[16] partial[15,16]
fumarase	activity	activity	BSA LEA protein	none[26] partial[26]
glucose-6-phospho-deHase	activity	absorbance	β-lactoglobulin LEA protein ribonuclease A	partial[24] partial[24] none[24]
*Hin*dIII	activity	DNA digestion	maltose sucrose trehalose	partial[15] partial[15] partial[15]
lactate deHase	activity	absorbance	α-crystallin β-lactoglobulin betaine BSA CAHS protein dextran glucose glycine Ficoll® flgM LEA protein maltose mannitol pathogenesis-related group 5 proteins polyethyleneimine polyethylene glycol ribonuclease A SH3 sucrose trehalose ubiquitin	partial[13] partial[24] none[14] full[10,14], partial[12,13,22] full[10,14] full[14] none[14] none[14] full[14] full[14] partial[12,13,22,24] partial[14] partial[14] none[22] partial[18] none[14] none[24] full[14] partial[14] partial,[11,13] full[10,12,14] full[14]
mCherry	conformational change	fluorescence	BSA LEA protein	none[17] partial[17]
T-REx293 proteome	aggregation	light scattering	dextran Ficoll® LEA protein	partial[17] partial[17] full[25], partial[17]

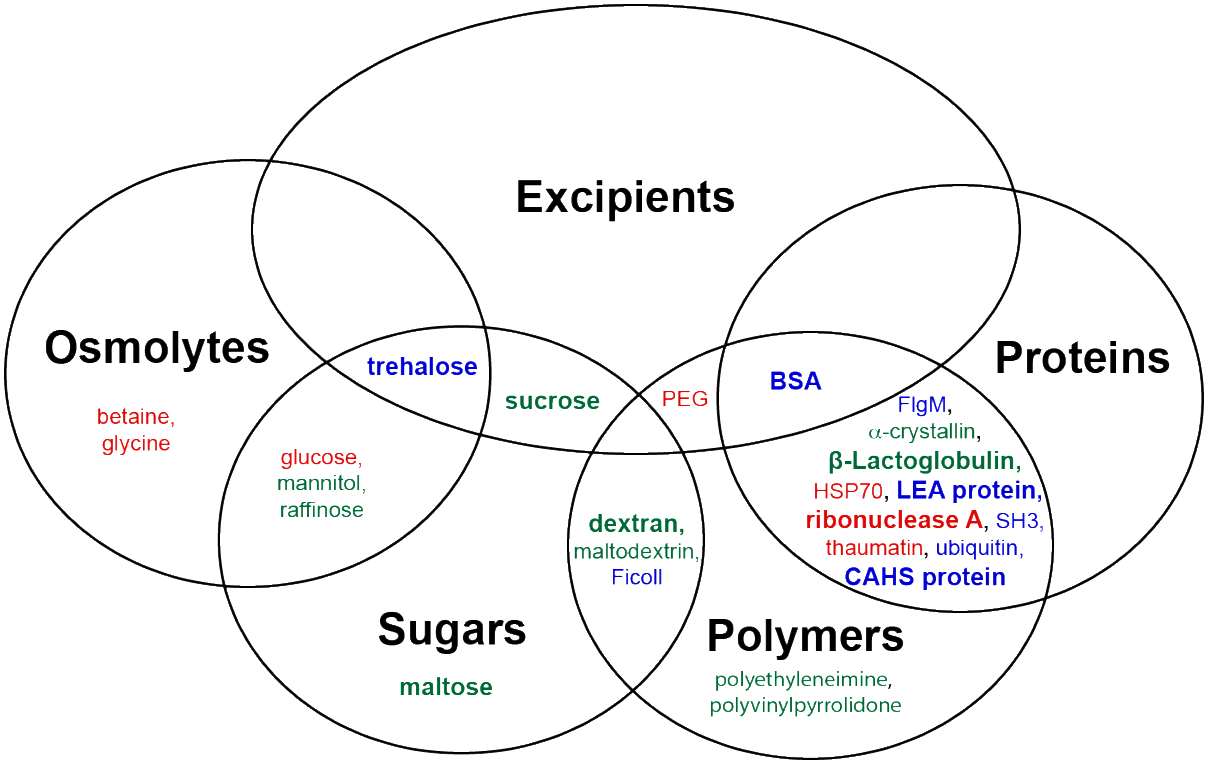


Figure 1.1. Venn diagram of additives tested as potential protectants against vacuum-drying and their categorization. Most additives reside in multiple categories. Additives in blue have at least one report of full protection. Additives in green have no reports of full protection but at least one report of partial protection. Additives in red have no reports of protection. Bolding represents two or more reports in the literature.

Freeze-thaw Stress

Degradative chemistry usually requires the dynamic motion present in liquid formulations. Therefore, it can be advantageous to freeze enzymes, but freezing often damages enzymes.

We have compiled reports of enzyme protection during freeze-thaw stress (Table 2). This table summarizes protection against the fewest number of freeze thaw cycles reported in each paper. Readers should be cautious comparing studies because several report that the cooling rates during freezing influence enzyme survival.[28-30] The highest degree of protection reported for each additive is further summarized in Figure 1.2.

In cells, trehalose protects both membranes and proteins against freeze-thaw damage.[31] Exogenous trehalose increases the tolerance of a variety of cells and organelles including *Lactobacillus bulgaricus*, *S. cerevisiae*, isolated thylakoids, and ram spermatozoa.[32-35] Heterologous expression of the *Staphylococcus aureus* hemolysin transmembrane pore allows trehalose uptake and improved cryopreservation of 3T3 fibroblasts and human keratinocytes.[36] However, reports of *in vitro* enzyme protection are inconsistent. There are conflicting reports for cryoprotection of lactate deHase.[11,37-42] Furthermore, trehalose fully protects phosphofructokinase[39] but fails to protect lipoprotein lipase.[14]

Like trehalose, inconsistent results are reported for many other sugars. One study reports full protection of lactate deHase by glucose, but another reports no cryoprotection by this sugar.[37,41] The same two studies test three different cyclodextrin variants -- β-cyclodextrin, 2,6-Di-O-methyl-β-cyclodextrin, and hydroxypropyl-β-cyclodextrin – and report no protection to full protection of the enzyme. [37,41] Most studies report full protection of lactate deHase by sucrose,[39-43] but other experiments report incomplete protection of lactate deHase and phosphofructokinase.[42,44] Similarly, maltose fully protects lactate deHase and partially protects phosphofructokinase against freeze-thaw stress.[39] Dextran is reported to cryoprotect fully both catalase and lactate deHase,[41,45] and Anchordoquy *et al.* report full protection of lactate deHase by Ficoll.[41] Despite this variability in these reports, at least one article reports full protection for every sugar molecule tested. It is likely that reports of partial or no protection result from an insufficient amount of sugar used, inhospitable buffer conditions, or other easily altered conditions.

The sugars trehalose, glucose, and cyclodextrin also function as osmolytes. Like sugars, reports of protection by osmolytes are inconsistent. Two reports observe partial protection of lactate deHase activity by betaine, one of which also reports protection of lactate deHase structural integrity *via* tryptophan fluorescence.[22,40] However additional experiments report no protection of lactate deHase but full protection of phosphofructokinase activity by betaine.[40,42] Similarly, ectoine and its derivatives demethylectoine, homoectoine, and hydroxyectoine provide full or partial protection to lactate deHase and full protection to phosphofructokinase.[39,40,42] Glutamate is reported to partially protect alcohol deHase, malate deHase, and pyruvate kinase.[42,46] Other studies, however, report full, partial, and no protection of lactate deHase,[37,46,47] and one study reports no protection of glucose-6-phosphodeHase.[46] Glycerol partially protects catalase activity and fully protects lactate deHase activity.[40,45] The N-acetylated diamino acids Nα-acetyllsyine, Nε-acetyllsyine, Nα-acetylornithine, and Nδ-acetylornithine fully protect the structure of lactate deHase as assessed by tryptophan fluorescence but only partially protects its activity.[40,42] The opposite is true of proline, which fully protects lactate deHase activity but only partially inhibits structural perturbation of this enzyme.[40,48] Trimethylamine-N-oxide fully protects lactate deHase activity and structure, but there is only one report.[42] Like sugars, there is at least one report of full protection for every osmolyte tested. Again, it is likely that reports of partial or no protection result from insufficient osmolyte concentration, inhospitable buffer conditions, or other easily modified conditions.

Similar to their behavior in desiccation tolerance, intrinsically disordered LEA proteins fully protect lactate deHase and citrate synthase *in vitro* and partially protect

ADP-glucose-pyrophosphorylase and glucose-6-phophodeHase in the soluble leaf proteome of *A. thaliana* from inactivation.[12,17,22,24,38,44,49-52] Two hydrophilins outside of the LEA protein family also protects lactate deHase activity.[38,53] Futhermore, an intrinsically disordered CAHS protein protects lipoprotein lipase against freeze-thaw-inactivation.[14] All three classes of intrinsically disordered proteins are more effective activity protectants than the globular protein BSA. Nevertheless, BSA protects the enzymes malate deHase and pyruvate kinase in addition to lactate deHase, citrate synthase, glucose-6-phophodeHase, and lipoprotein lipase.[12,14,22,43,44,46,49-52,54]

Among globular proteins, partial and full lactate deHase protection is reported for lysozyme and ovalbumin.[43,44,49,50] Partial cryoprotection of lactate deHase is reported for α-crystallin, apo-transferrin, cytochrome *c*, myoglobin, and phosphorylase b.[38,50] Ribonuclease A has no cryoprotective effect on ADP-glucose-pyrophosphorylase and glucose-6-phosphodeHase in the soluble leaf proteome of *A. thaliana*,[24] yet full, partial, and no protection are reported for lactate deHase *in vitro*.[24,38,44,50,51] Both full and no cryoprotection are reported for proteins from the pathogenesis-related protein family 5.[22,43] Like ribonuclease A, β-lactoglobulin has no cryoprotective effect on ADP-glucose-pyrophosphorylase and glucose-6-phosphodeHase in the soluble leaf proteome of *A. thaliana*,[24] but multiple groups report partial protection of lactate deHase activity.[24,44,50,51] Although full protection is reported for only a subset of these globular proteins, at least one study reports partial cryoprotection by each protein. It is likely that reports of no protection result from insufficient concentration, incompatible buffer conditions, or other facile variables. Furthermore, taken together, these results suggest that cryoprotection of proteins by other proteins is not a sequence specific property, but

rather a result of crowding. Consistent with this idea, lactate deHase, malate deHase, alcohol deHase, pyruvate kinase, phosphofructokinase, and β-galactosidase all retain more activity when frozen at higher concentrations in the absence of additives.[37,39,46,54-56]

In addition to these proteins, several synthetic polymers confer cryoprotection to enzymes. Polyethylene glycol fully protects both lactate deHase and phosphofructokinase,[28,37,41,57,58] and reports of full and partial cryoprotection of lactate deHase are reported for the related polymer polyethylene glycol dodecyl ether.[28,41]. Polidocanol, a short polyethylene glycol molecule attached to a hydrated carbon chain also partially cryoprotects lactate deHase.[37] Polyvinylpyrrolidone fully protects lactate deHase and catalase.[45,54] and polyethyleneimine partially protects lactate deHase against freeze-thaw.[18] The emulsifier polysorbate partially protects lactate deHase.[41] Poly L-lysine has no ability to protect lactate deHase, but only one concentration was tested.[38]

Some studies report increased enzyme protection with increased molecular weight of the synthetic polymer or sugar polymer additive.[18,41,58] Given the ability of numerous monosaccharides, disaccharides, and osmolytes to protect enzymes against freeze-thaw stress, additional studies are needed to determine the influence of macromolecular crowding.

Table 1.2. Ability of additives to protect proteins against freeze-thaw stress.

Test Protein	Assay	Output	Additives tested	Protection
ADP-glucose-pyrophosphorylase	activity	absorbance	β-lactoglobulin LEA protein ribonuclease A	none[24] partial[24] none[24]
alcohol deHase	activity	absorbance	BSA glutamate	none[46] partial[46]
catalase	activity	absorbance	dextran dimethyl sulfoxide glycerol polyvinylpyrrolidone	full[45] full[45] partial[45] full[45]
citrate synthase	activity aggregation	absorbance light scattering	BSA LEA protein BSA LEA protein	full[12] full[12] partial[12] full[12,17]
glucose-6-phosphodeHase	activity	absorbance	β-lactoglobulin BSA glutamate LEA protein ribonuclease A	none[24] partial[46] none[46] partial[24] none[24]
lactate deHase	activity	absorbance	α-crystallin apo-transferin β-lactoglobulin β-cyclodextrin betaine BSA CHAPS cytochrome *c* demethylectoine dextran DM-β-cyclodextrin ectoine Ficoll® glucose glutamate glycerol homoectoine HP-β-cyclodextrin hydrophilins hydroxyectoine LEA proteins lysine lysozyme maltose myoglobin Nα-acetyllsyine Nε-acetyllsyine Nα-acetylornithine Nδ-acetylornithine ovalbumin pathogenesis-related group 5 proteins phosphorylase b	partial[38] partial[50] partial[24,44,50,51] none[37] none,[39] partial[40,42] full,[22,43,44,49,52,54] partial,[46,50,51] none[53] partial[37] partial[50] partial[40] full[41] partial[37] full[39], partial[40] full[41] none,[37] full[41] full,[46] none,[37] partial[47] full[40], partial[56] partial[40] full,[41] partial[37] full,[38] partial[53] full[39,40] full,[22,38,49,52] partial[24,44,50,51] partial[47] full,[49] partial[50] full[39] partial[50] partial[40] partial[40] partial[40] partial[40] full[43], partial[44] full,[43] none[22] partial[50]

Test Protein	Assay	Output	Additives tested	Protection
			polidocanol	partial[37]
			polyethyleneimine	partial[18]
			polyethylene glycol	full[28,37,41,57,58]
			PEG dodecyl ether	full[28], partial[41]
			poly L-lysine	none[38]
			polysorbate	full[30], partial[41]
			polyvinylpyrrolidone	full[54]
			proline	full[40,48]
			ribonuclease A	full,[50] none,[24,38] partial[44,51]
			SDS	none[37]
			sucrose	full[39-41,43], partial[44]
			sucrose fatty acid monoester	partial[37]
			TMAO	full[42]
			trehalose	full,[11,39,41] none,[37] partial[38,40]
	aggregation	light scattering	BSA	partial[12]
			dehydrins	full[49]
			LEA protein	partial[12]
	structural perturbation	tryptophan fluorescence	betaine	partial[42]
			ectoine	full[42]
			hydroxyectoine	full[42]
			Nα-acetyllsyine	full[42]
			Nε-acetyllsyine	full[42]
			Nα-acetylornithine	full[42]
			Nδ-acetylornithine	full[42]
			proline	partial[48]
			sucrose	full[42]
			TMAO	full[42]
			trehalose	full[42]
lipoprotein lipase	activity	fluorescence	BSA	partial[14]
			CAHS protein	full[14]
			trehalose	none[14]
malate deHase	activity	absorbance	BSA	partial[46]
			glutamate	partial[46]
phosphofructokinase	activity	absorbance	betaine	full[39]
			ectoine	full[39]
			hydroxyectoine	full[39]
			maltose	partial[39]
			polyethylene glycol	full[57]
			sucrose	full[39]
			trehalose	full[39]
pyruvate kinase	activity	absorbance	BSA	partial[46]
			glutamate	partial[46]

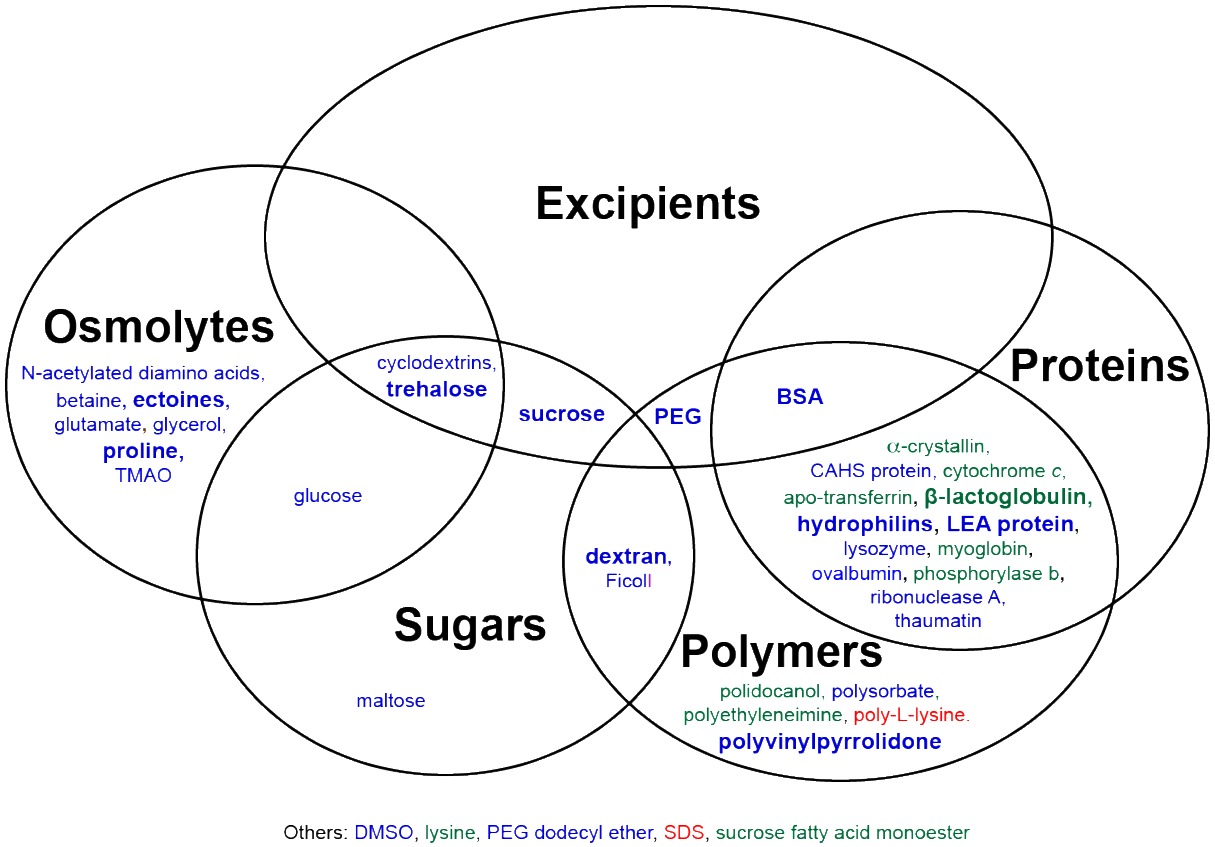


Figure 1.2. Venn diagram of additives tested as potential protectants against freeze-thawing and their categorization. Most additives fall into multiple categories. Additives in blue have at least one report of full protection. Additives in green have no reports of full protection but at least one report of partial protection. Additives in red have no reports of protection. Bolding represents two or more reports.

Freeze-drying Stress

In addition to vacuum-drying, water can be removed from frozen samples via lyophilization, which combines freeze-thaw stress and vacuum-drying stress. We have compiled reports of enzyme protection during freeze-dry stress (Table 1.3). The highest degree of protection for each additive is further summarized in Figure 1.3.

Trehalose exhibits mixed results. Formulating trehalose with *Lactobacillus reuteri* CICC6266 cells protects native lactate deHase and ATPase,[59] and trehalose provides full protection to β-galactosidase and L-asparaginase.[55,60] Alkaline phosphatase

lyophilized with trehalose even retains activity for up to 84 days.[61] However, trehalose confers only partial protection to lipase and mannitol deHase.[62,63] Trehalose was most frequently studied with lactate deHase, but at best, only partial protection is reported.[37,39,40,55,57,62,64] Furthermore, trehalose did not protect the enzymes phosphofructokinase and lipoprotein lipase against lyophilization-induced inactivation.[14,57]

Varied results are also reported for other sugars. Sucrose fully protects lactate deHase in *L. reuteri* CICC6266 cells, L-asparaginase activity in lyophilized formulations, and inhibits aggregation of monoclonal antibodies.[59,60,65] However, others report that sucrose partially protects lactate deHase and phosphofructokinase, and does not protect ATPase in *L. reuteri* CICC6266 cells.[39,40,59,66,67] Maltose fully protects L-asparaginase, but only partial protection is reported for lactate deHase and phosphofructokinase.[39,60,67] Glucose fully protects β-galactosidase and L-asparaginase, but does not protect lactate deHase or phosphofructokinase activity.[37,55,57,60,64] Similarly, lactose fully protects alkaline phosphatase and L-asparaginase, but no or partial protection are reported for lactate dehase.[57,60,61,64,67] Two studies examine five cyclodextrin variants -- α-cyclodextrin, β-cyclodextrin, γ-cyclodextrin, 2,6-Di-O-methyl-β-cyclodextrin, and hydroxypropyl-β-cyclodextrin – which confer, at best, only partial protection to β-galactosidase and lactate deHase.[37,55] Additional reports examine the sugar alcohols mannitol and sorbitol. Mannitol fully protects L-asparaginase and mixed results are reported for lactate deHase, and no protection is observed for alkaline phosphatase and phosphofructokinase.[57,60,61,64,66] Sorbitol partially protects lactate deHase, but no protection is observed when formulated with lipase.[62] Similarly to these

sugar alcohols, glucosylglycerol fully protects and glucosylglycerate partially protects mannitol deHase.[63] If we consider the greatest degree of protection reported, all these sugars can confer at least partial protection against freeze-drying.

The sugars trehalose, glucose, cyclodextrin, mannitol, sorbitol, glucosylglycerol, and glucosylglycerate are also classified as osmolytes. Reports of protection are less frequent among non-sugar osmolytes. Glutamate and proline fully protect β-galactosidase against freeze drying; although glutamate only partially protects LDH, and proline confers no protection to phosphofructokinase.[37,40,55] Betaine partially protects phosphofructokinase but does not protect lactate deHase or lipase.[39,40,62] However, chemical derivatives of betaine, dimethylthetin and homodeanol betaine, have a limited ability to protect lactate deHase and lipase.[62] Similarly, ectoine provides no protection to lactate deHase but partial protection to phosphofructokinase; while it's chemical derivative hydroxyectoine partially protects both enzymes.[39,40] Glycerol is the only osmolyte with no documented protective ability, at least in limited studies with lactate deHase and phosphofructokinase.[40]

Few proteins have been studied as protectants during freeze-drying and subsequent storage. Anchordoquy *et al.* report that BSA fully protects lactate deHase.[54] Piszkiewicz *et al.* report that BSA partially protects, and a CAHS protein fully protects, lipoprotein lipase.[14] Furthermore, several enzymes protect themselves against freeze-drying with increasing concentration.[37,54,55] Additional work is needed to determine if other proteins that protect enzymes against vacuum drying- and freeze thaw-stress are as protective against freeze drying-induced inactivation.

Slightly more data have been published for protection by synthetic polymers. Polyvinylpyrrolidone fully protects both catalase and lactate deHase.[54,68] Polyethylene glycol confers partial protection of β-galactosidase, partial or no protection of lactate deHase, and no protection of phosphofructokinase.[37,55,57,64] Polyethyleneimine partially protects lactate deHase activity.[18] Only polidocanol is ineffective against freeze drying.[37,55] Nevertheless, these studies are too limited to draw robust conclusions.

Table 1.3. Ability of additives to protect proteins against freeze-drying.

Test Protein	Assay	Output	Additives tested	Protection
alkaline phosphatase	activity	absorbance	lactose	full[61]
			mannitol	none[61]
			trehalose	full[61]
ATPase	activity	absorbance	skim milk	none[59]
			sucrose	none[59]
			trehalose	full[59]
β-galactosidase	activity	absorbance	α-cyclodextrin	none[55]
			β-cyclodextrin	none[55]
			γ-cyclodextrin	none[55]
			CHAPS	partial[55]
			DM-β-cyclodextrin	partial[55]
			glucose	full[55]
			glutamate	full[55]
			HP-β-cyclodextrin	partial[55]
			polidocanol	none[55]
			polyethylene glycol	partial[55]
			proline	full[55]
			SDS	none[55]
			sucrose fatty acid monoester	partial[55]
			trehalose	full[55]
catalase	activity	absorbance	alanine	full[69]
			arginine	partial[69]
			dimethyl formamide	none[69]
			dimethyl sulfoxide	none[69]
			cysteine	none[69]
			glycine	full[69]
			histidine	full[69]
			lysine	full[69]
			polyvinylpyrrolidone	full[68]
			serine	full[69]
			threonine	full[69]
			valine	none[69]
			4-hydroxyproline	full[69]
L-asparaginase	activity	absorbance	glucose	full[60]
			lactose	full[60]
			maltose	full[60]
			mannitol	full[60]
			sucrose	full[60]
			trehalose	full[60]
	aggregation	light scattering	glucose	full[60]
			lactose	full[60]
			maltose	full[60]
			mannitol	full[60]
			sucrose	full[60]
			trehalose	full[60]
lactate deHase	activity	absorbance	α-cyclodextrin	none[37]
			β-cyclodextrin	none[37]
			γ-cyclodextrin	none[37]
			betaine	none[39,40,62]
			BSA	full[54]
			CHAPS	partial[37]

Test Protein	Assay	Output	Additives tested	Protection
			dimethylthetin	none[62]
			DM-β-cyclodextrin	partial[37]
			ectoine	none[39,40]
			glucose	none[37,57]
			glutamate	partial[37]
			glycerol	none[40]
			homodeanol betaine	partial[62]
			HP-β-cyclodextrin	partial[37]
			hydroxyectoine	partial[39,40]
			lactose	none[57,64], partial[67]
			maltose	partial[39,67]
			mannitol	none[57,64], partial[66]
			polidocanol	none[37]
			polyethyleneimine	partial[18]
			polyethylene glycol	none,[57,64] partial[37]
			proline	none[40]
			polyvinylpyrrolidone	full[54]
			SDS	none[37]
			skim milk	partial[59]
			sorbitol	partial[62]
			sucrose	full,[59] partial[39,40,66,67]
			sucrose fatty acid monoester	partial[37]
			trehalose	none,[57,64] partial[37,39,40,59,62,67]
lipase	activity	pH (NaOH titration)	betaine	none[62]
			dimethylthetin	partial[62]
			homodeanol betaine	none[62]
			sorbitol	none[62]
			trehalose	partial[62]
lipoprotein lipase	activity	fluorescence	BSA	partial[14]
			CAHS protein	full[14]
			trehalose	none[14]
mannitol deHase	activity	absorbance	glucosylglycerate	partial[63]
			glucosylglycerol	full[63]
			trehalose	partial[63]
monoclonal antibodies	aggregation	light scattering	sucrose	full[65]
phosphofructokinase	activity	absorbance	betaine	partial[39,40]
			ectoine	partial[39,40]
			hydroxyectoine	partial[39,40]
			glucose	none[57,64]
			glycerol	none[40]
			maltose	partial[39]
			mannitol	none[57,64]
			polyethylene glycol	none[57,64]
			proline	none[40]
			sucrose	partial[39,40]
			trehalose	none,[39,57,64] partial[40]

Table footnote: References[57,64] are a part 1, part 2 combo of the same project. Data in [64] is likely just a summary of the more detailed experiments in [57].

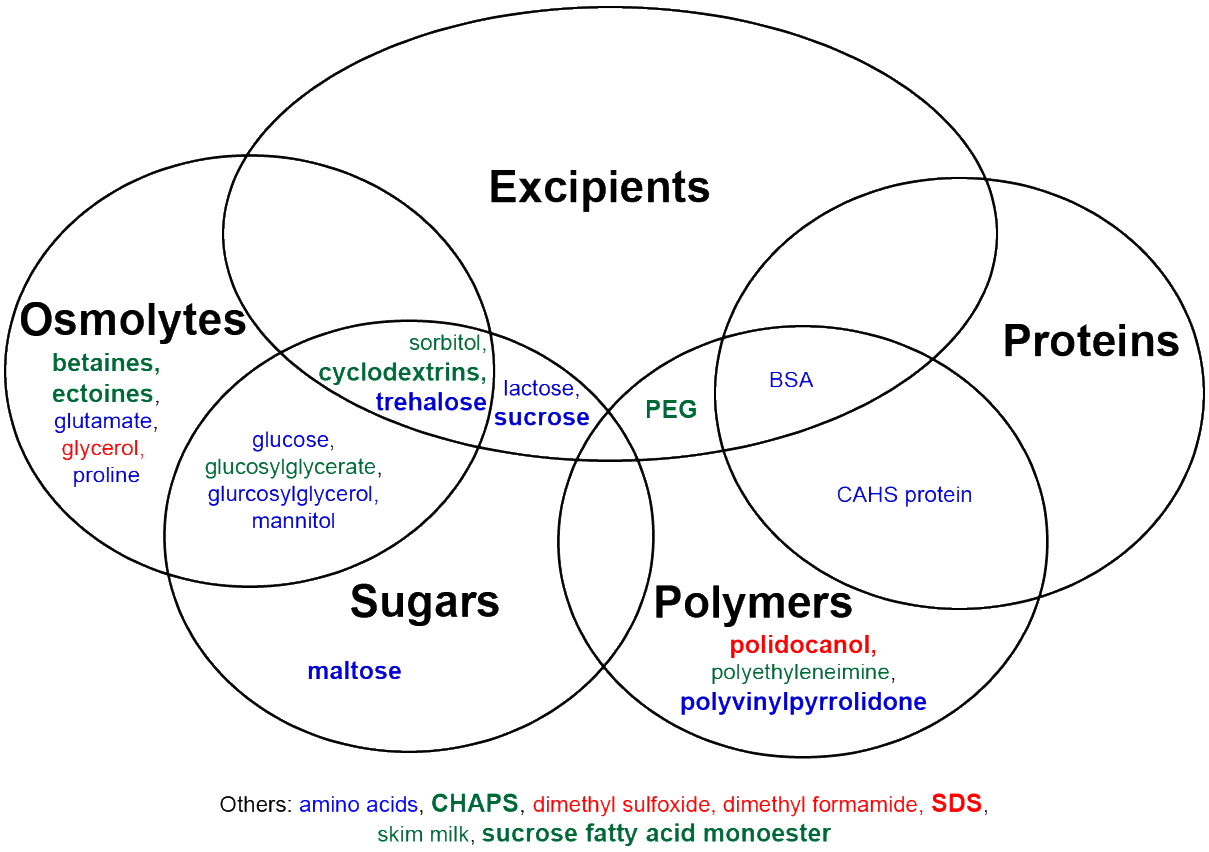


Figure 1.3. Venn diagram of additives tested as potential protectants against freeze-drying and their categorization. Most additives occupy multiple categories. Additives in blue have at least one report of full protection. Additives in green have no reports of full protection but at least one report of partial protection. Additives in red have no reports of protection. Bolding represents two or more reports in the literature.

Synergistic Protection

Several groups report protection as the result of synergistic interactions. Goyal *et al.* report protection of lactate deHase and citrate synthase against desiccation-induced inactivation by a mixture of LEA proteins and trehalose.[12] Tamiya *et al.* report synergistic protection of lactate deHase, malate deHase, glucose-6-phophodeHase, and pyruvate kinase against freeze/thaw by BSA combined with sodium glutamate.[46] Mannitol and glycine formulated in a 1:1 ratio protect lactate deHase from freeze-drying

inactivation.[29] This study, however, does not investigate the protective ability of these osmolytes individually.[29] Similarly, phenylalanine and arginine together protect lactate deHase against vacuum drying inactivation and inhibit granulocyte colony-stimulating factor aggregation, but these amino acids are not tested individually.[70] Carpenter *et al.* and Prestrelski *et al.* observe that polyethylene glycol, which protects against freeze/thaw, can be combined with trehalose, glucose, or lactose, which protect against water removal, to protect lactate deHase and phosphofructokinase against freeze-drying.[57,64] Miller *et al.* report that combining borate with trehalose synergistically improves long-term storage of lactate deHase after vacuum-drying.[11]

Each report investigates a limited set of potential synergistic interactions with little or no follow up. More thorough studies are needed to confirm the conclusions and understand the mechanism(s) leading to synergism.

Potential Mechanisms

Many studies suggest potential mechanisms for protection. Several groups suggest that vitrification, when additives form an amorphous glass rather than ordered crystals as they dry, can protect the enzymes encased by them and investigate this idea using scanning calorimetry to observe the glass transition temperature.[10,11,16,28,29,67,70,71] To further support this idea, several groups observe vitrification in desiccated organisms.[10,72,73] However, a few groups contest this theory.[41,57]

Numerous groups report inhibition of aggregation during vacuum drying,[7,12,17,24,25] feeze-thaw,[49] and freeze-drying.[55] Chakrabortee *et al.* propose the molecular shield hypothesis, in which shield molecules use physical interference to reduce the frequency of cohesive interactions between aggregating species.[17] This protection is conferred

through non-specific interactions as opposed to chaperone activity. Several studies consider this hypothesis directly[12,17,49] or describe a similar phenomenon.[58,60]

Other groups report damage to enzyme structure as a result of these stresses. Circular dichroism and Fourier transform infrared spectroscopy are used to observe damage to secondary structure.[24,48,58,64,66] Others report inhibition of enzyme subunit dissociation[41,54,58,74] and suggest that maintenance of quaternary structure can compensate for lack of water replacement during drying.[41,54] Several studies attribute dissociation to the precipitation of sodium phosphate buffer during freezing and resulting acidification of the sample.[46,47,54,56] However, this explanation fails to account for dissociation in other buffer conditions or other stresses.

Seguro *et al*. suggest that cryo-denaturation is most likely caused by ice crystal formation.[47] In addition to freeze-thaw protection, a few groups report inhibition or slowing of ice crystal formation by additives that may, in turn, prevent enzyme damage.[28,52,58] Hillgren *et al.* postulate that surfactants may protect enzymes by covering ice crystals as they form,[28] which could explain why 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS), polidocanol, polyethylene glycol dodecyl ether, polysorbate, and sucrose fatty acid monoester are able to protect some enzymes against freeze-thawing and freeze-drying induced inactivation.[28,30,37,41,55] Nevertheless, this explanation fails to explain protection against vacuum-drying.

Conclusions

Although dozens of studies have investigated how to protect enzymes against vacuum-drying, freeze-thaw, and freeze-drying stress, there remains much we do not understand. Some molecules have no protective ability in one report and full protective

ability in another. Often, results probably represent false negatives because an insufficient quantity of additive is used. For this reason, we recommend testing a range of additive concentrations. Additionally, most papers study only a small subset of additives. More studies are needed that directly compare a larger number of these additives under the same experimental conditions. These suggestions should also be applied to the investigation of synergistic interactions that enhance protection.

Researchers are further divided on the mechanisms behind protection. Additional work is needed to understand the importance of vitrification and molecular shield theory among other potential mechanisms.

CHAPTER 2: PROTECTING ACTIVITY OF DESICCATED ENZYMES[1]

Overview

Protein-based biologic drugs and many industrial enzymes are unstable, making them prohibitively expensive. Some can be stabilized by formulation with excipients, but most still require low temperature storage. In search of new, more robust excipients, we turned to the tardigrade, a microscopic animal that synthesizes cytosolic abundant heat soluble (CAHS) proteins to protect its cellular components during desiccation. We find that CAHS proteins protect the test enzymes lactate dehydrogenase and lipoprotein lipase against desiccation-, freezing-, and lyophilization- induced deactivation. Our data also show that a variety of globular and disordered protein controls, with no known link to desiccation tolerance, protect our test enzymes. Protection of lactate dehydrogenase correlates, albeit imperfectly, with the charge density of the protein additive, suggesting an approach to tune protection by modifying charge. Our results support the potential use of CAHS proteins as stabilizing excipients in formulations and suggest that other proteins may have similar potential.

Impact Statement

Protein-based drugs called biologics are among the most effective therapeutic treatments on the market. However, these drugs are unstable and require refrigerated storage, which makes them expensive. Manufacturers increase the shelf-life of biologics

[1] This chapter is in press in the journal Protein Science. The citation is as follows: Piszkiewicz S, Gunn KH, Warmuth O, Propst A, Mehta A, Nguyen KH, Kuhlman E, Guseman AJ, Stadmiller SS, Boothby TC, Neher SB, Pielak GJ (2019) Protecting activity of desiccated enzymes. Protein Sci 28:941-951.

by adding excipients (molecules that protect active ingredients) but most still require refrigeration. Discovering better excipients would make these products more affordable and accessible, particularly for poor and remote populations without access to refrigeration. Furthermore, some potential biologics may never reach the market because of instability. Better methods to protect and stabilize biologics could provide new treatment options for numerous diseases. Here we describe a family of proteins from tardigrades that increase the shelf stability of two enzymes. This family has potential as an excipient for biologics and for formulating industrially useful enzymes.

Introduction

Biologics, protein-based drugs derived from living organisms, are among the most effective therapeutic treatments on the market. However, they are unstable, have short half-lives, and require low temperature storage, which makes them expensive. Environmentally friendly industrial enzymes often confront similar challenges. Manufacturers increase the half-life of biologics and enzymes by adding excipients, molecules that stabilize the active ingredient.[75]

Trehalose is a common excipient and osmolyte that is synthesized by many desiccation tolerant organisms.[5-7,72] This non-reducing disaccharide can increase the mid-point denaturation temperature of a protein by as much as 18°C and its modified standard-state Gibbs free energy of denaturation by almost 5 kcal/mol at physiologically relevant temperatures.[76] However, trehalose also enhances autophagy,[77,78] and can interfere with drug efficacy in treatments to repress autophagy in autoimmune diseases like lupus.[79] Albumedix produces a protein-based excipient, recombinant human serum albumin, which is FDA approved in five biologics including the M-M-R®II vaccine, the type II diabetes drug Tanzeum®, and the hemophilia treatment Idelvion®. Nevertheless,

biologics still require storage at temperatures as low as -80°C, even after formulation.[80] Discovery of better excipients would make these products more affordable and accessible.

We turned to tardigrades as a potential source of novel excipients. These microscopic animals survive conditions ranging from -274°C to 151°C, from vacuum to 6000 atm, 1000-times more radiation than the average organism, 10 d exposed to outer space, 30 y frozen, and up to 10 y of desiccation.[81-85] Genomic and transcriptomic data suggest that, unlike many desiccation tolerant organisms, some species of tardigrades lack trehalose phosphatase,[10,86-90] and therefore cannot synthesize this sugar. Nevertheless, tardigrades produce several families of intrinsically disordered proteins (IDPs), including the cytosolic abundant heat soluble (CAHS) family.[91] IDPs have many functions and are thought to play a role in stress tolerance across all kingdoms of life,[17,19,20,22,92] and *in vitro* the intrinsically disordered late embryogenesis abundant (LEA) proteins protect enzymes against environmental stress.[12,17,22,24,49] These observations led us to investigate whether CAHS proteins could perform a similar function. We showed that CAHS proteins allow tardigrades to survive desiccation and have been detected only in these organisms.[10] Furthermore, recombinant expression of CAHS proteins in yeast and bacteria enhances the desiccation tolerance of both organisms by over 100-fold.[10]

We studied the protection of two enzymes by CAHS proteins. Lactate dehydrogenase (LDH, Figure 2.1), is a 150 kDa tetramer with an isoelectric point (pI) of 6.0 that is frequently studied in stress tolerance of *in vitro* samples.[10,12,22,24,29,37,46,49,54] Here, we expand our initial results[10] by quantifying the protective properties of

osmolytes, sugars, synthetic polymers, globular proteins, disordered proteins, and CAHS proteins at room temperature and 95°C. We also studied protection of the unstable enzyme lipoprotein lipase (LPL, Figure 2.2), a 110 kDa dimer with a pI of 8.5 that possesses therapeutic potential for treating hypertriglyceridemia.[93-95]

Results

Protecting LDH against desiccation induced inactivation

LDH is commercially available and sensitive to H_2O loss from vacuum-drying.[10-12,54] The enzyme was desiccated in the presence of osmolytes, sugars, synthetic polymers, globular proteins, disordered proteins, and CAHS proteins (Figure 2.1), with some occupying multiple categories (Figure 2.2), using a Genevac® EZ-2 Personal Evaporator. Protection was quantified as the percent activity of the desiccated samples compared to unstressed controls (Figure 2.3).

Trehalose, a non-reducing glucose disaccharide, is the most protective sugar, most protective osmolyte, and most protective small molecule tested (Figure 2.3A). Ficoll 70, a branched 70 kDa sucrose polymer containing non-reducing linkages, and dextran 20, a 20 kDa complex branched glucose polymer containing reducing linkages, are also protective. Sucrose, the non-reducing disaccharide and the monomer of Ficoll, protects only up to 70% of activity (Supplemental Figure 2.1). Maltose, the reducing glucose disaccharide comprising dextran, protects up to 30% of activity at concentrations between 10 g/L and 50 g/L, but is not effective at higher concentrations. The sugar alcohol and osmolyte mannitol protects no more than 10% of LDH activity. The monosaccharide glucose, the osmolytes glycine and betaine, and the polymer polyethylene glycol (PEG) 3350 are ineffective (data not shown).

All proteins tested outperform trehalose (Figure 2.3A and 2.3B). Bovine serum albumin (BSA), a homolog of the excipient human serum albumin, is the least protective protein and ubiquitin the most protective on a g/L basis. The disordered protein flgM[96] and the stabilized SH3 domain, SH3 T22G (SH3),[97] are less protective than ubiquitin but more protective than BSA. Lysozyme protects LDH activity at low concentrations, but at high concentrations it inactivates LDH in the unstressed controls (data not shown).

The g/L-concentration and mole ratio of additive required for 50% protection (Figure 2.4) were then calculated. The CAHS homologs tested fall between SH3 and BSA (Figure 2.3C, Figure 2.4A) on a g/L basis. When these data are converted from g/L to mole ratio, CAHS G is the most protective with a confidence interval of at least 67% (Figure 2.4B).

Protecting desiccated LDH against heat inactivation

Next, we tested the ability of trehalose, BSA, flgM, ubiquitin, CAHS D, and CAHS G to protect the desiccated enzyme at 95°C (Figure 2.5). We chose a common additive concentration of 20 g/L because all additives tested protect LDH at this concentration (Figure 2.3). Trehalose has no protective ability above 80°C and at 95°C almost all activity is lost in 5 min (Supplemental Figure 2.2). All proteins show some ability to protect the desiccated enzyme at 95°C (Figure 2.5). BSA is the least protective with activity disappearing in about 4 h. Ubiquitin is the most protective, preserving activity for 2 h at 95°C before a decline. The potency of CAHS D and G fall between flgM and BSA, similar to their behavior at room temperature (Figure 2.3)

Protecting LPL against lyophilization-induced inactivation and long-term storage

We also investigated the ability of a limited selection of additives to protect bovine LPL, an enzyme that normally requires storage at -80°C (Figure 2.6). LPL was frozen or lyophilized in solutions of trehalose, BSA, and CAHS D. Protection was measured as percent activity compared to unstressed controls. Trehalose does not protect the enzyme against inactivation by either freeze/thaw stress or lyophilization. BSA and CAHS D protect slightly better against freeze/thaw-inactivation than lyophilization induced-inactivation, but CAHS D is more protective against both processes.

LPL was then lyophilized from solutions of trehalose, BSA, or CAHS D and its activity monitored at 4°C as a function of time (Figure 2.7). Consistent with the observations described above, trehalose is not protective at 4°C whether the samples are lyophilized or in solution. In solution, BSA and CAHS D were both slightly protective, but activity diminished over a week. BSA and CAHS D provided longer-term stabilization for lyophilized LPL stored at 4°C, with activity gradually declining over two months. In both cases, CAHS D outperforms BSA.

Discussion

Trehalose outperforms other sugars, polymers, and osmolytes as a protectant of LDH

The non-reducing sugars trehalose and the sucrose polymer Ficoll 70 protect LDH better than the reducing sugars maltose, glucose, and the glucose polymer dextran 20. Non-reducing sugars may be more effective protectants because they lack the reactive carbonyl group of reducing sugars. However, this idea does not explain why the reducing sugar dextran 20 is more protective than the non-reducing sugar sucrose.

Additionally, sugar size does not have a consistent effect. Both sugar polymers outperform the disaccharides sucrose and maltose but not the disaccharide trehalose. These observations may explain why trehalose is one of the most widely used excipients. The sugar alcohol and osmolyte mannitol protects poorly against desiccation-induced LDH inactivation. The non-sugar osmolyte betaine, despite its ability to stabilize proteins *in vitro* and in cells,[98-100] does not protect LDH against desiccation-induced inactivation. The same is true for the amino acid and osmolyte glycine.[99] Similarly, despite its use as an excipient and its ability to protect LDH against freeze/thaw and freeze/dry stress,[37] the synthetic polymer PEG 3350 does not protect LDH against desiccation-induced inactivation. With the exception of glucose, all sugars have some ability to protect LDH against desiccation-induced inactivation, but we find no other clear patterns among sugars, polymers, and osmolytes.

Proteins outperform trehalose

We chose the proteins ubiquitin, flgM, SH3, and BSA because they are easy to produce and store, and they represent two protein classes: globular and disordered. These proteins protect LDH against desiccation-induced inactivation at room temperature and 95°C more effectively than trehalose and other sugars, polymers, and osmolytes (Figure 2.3-2.5). BSA also protects LPL against freeze/thaw and lyophilization-induced inactivation Figure 2.6-2.7). By contrast, trehalose does not protect LDH from these stresses.

Protein protection and physical properties

We sought to explain the protective properties of proteins (Table 2.1). With the exception of SH3, their protective ability, measured in g/L, directly correlates to the total charge at pH 7 divided by the number of amino acid residues, which we call the

sequence charge density (Figure 2.8A). With two exceptions (discussed below), the more positive its sequence charge density, the better the protein protects negatively charged LDH (pI 5 to 6, depending on the isozyme).

SH3 deviates from this pattern, possibly because of its small size or its large surface hydrophobicity (Table 1). Lysozyme is the other exception. Its protective ability cannot be quantified because it inactivates LDH even in unstressed control solutions, consistent with its generally destabilizing effect.[101-104] In solution, test proteins are stabilized by crowders of the same charge and destabilized by crowders of the opposite charge.[98,101-111] If negatively charged CAHS proteins function by protecting more positively charged proteins against desiccation, then it is likely that the mechanism of desiccation tolerance differs from the mechanism that stabilizes test proteins under crowded conditions in solution.

We suggest that CAHS proteins (pI ~6) are best at protecting proteins with isoelectric points greater than 6. This idea holds for LDH and LPL, and perhaps tardigrades. In the species *Hypsibius exemplaris*, from which CAHS D and G are derived, 80% of proteins in predicted open reading frames have a higher pI than CAHS proteins (Figure 2.8B), consistent with our suggestion. This trend is also true for LEA proteins (pI ~5), an intrinsically disordered protein family required for desiccation tolerance in *Caenorhabditis elegans,*[112,113] which also protects enzymes against environmental stress.[12,22,24,114,115] 91% of proteins in predicted open reading frames of *C. elegans* have a higher isoelectric point than LEA proteins (Figure 2.8C).

CAHS proteins protect against multiple stresses

CAHS D and G protect LDH against desiccation-induced inactivation and heat-induced inactivation of the desiccated enzyme but do not outperform the control

proteins ubiquitin, flgM, and SH3 on a g/L basis. CAHS proteins and BSA also protect LPL against freeze/thaw. Most noticeably, CAHS proteins outperform BSA in stabilizing LPL after lyophilization and storage at 4°C. Tardigrades require CAHS proteins to survive desiccation, but not freeze/thaw stress,[10] suggesting redundant strategies to protect their cellular components against freeze/thaw stress. CAHS proteins may arrest formation of ice crystals, or they may protect enzyme activity orthogonally to antifreeze proteins.[116] Either way, CAHS proteins may be valuable for organ preservation.[117-120] Other proteins with no known link to freeze/thaw or desiccation tolerance may also have these protective properties.

Conclusions

Intrinsically disordered CAHS proteins from tardigrades can protect test enzymes against desiccation-, freezing-, and lyophilization- induced deactivation, consistent with the idea that protection of enzymes and other globular proteins is part of their native function. Our data also show that a variety of globular and disordered protein controls with no known link to desiccation tolerance can protect test enzymes. LDH protection correlates, albeit imperfectly, with the sequence charge density of the protein additive, suggesting a method to tune the protective ability of CAHS proteins or other protective proteins by modifying their charge.

CAHS proteins may also be optimized in other ways to confer desiccation tolerance. For instance, they may be more effective at long-term protection against temperature fluctuations, as would be experienced with the changing of seasons. CAHS proteins may protect membranes in addition to proteins. CAHS proteins could also be inert to essential biochemical pathways compared to other proteins. Additionally, their intrinsic disorder and the disorder of other proteins linked to desiccation tolerance may

facilitate degradation after rehydration. Additional studies are needed to explore these possibilities. Nonetheless our results support the potential use of CAHS proteins as a stabilizing excipient in biologic formulations.

Materials and Methods

Commercial additives

Betaine and Ficoll 70 were purchased from Sigma Life Science. PEG 3350 and sucrose were purchased from Sigma Chemical. Bovine serum albumin and lysozyme were purchased from Sigma. D-Glucose was purchased from MP Biomedicals. D-Maltose was purchased from Fluka Biochemika. D-Mannitol was purchased from Acros Organics. D-Trehalose was purchased from Aldrich Chemical and Acros Organics. Dextran 20 was purchased from Alfa Aesar. Glycine was purchased from Fisher Chemical. H_2O with a conductivity of 17 $M\Omega cm^{-1}$ was used for protein purification and LDH assays.

CAHS D purification

Plasmids for CAHS D, also known as CAHS 94063, were engineered and transformed into BL21star(DE3) cells as described.[10] A single colony was used to inoculate 10 mL of Lennox broth (LB, 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) supplemented with the antibiotic kanamycin to a final concentration of 60 μg/mL. The culture was shaken at 37°C overnight (New Brunswick Scientific I26 incubator, 225 rpm). This 10 mL culture was used to inoculate 1 L of kanamycin supplemented LB.

One-L cultures were shaken at 37°C until the optical density at 600 nm was greater than 0.4 but less than 0.8 (BioRad SmartSpec Plus spectrophotometer). Isopropyl β-D-1-thiogalactopyranoisde (1mM final concentration) was added to induce

expression. After 4 h, cells were pelleted at 1,000*g* at 10°C for 30 min (Sorvall Instruments RC-3B). Pellets were stored at -20°C.

Three cell pellets were resuspended in 15 mL of 25 mM Tris/HCl (pH 7.0) supplemented with 50 µL protease inhibitors (Sigma-Aldrich P2714 containing 40 mM AEBSF, 6 µM aprotinin, 2.3 mM bestatin, 280 µM E-64, 20 µM leupeptin, and 20mM EDTA) per cell pellet and then heat shocked at 95°C for 15 min. The heat-insoluble fraction was removed by centrifugation at 20,000*g* for 30 min (Du Pont RC-5B) at room temperature. The supernatant was mixed with two volumes of 8 M urea containing 50 mM sodium acetate (pH 4.0), which had been passed through a 0.45 µm filter (Millipore SLGVM33RS). This and all other urea solutions were deionized with 5 g/L Dowex® MB Mixed Ion Exchange Resin (Sigma) before filtering out the resin with a 0.22 µm filter (Corning Inc. 431161) and adding buffer salts.

Cation exchange chromatography was performed at room temperature (GE AKTA Start, 5 mL GE HiTrap SP HP) in 8 M urea, 50 mM sodium acetate (pH 4.0) with a 28-column-volume gradient of 0% to 50% 1 M NaCl. SDS-PAGE (Bio-Rad 4-20% Criterion™ TGX™ Gels) was used to identify fractions containing pure CAHS D. The fractions were pooled, filtered (0.45 µm), and transferred to 10,000 MWCO dialysis tubing (Fisher 68100). Samples were dialyzed against 2 M urea, 20 mM Tris/HCl (pH 7.5) for a minimum of 3 h followed by four changes of 20 mM Tris/HCl (pH 7.5) and one change of H_2O for at least 3 h each. The samples were flash frozen in a CO_2(s)/ethanol bath and lyophilized for 48 h (Labconco FreeZone) before storage at room temperature.

The purity of CAHS D was confirmed using a ThermoScientific Q Exactive HF-X mass spectrometer. Lyophilized CAHS D was resuspended at a concentration of ~1 g/L

protein in autoclaved H_2O and filtered (0.20 µm, Millipore SLHVM33RS). The solution was diluted 1:1 with acetonitrile before injection.

CAHS G purification

CAHS G, also known as CAHS 89226,[10] was expressed in BL21star(DE3) cells as described above. Two cell pellets were resuspended in 20 mL of 25 mM Tris/HCl (pH 7.0) supplemented with protease inhibitors and heat shocked as described above. The supernatant was mixed with 2 volumes of 3 M urea, 20 mM Tris/HCl (pH 9.0) that had been passed through a 0.45 µm filter.

Anion exchange chromatography was performed at room temperature on the AKTA Start (5 mL GE HiTrap SP Q) in 3 M urea, 20 mM Tris/HCl (pH 9.0) with a 24-column-volume gradient of 0% to 100% 150 mM NaCl. SDS-PAGE was used to identify the fractions containing pure CAHS G. These fractions were pooled, filtered (0.45 µm), and transferred to 10,000 MWCO dialysis tubing. Samples were dialyzed against 20 mM Tris/HCl (pH 7.5) for a minimum of 4 h followed by five changes of H_2O for at least 3 h each. The samples were flash frozen, lyophilized, and stored as described for CAHS D. Purity was determined only by SDS PAGE because CAHS G is insoluble under the conditions used for mass spectrometry.

FlgM

A plasmid harboring the FlgM gene[121] was used to express the protein in *E. coli* BL21star(DE3) cells using the protocol above, except that antibiotic ampicillin was used for selection (100 µg/mL final concentration). Three pellets were resuspended in 15 mL of 50mM Tris/HCl (pH 7.5) supplemented with protease inhibitors. The heat-soluble fraction was collected as described above.

Anion exchange chromatography was performed at 4°C (GE AKTA FPLC, GE Q Sepharose column, 1.6 cm × 10 cm) in 50 mM Tris/HCl (pH 7.5) using a gradient of 2.5% to 50% 2 M NaCl. FlgM does not bind. The flgM in the flow through was further purified by size-exclusion chromatography (GE Superdex 75 column, 1.6 cm x 600 cm eluted with 50 mM Na_2HPO_4, 20 mM KH_2PO_4, 9 mM NaCl, pH 7.4). Purified protein was dialyzed against H_2O for 4 h at room temperature or overnight at 4°C. Dialyzed samples were flash frozen in a CO_2(s)/ethanol bath and lyophilized.

SH3

The stabilized T22G mutant[97] was produced by site-directed mutagenesis of the pET11d plasmid containing the drkN SH3 gene using the Agilent QuikChange kit and the following primers: forward 5' GAC GAC GAG CTG AGT TTT CGC AAA GGT CAG ATT CTA AAG ATA TTA AAT ATG G 3' and reverse 5' C CAT ATT TAA TAT CTT TAG AAT CTG ACC TTT GCG AAA ACT CAG CTC GTC GTC 3'. The plasmid was used to express the protein in *E. coli* BL21star(DE3) cells using the protocol for CAHS expression except that ampicillin was used for selection (100 µg/mL final concentration). Six pellets were combined in 10 mL of 50 mM Tris/HCl (pH 7.5) supplemented with protease inhibitors and lysed by sonication (10% amplitude, 4 s on, 2 s off for 15 min). Anion exchange was performed as described for flgM. SH3 does bind to anion exchange resin. SH3 containing fractions identified by SDS PAGE were further purified by size exclusion chromatography as described for flgM. Purified protein was dialyzed and lyophilized a described for flgM.

Ubiquitin

A plasmid harboring the gene encoding histidine-tagged ubiquitin[111,122] was used to express the protein in *E. coli* BL21star(DE3) cells using the protocol described for the

CAHS proteins except ampicillin (100 µg/mL final concentration) was used for selection. Three pellets were resuspended in 15 mL of 50 mM Na_2HPO_4, 500 mM NaCl, 15 mM imidazole (pH 7.6) supplemented with protease inhibitors and purified as described.[111]

Lactate dehydrogenase assays

The assay is based on published protocols.[10,12] L-LDH from rabbit muscle (Roche) was diluted to 0.1 g/L in 100 µL of 25 mM Tris/HCl (pH 7.0) containing various concentrations of CAHS D, CAHS G, ubiquitin, SH3, flgM, or other additives. Concentrations of CAHS D, CAHS G, ubiquitin, and SH3 were determined using the Bradford assay.[123] Before addition of LDH, CAHS proteins were resuspended at high concentration by heat shock at 95°C. FlgM does not react in a linear fashion with the Bradford reagent, so we determined the concentration spectrophotometrically[124] (ε_{280} = 1,490 M^{-1} cm^{-1}) despite its lack of tryptophans. BSA concentration was also quantified spectrophotometrically (ε_{280} = 43,824 M^{-1} cm^{-1} according to supplier). Concentrations of lysozyme were based on mass.

Half of each sample was stored at 4°C. The other half was dehydrated in a Genevac® EZ-2 Personal Evaporator using the aqueous setting (time to final stage: 16 h; final stage: 0 h) without added heat.

Protection assay samples were immediately rehydrated with 250 µL of H_2O. Heat tolerance assay samples containing protein additives were exposed to 95°C (Fisherbrand™ Isotemp™ heat block) for various times, cooled on ice, and rehydrated with 250 µL of H_2O. Heat tolerance assay samples containing trehalose were exposed to a fixed temperature for 5 min before cooling to 4°C (Eppendorf® Mastercycler Personal) and resuspension in 250 µL of H_2O. Control samples were diluted with 200 µL of H_2O.

If necessary for resuspension, samples were shaken (New Brunswick Scientific I26 incubator, 225 rpm) at 37°C for 10 min. Otherwise, samples were kept on ice until assayed. Enzyme activity was determined as described.[10] Experiments were performed in triplicate.

Lipoprotein lipase assays

Lipoprotein lipase was purified from fresh, unpasteurized cow's milk as described.[125] Samples containing 27.5 ng of LPL (100 µL of 2.5 nM LPL) were mixed with additive. Excipients were resuspended in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4) and mixed with LPL prior to flash freezing and lyophilization. Samples were brought to equal volumes with PBS, flash frozen, and lyophilized in a Savant Speed Vac attached to a lyophilizer. Positive controls were flash frozen but not lyophilized and stored at -80°C until lyophilization was complete. Following lyophilization, positive controls were adjusted to the same condition as their lyophilized counterpart.

Samples were resuspended in 100 µL LPL assay buffer, which has a final concentration of 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.2% fatty-acid free BSA (Sigma), and 1 mM sodium deoxycholate (Sigma). Immediately prior to measuring activity, 10 µM of the fluorescent substrate 1,2-di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresofurin ester) (DGGR, Sigma) in 0.01% Anzergent 3-16 (Anatrace) was added.

Activity was measured as described.[93] Assays were conducted at 37°C in triplicate. The activities were corrected by subtracting a buffer control comprising DGGR and the appropriate concentration of CAHS, BSA, or trehalose, but without LPL. Data were normalized to unstressed LPL.

Histograms of pI values for predicted open reading frames

The *C. elegans* (UP000001940) proteome was obtained from Uniprot.org. The *H. exemplaris* (formerly *H. dujardini*) proteome (Hypsibius_dujardini_nHd.3.1.5.proteins.fa) was obtained from tardigrades.org. Isoelectric points were calculated using ipc-1.0.[126]

Table 2.1. Ability of additives to protect LDH and some of their physical properties.

Additive	g/L for 50% activity	Mole ratio for 50% activity	pI	MW (kDa)	# residues	Hphob[a]	Hphob[a] density	Charge at pH 7	Sequence charge density, pH 7	Surface Hydro-phobicity[127]
BSA	6.4	130	4.8	66	583	-279.2	-0.48	-13.9	-0.024	0.545
CAHS D	4.0	220	6.0	25.5	227	-223.5	-0.99	-3.1	-0.014	n/d[b]
CAHS G	3.6	110	5.9	45.5	414	-341.5	-0.82	-5.1	-0.012	n/d[b]
SH3	2.3	460	4.8	6.8	59	-85.8	-0.95	-5.6	-0.095	0.596
FlgM	1.4	180	9.1	10.6	97	-59.8	-0.62	1.9	0.020	n/d[b]
Ubiquitin[c]	0.84	140	8.5	10.4	90	-85.8	-0.95	2.4	0.027	0.491
Lysozyme	n/d[e]	n/d[e]	9.0	14.3	129	-60.9	-0.47	7.9	0.061	0.471
LDH – 1[d]	n/a[f]	n/a[f]	6.0	147	1336	-186.6	-0.002	-18	-0.013	n/a[f]
LDH – 2[d]	n/a[f]	n/a[f]	6.5	146	1334	-347.4	0.011	-9	-0.007	n/a[f]
LPL	n/a[f]	n/a[f]	8.5	107	956	-499.8	-0.15	11	0.012	n/a[b, f]

[a] Hphob, Kyte and Doolittle hydrophobicity.[21]
[b] n/d, not determined because there is no PDB.
[c] Calculations account for His tag.[111,122]
[d] Isozymes LDH – 1 and LDH – 2 are both present in commercial LDH.
[e] n/d, not determined because lysozyme inactivates LDH in solution.
[f] n/a, not applicable because these are test enzymes, not additives.

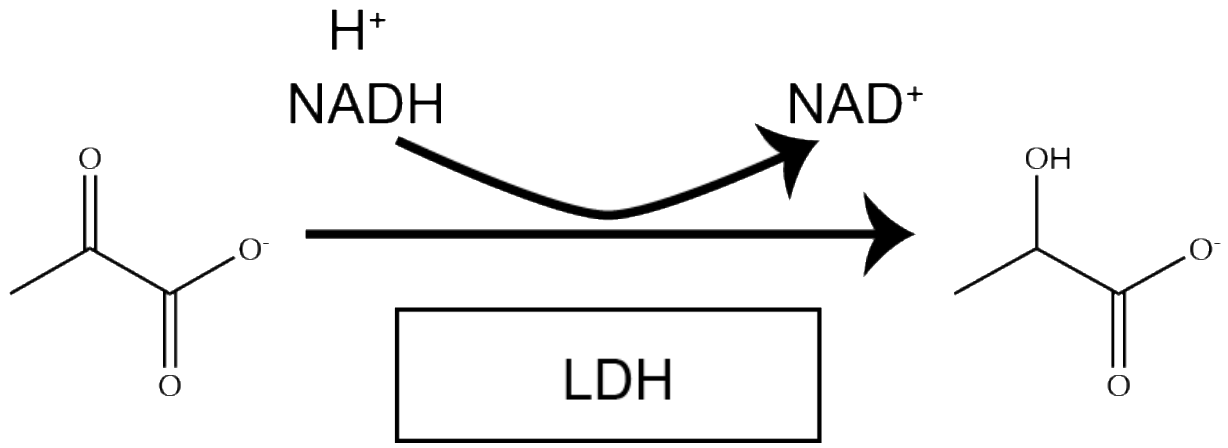


Figure 2.1. LDH reaction. A hydride from NADH and a proton are transferred to pyruvate, forming the reduced product, lactate, and the oxidized cofactor NAD^+.

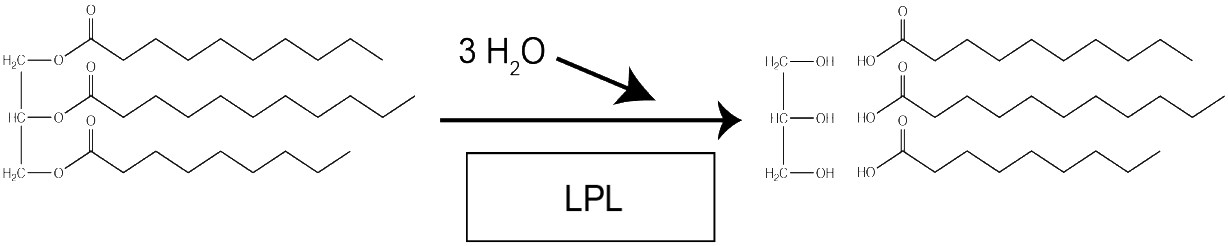


Figure 2.2. LPL reaction. Triglycerides are hydrolyzed to fatty acids and glycerol.

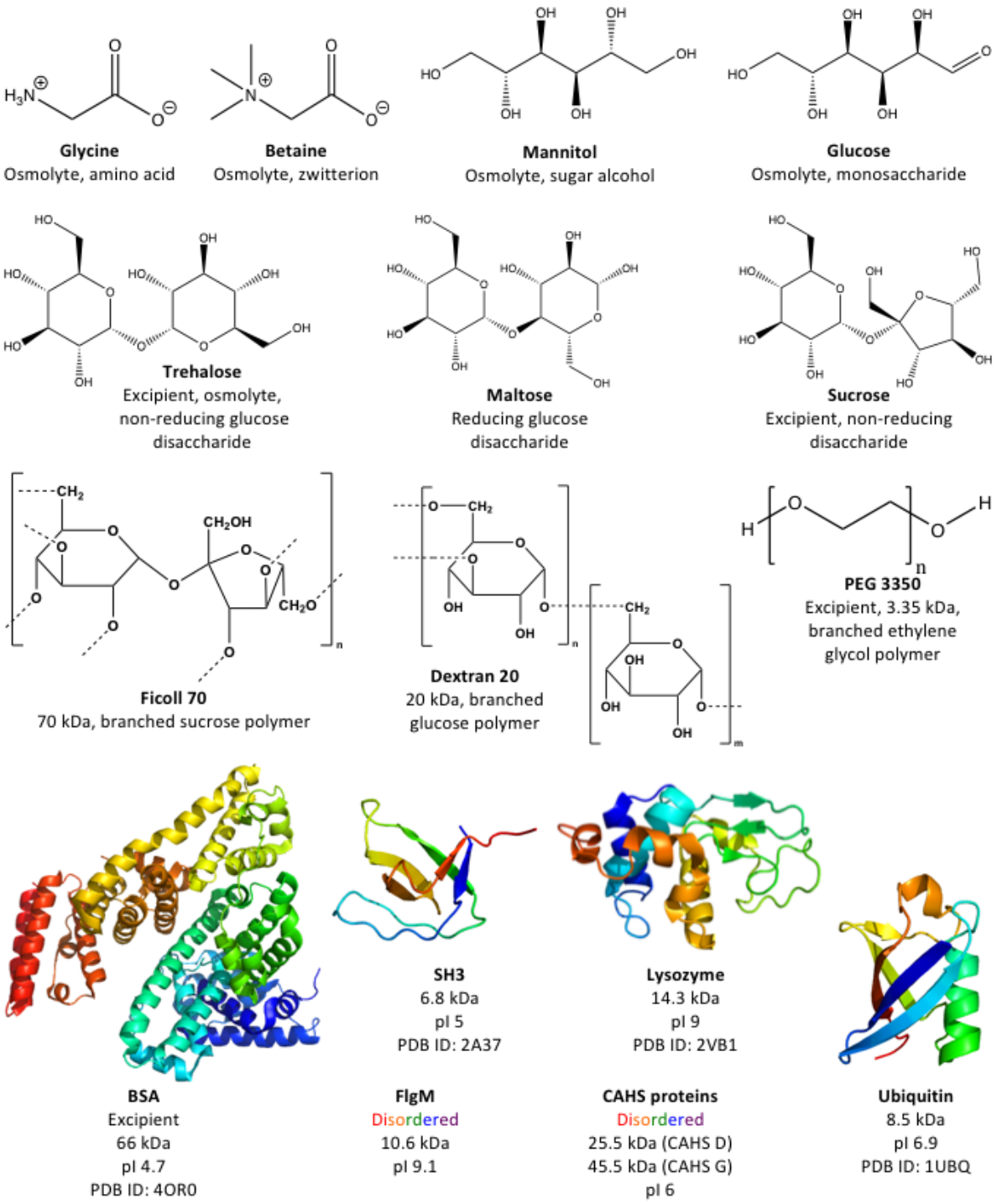


Figure 2.3. Additives tested as potential protectants.

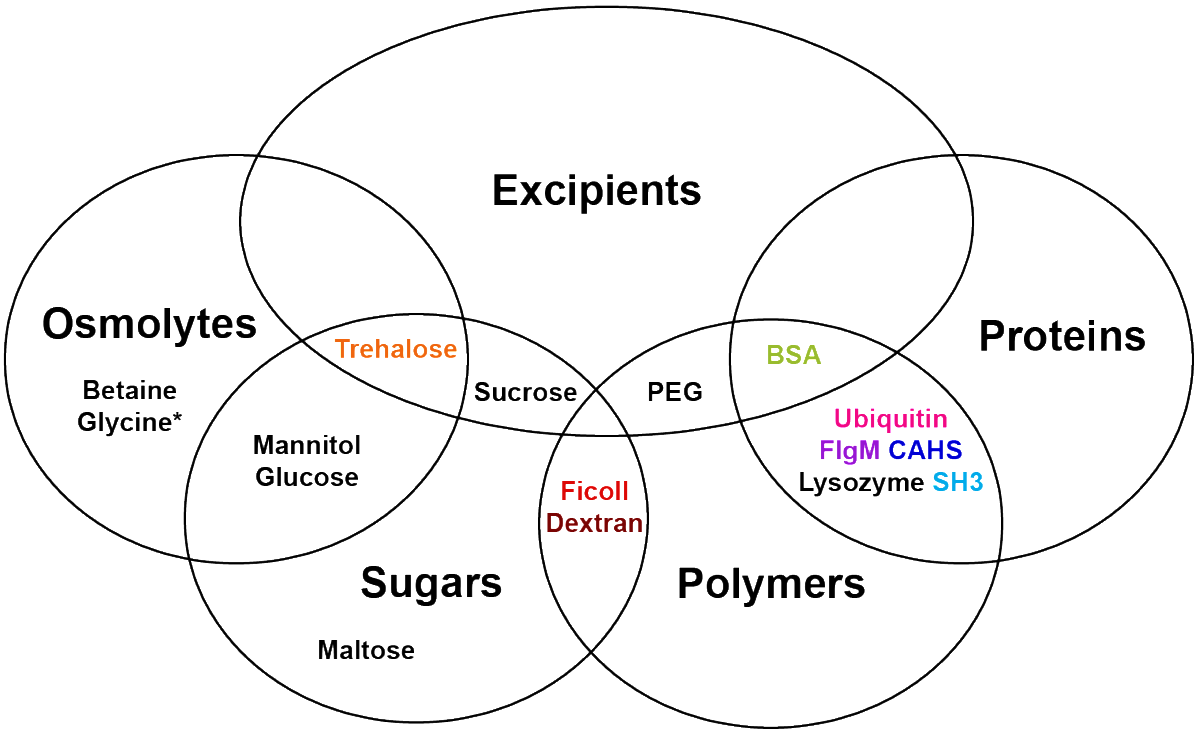


Figure 2.4. Venn diagram of additives tested as potential protectants and their categorization**.** Most additives fall into multiple categories. *Glycine is an amino acid and an osmolyte.

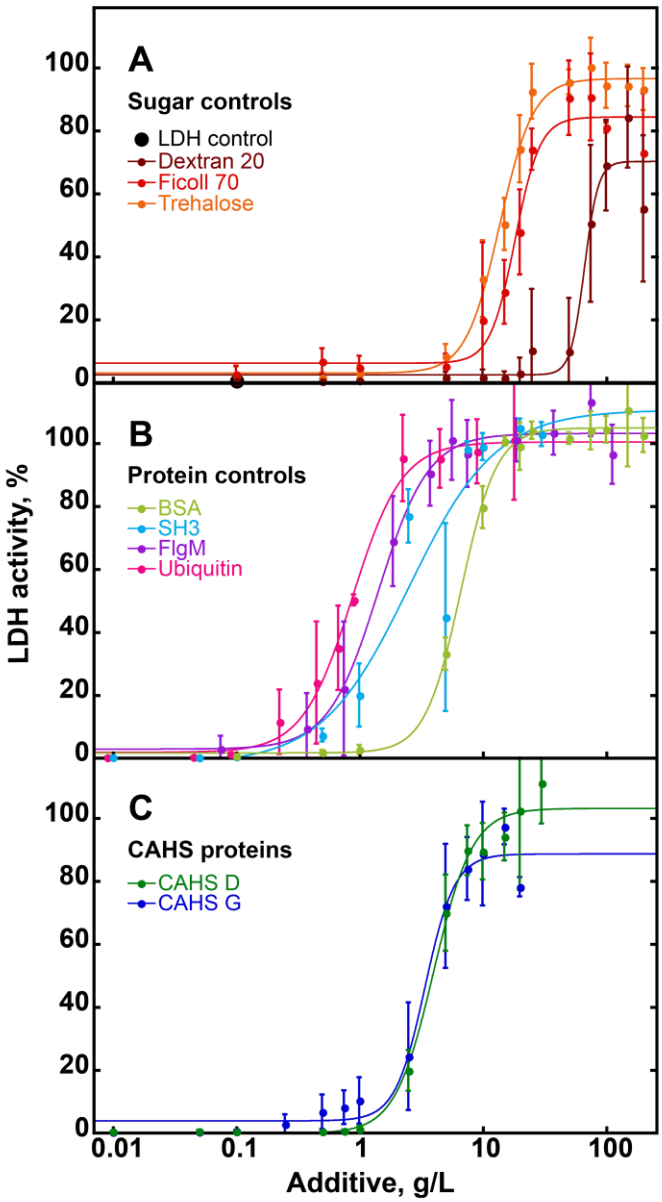


Figure 2.5. Protection of LDH activity against desiccation-induced inactivation. Buffered LDH (0.10 g/L) was desiccated and rehydrated by itself and with additives. Percent activity was determined by comparison to a sample of the same solution stored at 4 °C. Uncertainties are the standard deviations of the mean from triplicate measurements. Data for the less effective additives sucrose, maltose, and mannitol are given in Supporting Information Supplemental Figure 2.1. “LDH control” represents the activity in the absence of additive or in the presence of glucose, glycine, betaine or PEG 3350 (i.e., these additives are ineffective). Sigmoidal curves were added as a visual guide and used to calculate the concentration of additive required for 50% protection (Figure 2.4). Data for trehalose and BSA have been published.[10] Data for CAHS D and G are slightly different from our previous report[10] because, as described in the Materials and Methods, we improved the purification protocol.

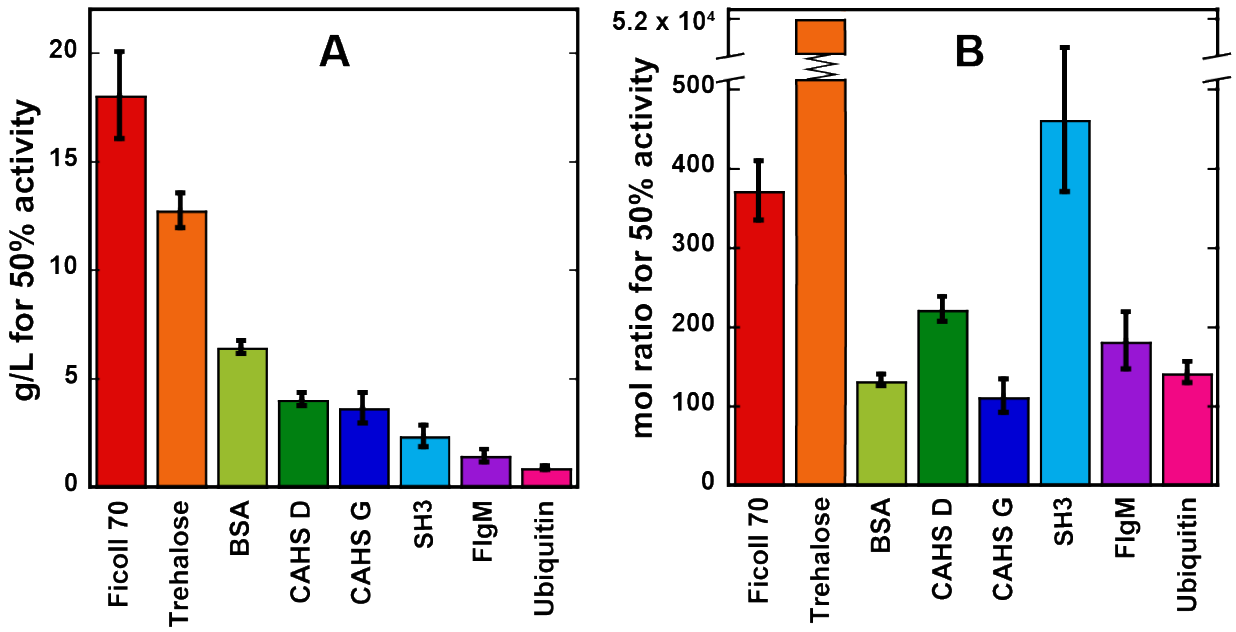


Figure 2.6. Additive concentrations affording 50% LDH protection from desiccation-induced inactivation as determined from the sigmoidal fits of the data in Figure 2.3. (A) g/L concentrations. Uncertainties are the standard deviations of the mean from triplicate measurements (B) Mole ratios of additive to LDH. The upper and lower uncertainties for trehalose are 55,000 and 49,000.

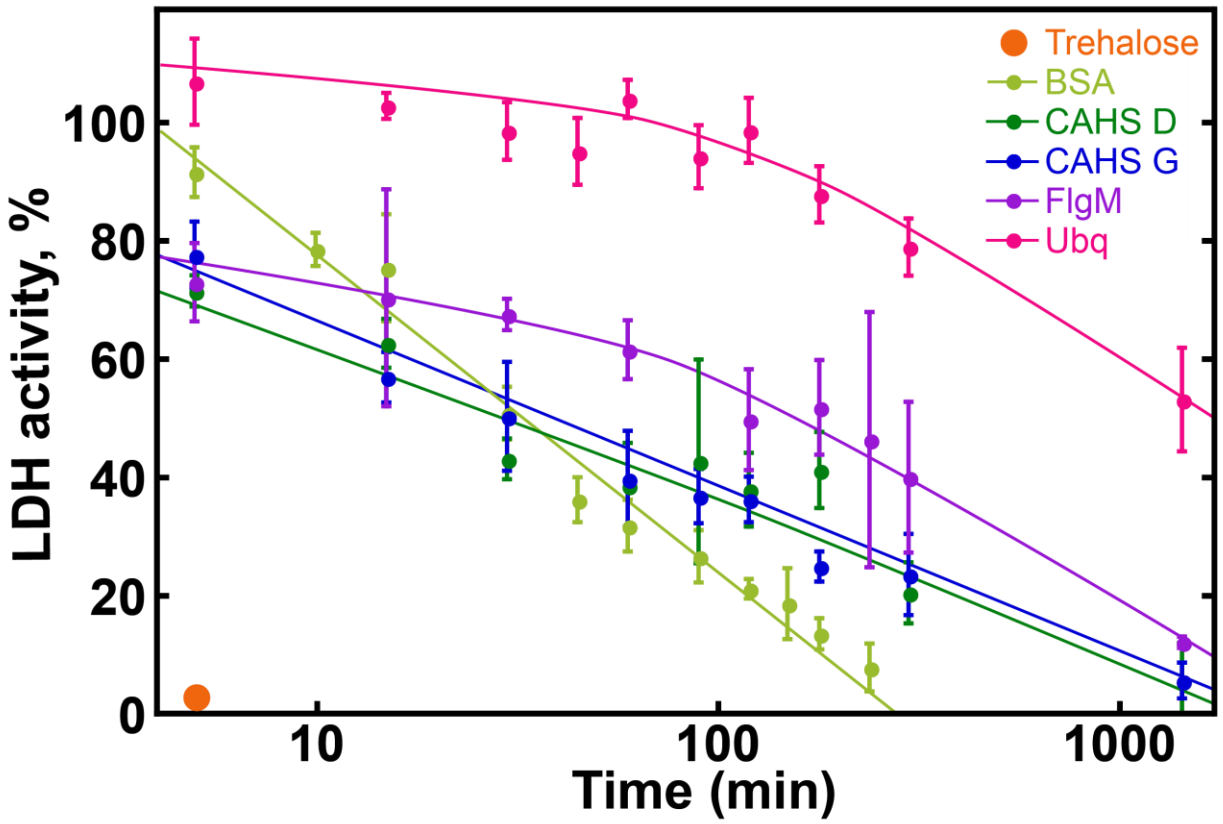


Figure 2.7. Protecting dried LDH from heat-inactivation.
Buffered LDH (0.10 g/L) was desiccated in the presence of 20 g/L protectant before exposure to 95 °C. After rehydration, the percent activity was determined by comparison to a control comprising the same solution stored at 4 °C. Uncertainties are the standard deviations of the mean from triplicate measurements. Curves were added as a visual guide but have no theoretical significance.

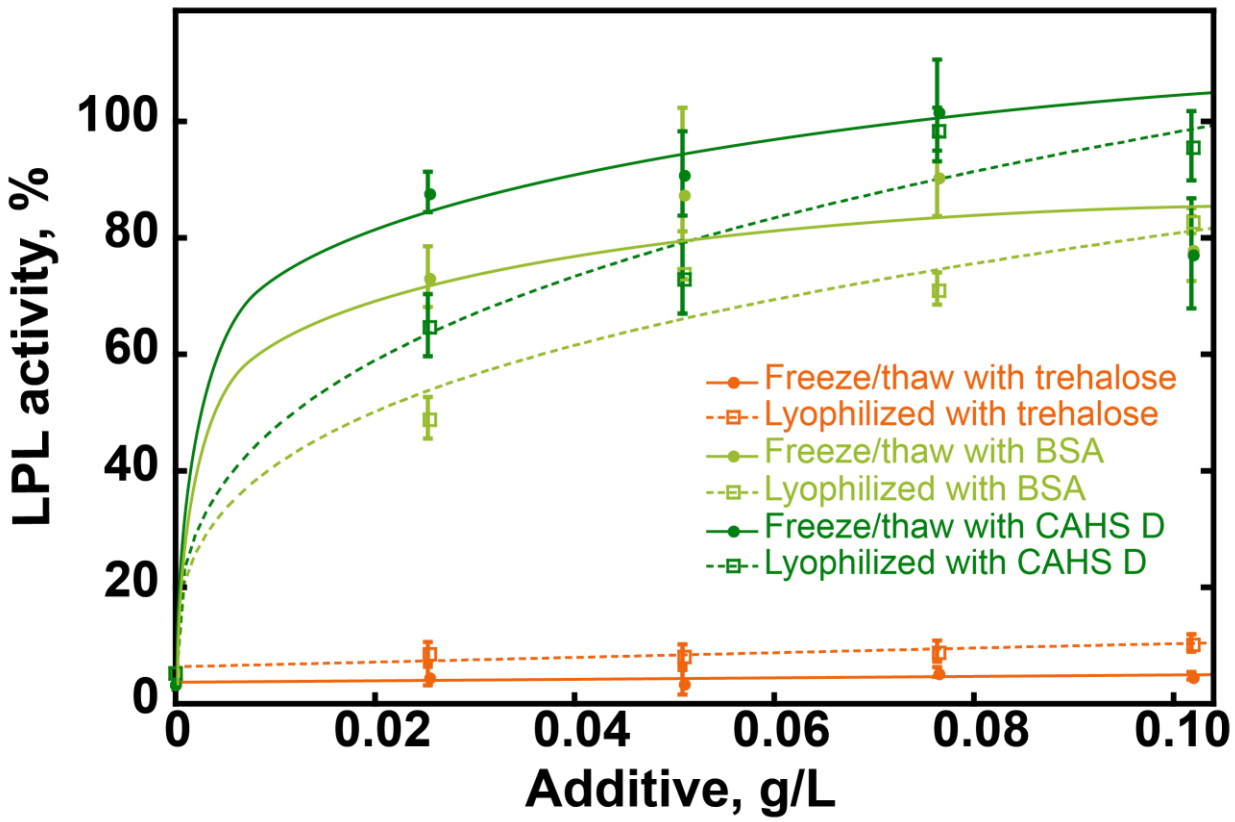


Figure 2.8. Protecting LPL against freeze/thaw and lyophilization induced inactivation. Buffered LPL (0.28 mg/L) was formulated with various concentrations of additives before flash freezing. Freeze/thaw samples were stored at -80°C. Other samples were lyophilized. Normalized activity was determined by comparison to an LPL solution stored at -80°C without freeze/thaw stress. Curves were added as a visual guide but have no theoretical significance.

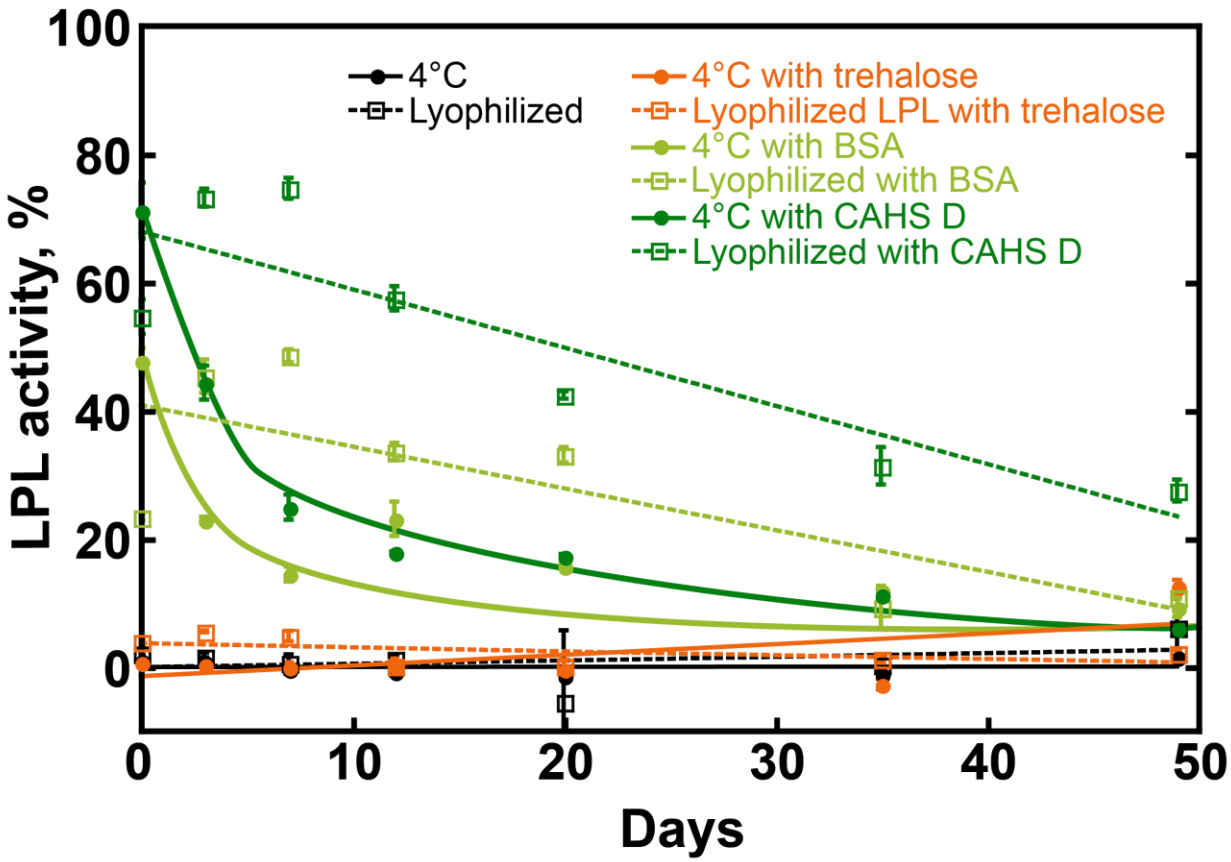


Figure 2.9. Protecting LPL against lyophilization-induced inactivation. Buffered LPL (0.28 mg/L) was formulated with 0.026 g/L additive. Samples were stored at either 4°C or flash frozen and lyophilized before storage at 4°C. Normalized activity was determined by comparison to a control comprising the same solution stored at -80°C. Curves were added as a visual guide but have no theoretical significance.

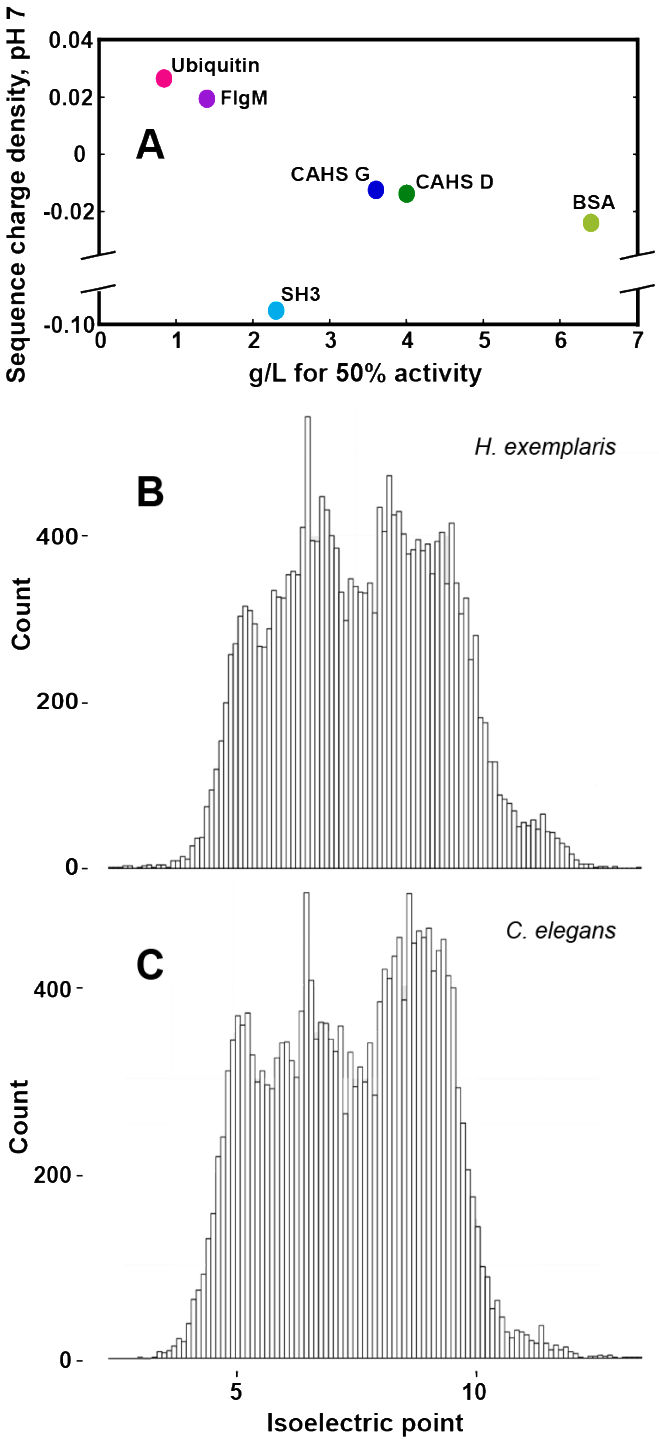
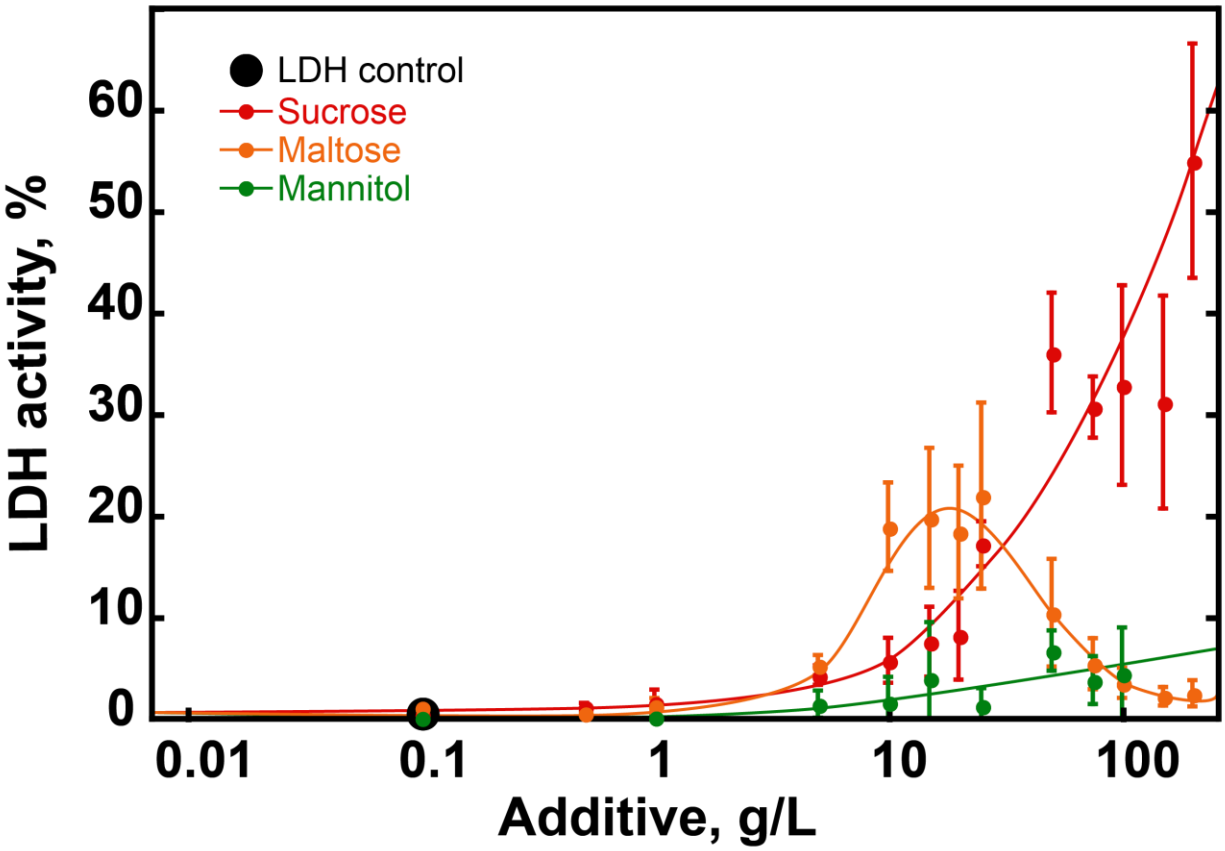
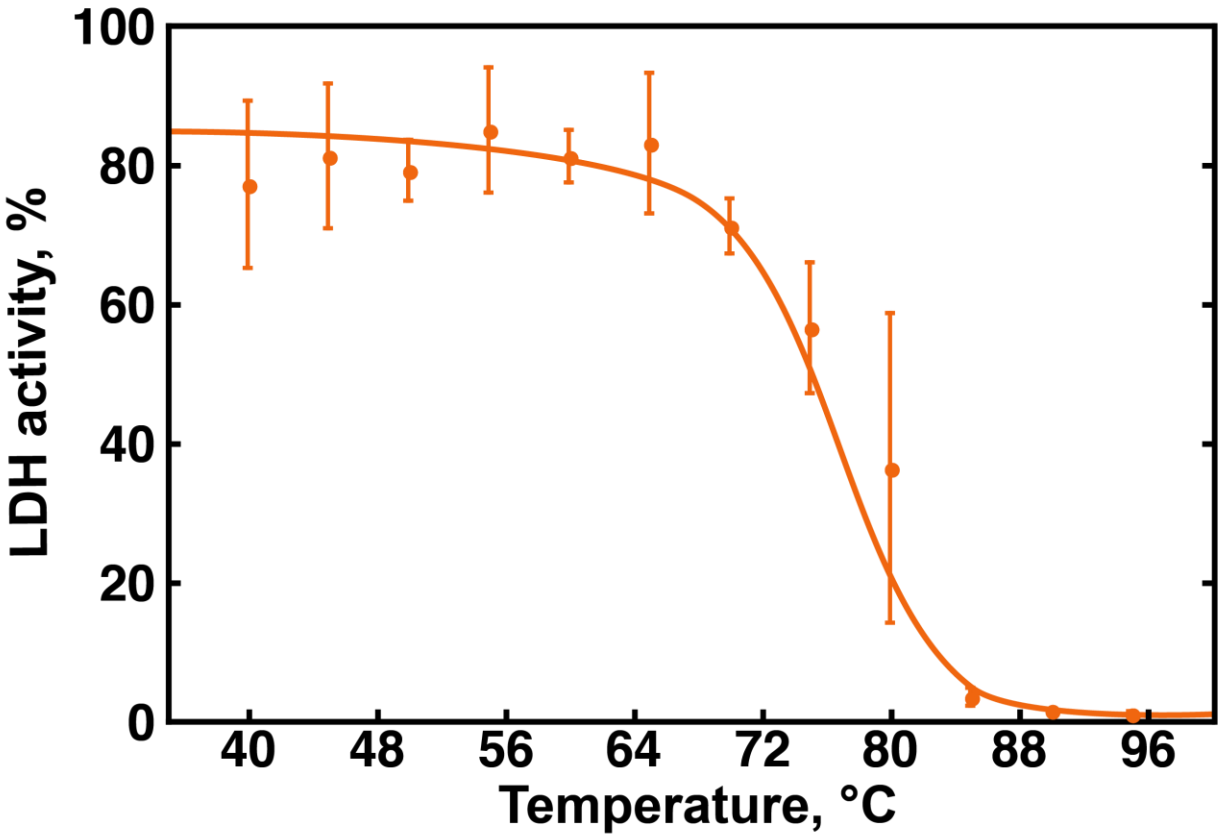


Figure 2.10. (A) Charge at pH 7 divided by the number of residues in each protein plotted against the concentration for 50% protection of LDH activity. (B) Histogram of pI values at 0.1 intervals for predicted open reading frames in the genome of *H. exemplaris*.[128,129] (C) Histogram of pI values at 0.1 intervals for predicted open reading frames in the genome of *C. elegans*.[130,131]



Supplemental Figure 2.1. Protecting LDH with sugars.
Buffered LDH (0.10 g/L) was desiccated and rehydrated by itself or with additives. Percent activity was determined by comparison to a control of the same solution stored at 4 °C. Uncertainties are the standard deviation of the mean from triplicate measurements. Curves were added as a visual guide but have no theoretical significance.



Supplemental Figure 2.2. Trehalose protects dry LDH from heat inactivation. Buffered LDH (0.10 g/L) was desiccated in the presence of 20 g/L trehalose before 5 min exposure to a fixed temperature. After cooling to room temperature and rehydration, the percent activity was determined by comparison to a control comprising the same solution stored at 4 °C. A smooth curve was added as a visual guide but has no theoretical significance.

CHAPTER 3: STRUCTURAL STUDIES OF CYTOSOLIC ABUNDANT HEAT SOLUBLE PROTEINS FROM TARDIGRADES[2]

Overview

Tardigrades require cytosolic abundant heat soluble (CAHS) proteins, a family unique to tardigrades, to survive desiccation. Previous work has suggested that these proteins are intrinsically disordered; however, these observations were qualitative rather than quantitative. I have used nuclear magnetic resonance spectroscopy (NMR), rheology, and synchrotron circular dichroism spectropolarimetery (SRCD), to quantitatively show that CAHS protein structure is not that simple. In dilute solution, CAHS proteins are intrinsically disordered but have transient structure that unfolds on the NMR timescale. As CAHS proteins are concentrated, these transient structural elements are more likely to come into contact, and the resulting intermolecular interactions result in gelation.

Introduction

Tardigrades (water bears) comprise a phylum of microscopic animals renowned for their ability to survive a vast array of environmental extremes, including-essentially complete desiccation for up to a decade.[132] Wright *et al.* observed that different tardigrade species survive drying at different rates, but all species tested die if dried too quickly.[133] This trend suggests that tardigrades need time to produce protectants, a

[2] Part of this chapter is adapted from the article in the journal *Molecular Cell*. The original citations is as follows: Boothby TC, Tapia H, Brozena AH, Piszkiewicz S, Smith AE, Giovannini I, Rebecchi L, Pielak GJ, Koshland D, Goldstein B (2017) Tardigrades use intrinsically disordered proteins to survive desiccation. Mol Cell 65:975-984 e975.

theory supported by the evidence that *de novo* transcription and translation are required for the tardigrade *Hypsibius dujardini* to robustly survive desiccation.[134]

Boothby *et al.* show that tardigrades upregulate the expression of genes encoding cytosolic abundant heat soluble (CAHS) proteins in response to dehydration stress.[10] This protein family was first identified in a proteomic analysis of tardigrades, and is unique to the phylum.[23,91] Disruption of gene expression for several CAHS proteins, using RNAi, results in severely diminished desiccation tolerance.[10] Furthermore, the heterologous expression of CAHS proteins in both prokaryotic and eukaryotic cells is sufficient to increase their desiccation,[10] and purified CAHS proteins protect desiccation-sensitive proteins in vitro.[10,14] Purified and heterologously expressed CAHS proteins also vitrify upon desiccation, and this vitreous state mirrors the protective capabilities of these proteins.[10] CAHS proteins may function through a similar mechanism to trehalose, a non-reducing disaccharide essential for some organisms to survive desiccation that is thought to protect organisms by vitrifying their cellular contents.[5,7,72] However, to understand the function of CAHS proteins we must also understand their structure.

On the basis of heat solubility experiments and circular dichroism spectropolarimetery, CAHS proteins are thought to be intrinsically disordered.[23,91] In the past two decades, myriad cellular roles for intrinsically disordered proteins (IDPs) have emerged, including roles in transcription, post-translational modification, development, and cellular organization.[135-138] Several families of IDPs, including late embryogenesis abundant proteins and hydrophilins, have known or suspected roles in stress tolerance in organisms spanning all kingdoms of life.[17,19,20,22,114] Boothby *et al.* took a bioinformatic

approach to characterize the widespread disorder of CAHS proteins, and these predictions support the conclusion that these proteins are largely disordered.[10] I have used nuclear magnetic resonance spectroscopy (NMR), rheology, and synchrotron circular dichroism spectropolarimetery (SRCD), to show that CAHS protein structure is not that simple.

NMR Experiments Reveal CAHS Proteins are Disordered

IDPs lack persistent secondary structure,[92] which we confirmed for CAHS proteins using NMR. To do this we mapped the chemical environment of the covalent bond between each backbone amide nitrogen-15 and its attached proton with a heteronuclear single-quantum coherence spectrum (HQSC). In this experiment, each bond gives rise to a feature called a cross peak at the chemical shift coordinates of the two nuclei for each non-proline residue. For structured proteins such as ubiquitin, the cross peaks occur over a range of 7.5 to 10 ppm in the proton dimension (Figure 3.1, top). For α-synuclein, a known disordered protein, and for CAHS proteins, the cross peaks occur over a narrower window (Figure 3.1, top), from 8.0 to 8.6 ppm, which coincides with the range for amide protons in the central residue of unstructured tripeptides.[139] To further test our conclusion that these proteins are disordered, we assessed backbone proton-deuterium exchange. Amide protons in tripeptides exchange with deuterons from D_2O in seconds[140] but can be protected in the interior of stable globular proteins for days to weeks.[141] After acquiring an HSQC spectrum (Figure 3.1), we removed two aliquots from each sample. One aliquot was diluted 10-fold with H_2O, and the other was diluted 10-fold with D_2O. For the disordered α-synuclein and CAHS proteins, nearly all the amide protons were exchanged for deuterons within 20 min, as

shown by the decrease in intensity of the one-dimensional proton spectrum (Figure 3.1). In contrast, less exchange was observed for the structured protein ubiquitin in 20 min (Figure 3.1). These data, combined with published heat solubility, circular dichroism, and bioinformatics approaches, show that many, if not all, tardigrade CAHS proteins are disordered,[10,91] at least in the tested concentration range.

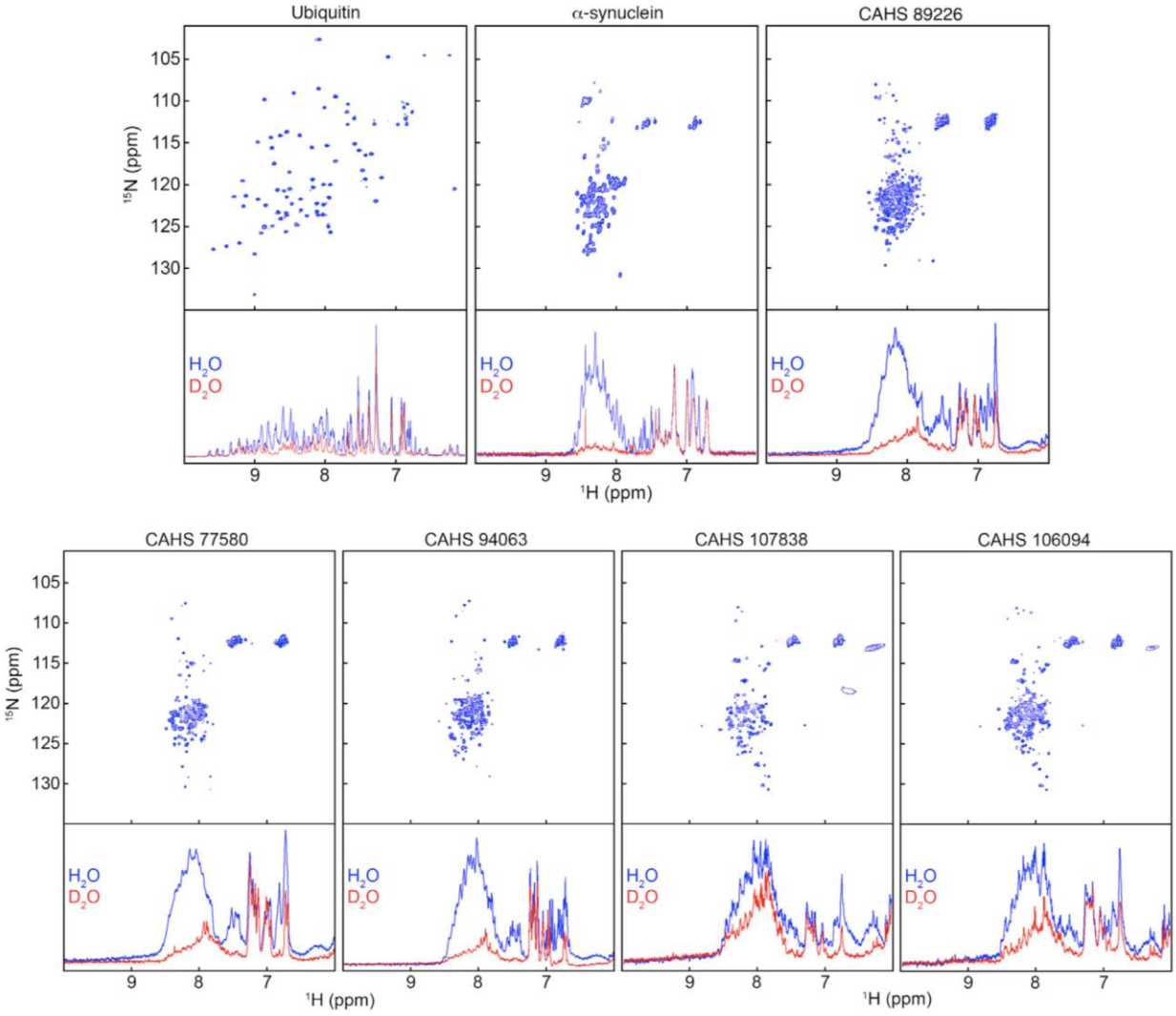


Figure 3.1. Tardigrade cytosolic abundant heat soluble proteins are disordered**.** Top: two-dimensional ^{15}N-^{1}H HSQC spectra of ubiquitin (a globular protein), α-synuclein (a known disordered protein), and tardigrade CAHS proteins in 90:10 (vol/vol) H_2O:D_2O 50 mM sodium phosphate (pH 7.0). Bottom: after the spectra were acquired, two aliquots were diluted 10-fold with either buffered 90:10 (vol/vol) H_2O:D_2O or buffered D_2O and one-dimensional proton spectra acquired 20 min later.

CAHS Proteins Form Gels

We stumbled upon an unexpected physical property while purifying CAHS proteins for the above NMR experiments: CAHS proteins form thermoreversible gels. This property is so robust that the lysate from sonication of *E. coli* cells overexpressing CAHS homologues gelled almost immediately, and the inverted lysate resisted gravity for over 24 h (Figure 3.2). We did not observe gelation in lysates that had overexpressed the disordered protein α-synuclein, from which we can conclude gelation is not a general property of disordered proteins.

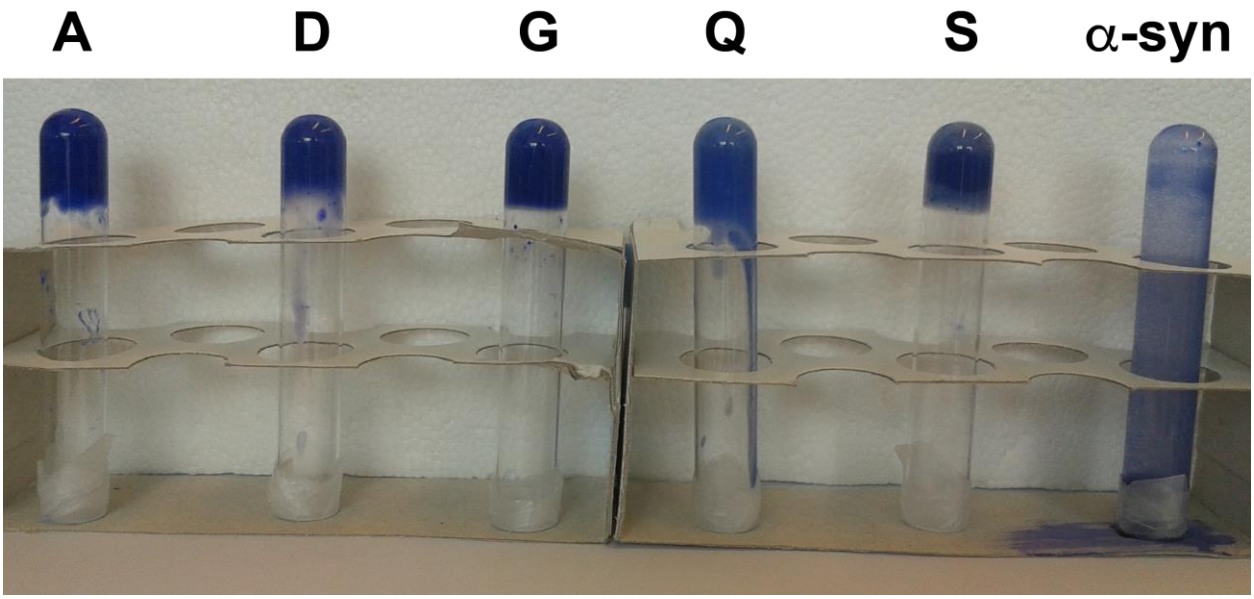


Figure 3.2. CAHS proteins gel in lysates of *E. coli* lysates.
After heterologous over expression in, cells were mixed with Coomassie brilliant blue dye, lysed by sonication, and inverted for 24 h.

To quantitatively study the gel properties of CAHS proteins, we have turned to rheology: the study of the flow and deformation of materials.[142] Gels are viscoelastic materials, meaning they have properties of both solid sand fluids, which can be quantified in an oscillatory shear experiment using a cone and plate rheometer. Gel

formation is defined to occur when the storage modulus, representing the elastic stress response, exceeds the loss modulus, which represents the viscous response.[142]

A CAHS D sample was heated to 95°C before deposition onto the instrument stage, and the storage and loss module were measured as the sample cooled (Figure 3.3). At 50 s, the storage modulus becomes greater than the loss modulus, meaning the sample was more of a solid than a liquid and a gel had formed.

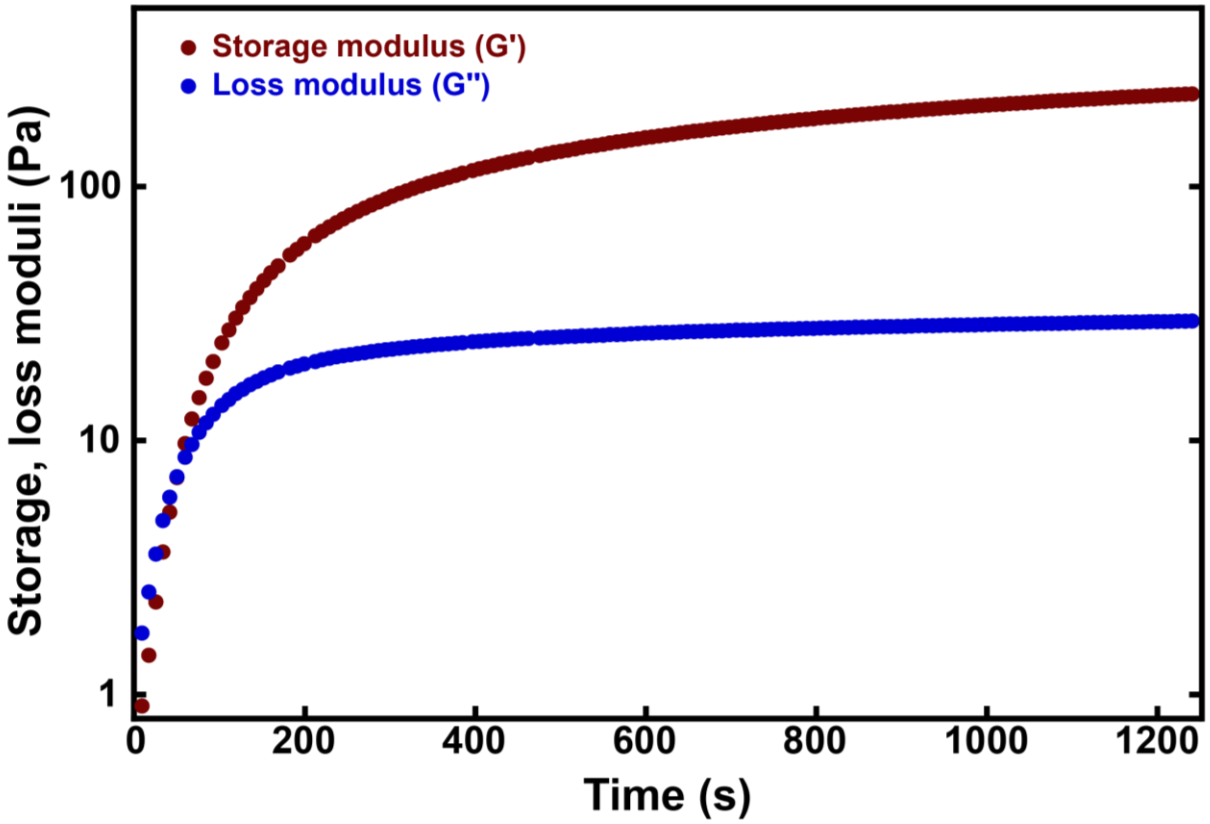


Figure 3.3. Oscillatory shear rheology of CAHS D.
Gelation occurs when the storage modulus (red) exceeds the loss modulus (blue).

SRCD Experiments Reveal Concentration-Dependent Structural Changes of CAHS Proteins

Solution NMR spectroscopy relies on the tumbling of molecules in solution, and therefore cannot be used to study protein structure within CAHS gels. Therefore, we

turned to circular dichroism spectropolarimetery. Although Yamaguchi *et al.* reported a circular dichroism spectrum for a CAHS protein, these data were analyzed only qualitatively for intrinsic disorder.[91] Previous bioinformatic studies suggest some members of disordered protein families are folded in the desiccated state,[143] and therefore CAHS proteins might gain structure as they concentrate and gel. Software like the DichroWeb on-line analysis tool[144-146] can be used to quantify secondary structure from circular dichroism spectropolarimetery data.[147] I set out to confirm my NMR results by circular dichroism spectropolarimetery and quantify the secondary structure content of CAHS proteins in solution and gels.

Preliminary experiments revealed that the circular dichroism spectrum of CAHS proteins, and therefore CAHS secondary structure, changes with concentration. However, circular dichroism spectropolarimetery relies on the transmission of light, and we do not have the equipment at UNC Chapel Hill to collect accurate CD data for highly concentrated samples that absorb or scatter greater than 99.9% of light. The high flux of light produced by a synchrotron can penetrate concentrated liquids, gels, and even powders, while allowing data acquisition at shorter wavelengths, which is valuable to studies of disordered proteins that have a spectral signature below 200 nm.[148,149] Therefore, we launched a collaboration with the creator of DichroWeb, Professor Bonnie Wallace, at Birkbeck College, University of London. The Wallace lab has assisted with collection of high-quality synchrotron circular dichroism spectropolarimetery (SRCD) data.

Consistent with other proteins that protect against desiccation stress,[150] these data reveal that as CAHS concentration increases, so does α-helical content as seen by

the decrease in $\Delta\varepsilon$ at 222 nm (Figure 3.4).[151,152] Directly comparing DichroWeb analysis of a relatively dilute 6 g/L sample to a highly concentrated 100 g/L sample shows that regardless of concentration, roughly 1/3rd of CAHS D is disordered (Figure 3.5). However, we observe at least as much structure as disorder, and α-helical content increases with concentration and gel formation.

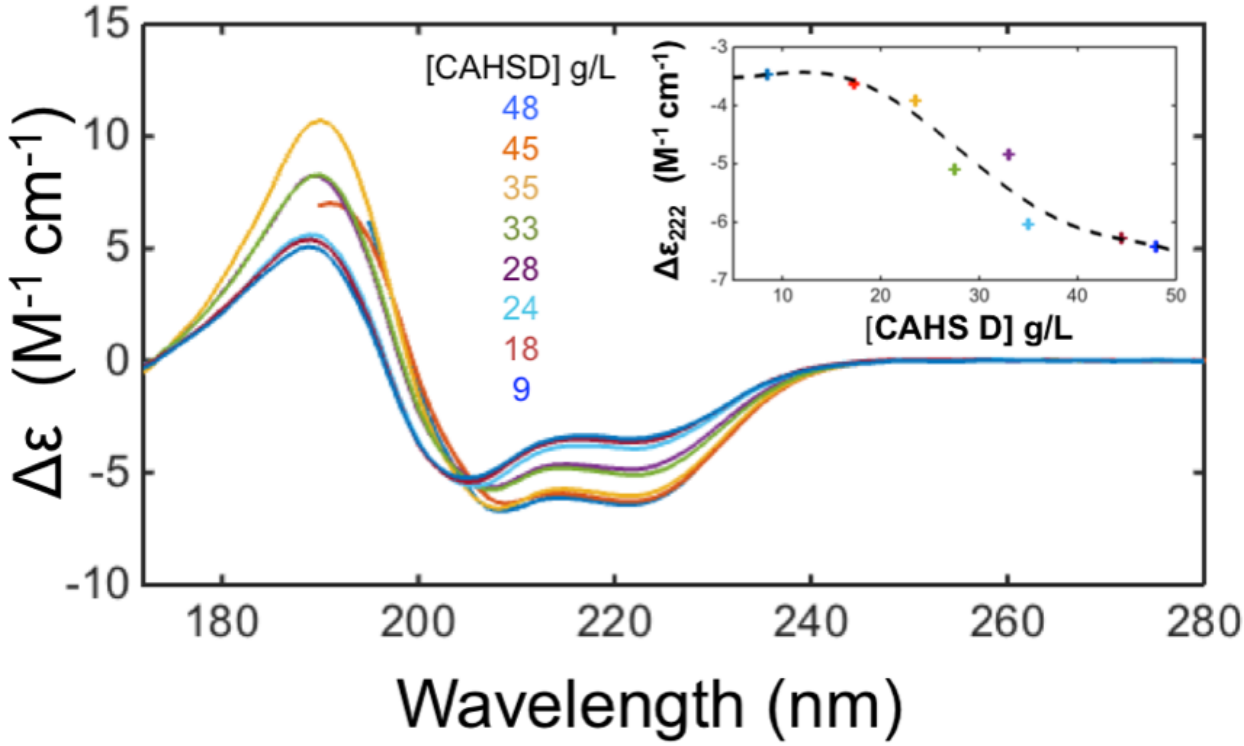


Figure 3.4. Synchrotron circular dichroism spectra of CAHS D as a function of concentration (pH 7.4, 20°C). Inset: $\Delta\varepsilon_{222}$ as a function of concentration indicates that α-helix content increases with increasing concentration.

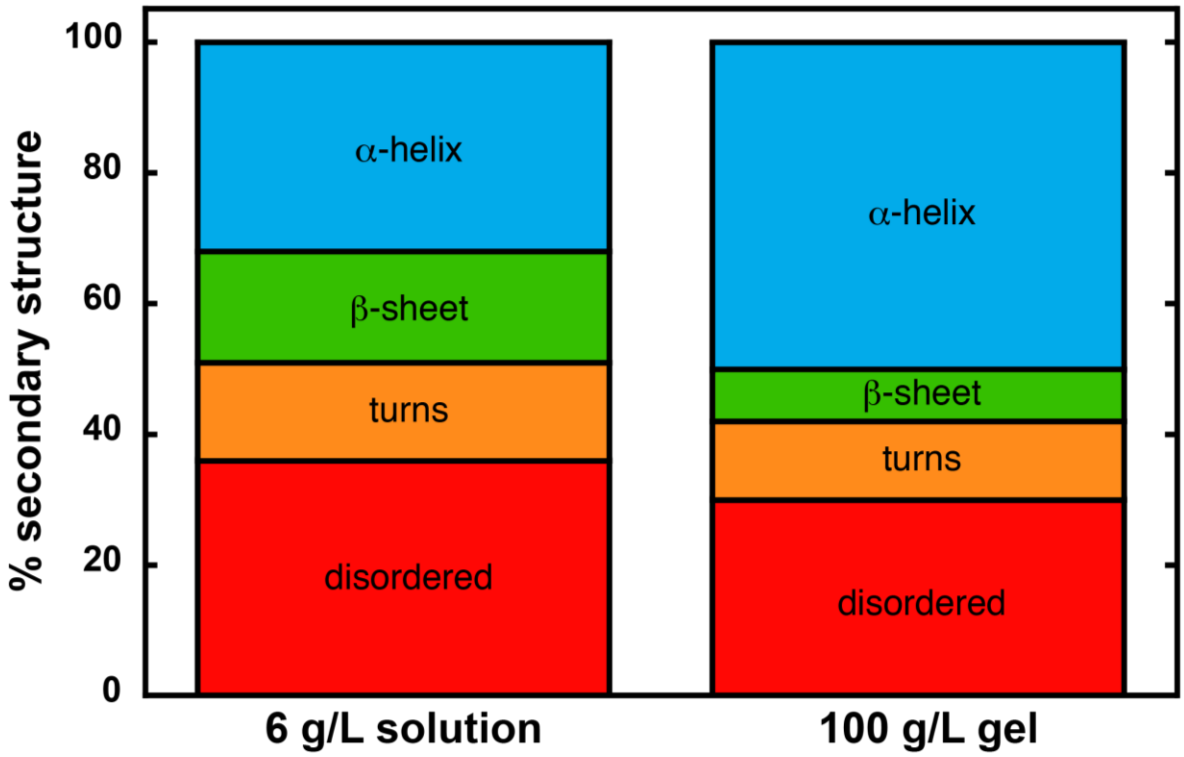


Figure 3.5. Dichroweb analysis of CAHS (<5% uncertainty).

Conclusions

For dilute solutions, NMR experiments demonstrate that CAHS proteins are disordered, yet SRCD experiments demonstrate that nearly half of the CAHS protein comprises α-helices and β-sheets. These results are not necessarily contradictory. Circular dichroism and our HSQC experiments observe the average ensemble of the CAHS protein. However, these techniques observe proteins on different time scales. We can conclude that the structure observed by SRCD of dilute CAHS is transient on the NMR timescale. Our one-dimensional hydrogen-deuterium exchange experiment provides information about the frequency at which CAHS unfolds. As CAHS is concentrated, these transient structural elements are more likely to come into contact.

Intermolecular interaction could stabilize transient α-helices, resulting in gelation and the increased helical content observed by SRCD.

Methods

Protein expression and purification for NMR experiments

E. coli codon-optimized gBlocks encoding tardigrade CAHS proteins were synthesized (Integrated DNA Technologies) and cloned into the pET28b expression vector. BL21star (DE3) *E. coli* were transformed with pET28b + CAHS plasmids. Note that CAHS 77580 is also known as CAHS A, CAHS 89226 is also known as CAHS G, CAHS 94063 is also known as CAHS D, CAHS 106094 is also known as CAHS Q, and CAHS 107838 is also known as CAHS S.

A single colony was used to inoculate 10 mL of Lennox broth (LB, 10 g/L, tryptone, 5 g/L yeast extract, 5 g/L NaCl) supplemented with 60 mg/mL (final concentration) of kanamycin. The culture was shaken at 37°C overnight (New Brunswick Scientific Innova I26, 225 rpm). Three cultures were used to inoculate 1 L of supplemented M9 media (50 mM Na_2HPO_4, 20 mM KH_2PO_4, 9 mM NaCl, 4 g/L glucose, 1 g/L $^{15}NH_4Cl$, 0.1 mM $CaCl_2$ 2 mM $MgSO_4$, 10 mg/L thiamine, 10 mg/L biotin, and 60 mg/mL of kanamycin).

The 1 L cultures were shaken at 37°C until the optical density at 600 nm reached 0.5. Isopropyl β-D-1-thiogalactopyranoside (1 mM final concentration) was then added to induce expression. After 4 h, the cells were pelleted at 1,000*g* at 10°C for 30 min. The cell pellets were stored at -20°C. Pellets were resuspended in 12.5 mL of 50 mM HEPES, 50 mM NaCl (pH 8.0) supplemented with half a Roche cOmplete EDTA-free protease inhibitor tablet (Sigma-Aldrich Cat. #4693159001). Cells were then lysed by

heat shock at 95°C for 15 min. Lysates were cooled at room temperature for 30 min. Insoluble components were removed by centrifugation at 20,000*g* and 10°C for 30 min.

$MgCl_2$ (final concentration 2 mM) was added to the heat soluble fraction before digestion with 1250 units of Benzonase (Sigma- Aldrich) at 37 °C for 1 h. Benzonase was then inactivated by heating to 95 °C. After cooling to room temperature, the lysate was sterile filtered using a 0.45 μm filter and transferred to 10,000 MWCO dialysis tubing. Samples were dialyzed against 50 mM sodium phosphate (pH 7.0) overnight followed by dialysis against three changes of 17 $M\Omega cm^{-1}$ H_2O for at least 3 h each. The dialysate was again filtered before being flash frozen in CO_2(s)/ethanol and lyophilized for 48 h (Labconco FreeZone). Purity was determined by SDS PAGE, DNA electrophoresis, and an ethidium bromide fluorescence assay. α-synuclein and ubiquitin were as described.[111,153]

NMR

Purified CAHS proteins were dissolved at 10 g/L in 50 mM sodium phosphate (pH 7.0), 90:10 (vol/vol) H_2O:D_2O by boiling and then centrifuged at 14,000g for 10 min to remove undissolved material. ^{15}N-^{1}H HSQC spectra were acquired at 298 K on an 850 MHz Bruker Avance III spectrometer equipped with a TCI cryoprobe. Sweep widths were 11,000 Hz and 3,500 Hz in the ^{1}H and ^{15}N dimensions, respectively. Each spectrum comprised 256 increments of 24 scans per increment. One-dimensional spectra were acquired 20 min after sample preparation using a ^{1}H sweep width of 13,500 Hz and comprised 128 scans. Each pair of H_2O/D_2O spectra was normalized using the methyl resonances at 0.8 ppm.

Purified ubiquitin (2 mM) was resuspended in 50 mM sodium phosphate (pH 7.0), 95:5 (vol/vol) H_2O:D_2O and centrifuged at 20,000*g* for 5 min to remove undissolved material. ^{15}N-^{1}H HSQC spectra were acquired at 298 K on the 850 MHz spectrometer. Sweep widths were 14,000 Hz and 3,500 Hz in the ^{1}H and ^{15}N dimensions, respectively. Each spectrum comprised 256 increments of 4 scans per increment. One-dimensional spectra were acquired 20 min after sample preparation using a ^{1}H sweep width of 14,000 Hz and comprised 128 scans. Each one-dimensional spectrum was normalized using the methyl resonance at -0.15 ppm, and all spectra are referenced to DSS at 0 ppm.

Purified α-synuclein (0.1 mM) was resuspended in 50 mM sodium phosphate (pH 7.0), 95:5 (vol/vol) H_2O:D_2O and centrifuged at 20,000*g* for 5 min to remove undissolved material. ^{15}N-^{1}H HSQC spectra were acquired at 298 K on the 850 MHz spectrometer. Sweep widths were 14,000 Hz and 3,500 Hz in the ^{1}H and ^{15}N dimensions, respectively. Each spectrum comprised 256 increments of 4 scans per increment. One-dimensional spectra were acquired 20 min after sample preparation using a ^{1}H sweep width of 14,000 Hz and comprised 128 scans. Each one-dimensional spectrum was normalized using the methyl resonance at 1 ppm, and all spectra are referenced to DSS at 0 ppm.

Sonication assay

Protein was expressed as described for NMR experiments. However, after 4 h of expression each culture was pelleted, drained of as much media as possible, and resuspended in 200 μL protease inhibitors (Sigma-Aldrich P2714 containing 40 mM AEBSF, 6 μM aprotinin, 2.3 mM bestatin, 280 μM E-64, 20 μM leupeptin, and 20mM EDTA). A spatula tip of brilliant blue dye was mixed into 1 mL of the resulting cell slurry

with a short pulse on a vortex mixer. Samples were transferred to glass test tubes in an ice bucket. Cells were lysed by six cycles of sonication (25% amplitude, 2 s on, 2 s off for 10 s) with 30 s between cycles. After sonication, samples were left on ice for 5 min before inversion. Gelled samples remained at the bottom of the tubes at room temperature until photography 24 h later.

Cone and plate oscillatory shear rheology

CAHS D, expressed and purified as described for NMR experiments, was resuspended to approximately 30 g/L in 15 mM Tris, pH 7.0 by heat shock at 95 °C. The melted sample was transferred to the stage of an ARES-G2 rotational rheometer (TA instruments) equipped with a 50 mm, 0.2 rad cone. Data collection began within 10 s of pipetting the sample, and a solvent trap was used to prevent evaporation during the experiment. Gelation of the sample was measured at 6.28319 rad/s as the sample cooled to room temperature. The gel point is the crossover between the storage- and loss-moduli.

Protein expression and purification for SRCD experiments

CAHS D was expressed and purified as described in Chapter 2. Tubes of lyophilized protein were sealed with parafilm and shipped at room temperature to Bonnie Wallace's lab at Birkbeck College of London.

Synchrotron radiation circular dichroism (SRCD) spectroscopy

CAHS D was resuspended in 50 mM sodium phosphate, pH 7.0 by heat shock at 95 °C. The amino acid sequence was used to calculate the extinction coefficients at 205 nm and 280 nm. The concentration is reported as the average calculated from the absorbance at 280 nm as determined using a Nanodrop 1000 spectrophotometer and

the absorbance at 205 nm as determined from the high tension (HT) signal of the SRCD spectrum.

Spectra were collected by Dr. Andy Miles of the Wallace lab on beamline CD1 at the Institute of Storage Ring Facilities, University of Aarhus, Denmark. The 100 g/L sample was measured in a 0.00042 cm pathlength cell. Samples between 17- and 48-g/L were measured in a 0.00066 cm pathlength CaF_2 cell. Spectra for more dilute samples were measured in a 0.001 cm Suprasil quartz cell from Hellma Ltd. Samples were warmed to 45 °C when needed to lower the viscosity while loading the cell. Data were collected at 20 °C from 260 nm to 170 nm using a 1 nm step size and a dwell time of two s. For highly concentrated samples, the data were truncated to exclude points with HT voltage greater than the cutoff criterion of 5.5.[154] Spectra were calibrated against a spectrum of camphoursulfonic acid.[155] Secondary structure was quantified using the sp175 data set[156] in the DichroWeb online analysis tool.[144-146] The 6 g/L sample, which produced usable data down to 170 nm, was analyzed using the CONTIN[157,158] and CDSSTR[159-161] algorithms. The results were averaged. The 100 g/L sample, which only produced usable data down to 200 nm, was analyzed using the SELMAT algorithm.[162,163] Because this data does not extend below 200 nm, the resulting analysis should be viewed cautiously.

CHAPTER 4: EXCIPIENTS INHIBIT UNFOLDING OF A GLOBULAR PROTEIN DURING DESICCATION

Overview

To understand the survival mechanisms of desiccation tolerant organisms and improve formulations of biologic drugs and industrial enzymes, we must understand how proteins behave in the absence of water. Using hydrogen-deuterium exchange experiments, I have shown that the test protein GB1 unfolds in the lyophilized state. The FDA-approved excipient mannitol is unable to protect GB1 during lyophilization. However, the FDA-approved excipient trehalose and a CAHS protein from desiccation tolerant tardigrades inhibit hydrogen-deuterium exchange of lyophilized GB1. It is unclear if trehalose and CAHS inhibit unfolding of GB1 during lyophilization or if they shield lyophilized GB1 from water vapor. Nevertheless, these data show that trehalose and CAHS protein inhibit exchange via different mechanisms.

Introduction

Biochemical studies usually focus on the behavior of biomolecules in aqueous environments such as dilute buffers, crowded environments, or inside cells. However, biomolecules also survive years of desiccation in a variety of species, including tardigrades, nematodes, and rotifers.[82] Desiccating biologic drugs and industrial enzyme reduces the weight of these products and often allows them to be stored at room temperature, which drastically reduces shipping costs. Furthermore, enzyme powders are ideal for industrial synthesis and degradation reactions in non-aqueous media.[4] I set

out to better understand the behavior of desiccated proteins using nuclear magnetic resonance spectroscopy (NMR).

Conventional NMR experiments rely on the tumbling of molecules in solution to produce sharp, distinct peaks. Desiccated proteins do not have the motion necessary for these experiments. Solid state NMR can be used to study desiccated proteins samples,[164] but UNC-Chapel Hill lacks a modern solid-state spectrometer. We bypassed these problems by using a solution-based NMR-detected hydrogen-deuterium exchange experiment for evaluating the structure of desiccated proteins.[165]

Hydrogen-deuterium exchange is commonly used to determine if a protein is folded.[10,141,166,167] Solvent exposed amide protons readily exchange with deuterons from D_2O,[140] while amide protons that are only exposed upon unfolding are less likely to exchange.[141] Rather than observe hydrogen-deuterium exchange in solution, Desai *et al.* used a desiccated protein that has its amide- (and other exchangeable-) protons exchanged for deuterons. The desiccated protein is then exposed to H_2O vapor.[165] Their one-dimensional ^{1}H NMR data shows that six amide deuterons buried in the interior of folded bovine pancreatic trypsin inhibitor exchange with protons in the vapor.[165] In other words, the protein unfolds while desiccated. The authors also show that formulation with sorbitol, an FDA-approved excipient, blocks exchange of those amide deuterons.[165]

We modified their protocol to examine the desiccated, ^{15}N-enriched B1 domain of streptococcal protein G (GB1,[168] 6.2 kDa, pI 4.5) using a two-dimensional ^{15}N-^{1}H heteronuclear single-quantum coherence (HSQC) experiment. This approach allows us

to study GB1 formulated with an excess of additive as long as that additive is not ^{15}N-enriched.

GB1 Unfolds During Desiccation

Our lab has used two-dimensional ^{15}N-^{1}H HSQC experiments to monitor hydrogen-deuterium exchange of and calculate the free energy of opening for GB1 residues in buffer, in crowded solutions, and in living cells.[103,169-172] Regardless of the aqueous environment, at least fourteen residues are protected in the interior such that hydrogen-deuterium exchange only occurs upon global unfolding of the protein.[103,173] In solution, global unfolding of this highly stable protein is infrequent, and full exchange of these secluded residues takes hours.[103]

I followed up on these results by probing GB1's structure in the desiccated state with an experiment based on that described by Desai *et al*. [165] and preliminary experiments by Dr. Rachel Cohen. First, all exchangeable protons in GB1 are exchanged for deuterons by repeated cycles of dissolution in D_2O, heating to promote unfolding and exchange of interior exchangeable protons, and lyophilization. The desiccated, deuterium-exchanged protein cake was placed in a 70% relative humidity chamber.[165,174] Backbone-amide deuterons are then allowed to exchange with protons from the H_2O vapor. After 24 h, exchange was quenched by dissolving the protein in a pH 3.5 deuterated buffer, and this snapshot was observed with a solution-state two-dimensional ^{15}N-^{1}H HSQC experiment. Each backbone nitrogen and attached proton result in a cross peak with chemical shift coordinates reflecting the environment of these nuclei whose volume is proportional to the concentration of protons attached to the amide ^{15}N. The results are compared to a control of protonated GB1 resuspended in

deuterated quench buffer, which shows the maximum proton signal (Figure 4.1-4.4, black)

At 0 h of exchange, deuterated GB1 gives rise to few cross peaks (Figure 4.1, cyan). These small peaks correspond to surface residues that pick up residual protons from the quench buffer as noted by Desai *et al*.[165] Exchange of deuterons for protons increases cross peak volume. Without additives, all observable GB1 residues exchange with the H_2O vapor (Figure 4.1, red) including thirteen residues that are classified as global unfolders, that is sites where the backbone amide is only exposed on complete unfolding in solution.[103,173] These results demonstrate that, like bovine pancreatic trypsin inhibitor protein,[165] GB1 unfolds in the desiccated state.

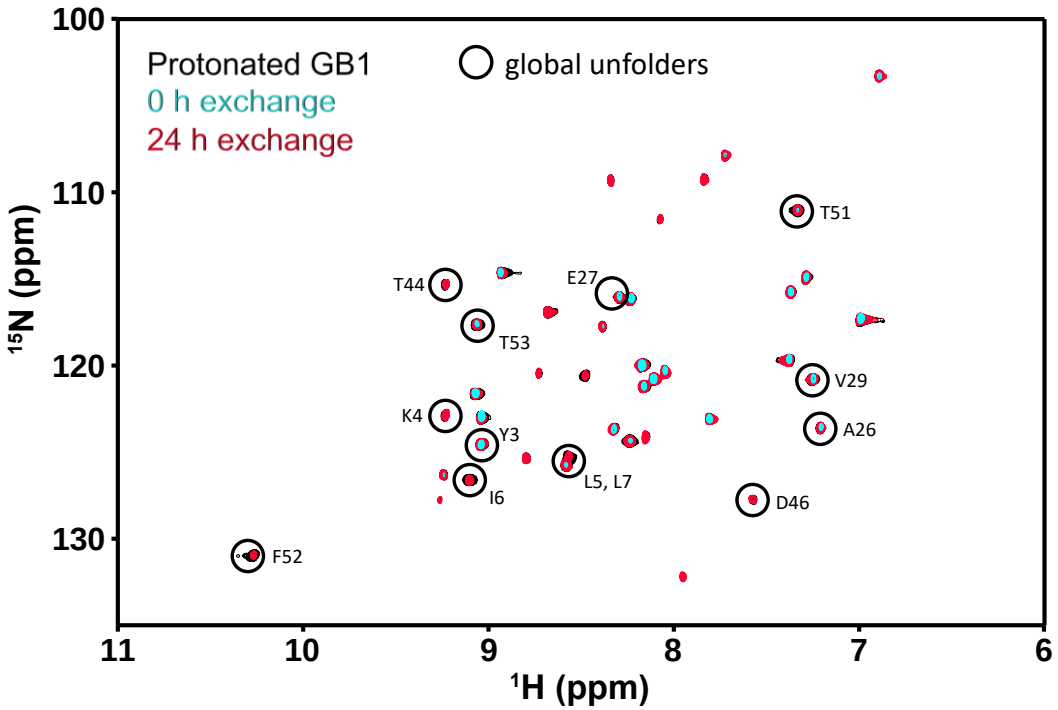


Figure 4.1. GB1 unfolds upon desiccation. ^{15}N-^{1}H HSQC spectra of protonated, lyophilized GB1 (black); deuterated, lyophilized GB1 after 0 h in a humidity chamber (cyan); and deuterated, lyophilized GB1 after 24 h in a humidity chamber (red). Global unfolders are circled and labeled.

Desai *et al.* observed that a 1:1 mass:mass formulation of bovine pancreatic trypsin inhibitor protein and the FDA-approved excipient sorbitol inhibits exchange of deuterons for protons in the desiccated state.[165] Based on this observation, we formulated GB1 with the sugar alcohol mannitol, which unlike sorbitol we had in supply. However, even at a 20:1 mass ratio of mannitol to GB1, mannitol does not inhibit exchange of the global unfolders (Figure 4.2, red). Consistent with our result, others show that mannitol does not protect enzymes against lyophilization-induced inactivation.[57,61,64] However, another study reports full protection of L-asparaginase by mannitol, suggesting that buffer conditions or other variables influence protection.[60]

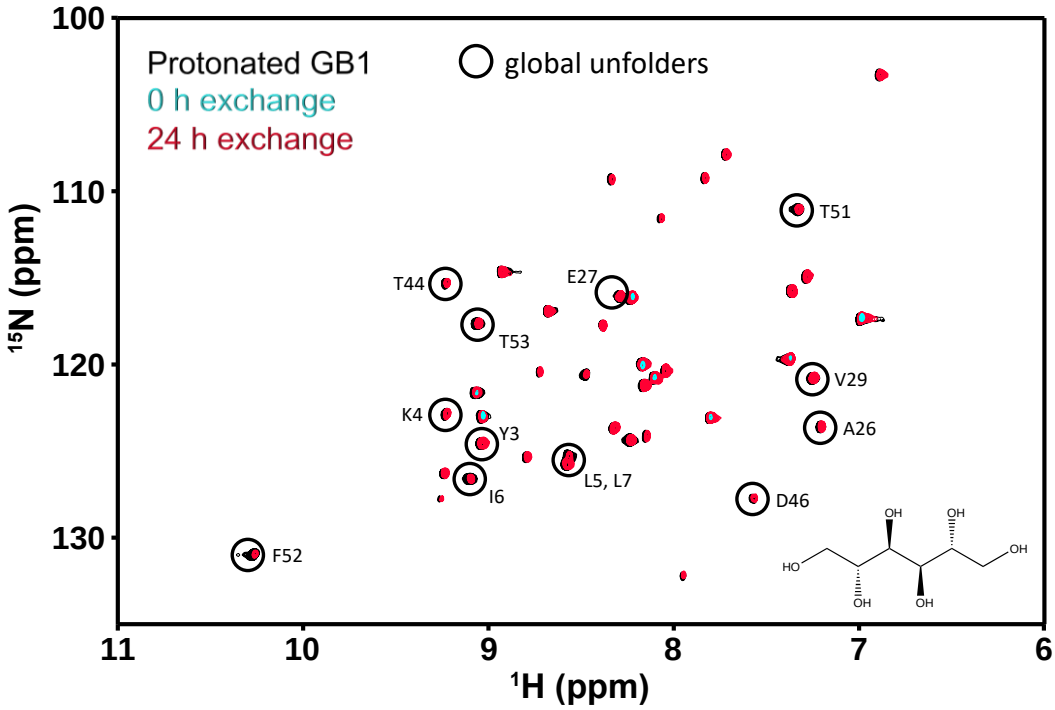


Figure 4.2. Mannitol does not protect lyophilized GB1.
^{15}N-^{1}H HSQC spectra of protonated, lyophilized GB1 (black); deuterated GB1 lyophilized 10:1 by mass with mannitol after 0 h in a humidity chamber (cyan); and deuterated GB1 lyophilized 10:1 by mass with mannitol after 24 h in a humidity chamber (red). Global unfolders are circled and labeled. Inset: structure of mannitol.

Trehalose and CAHS Proteins Inhibit Amide-Proton Exchange of Desiccated GB1

Formulation with mannitol does not inhibit hydrogen-deuterium exchange of GB1, but several other molecules protect GB1. The non-reducing disaccharide trehalose is a well-studied lyoprotectant. Many reports find that trehalose protects enzymes against lyophilization-induced inactivation,[37,39,40,55,59-63] although there are a few exceptions.[14,57,64] I formulated GB1 with trehalose at a 1:1 mass ratio and observed inhibition of exchange (Figure 4.3).

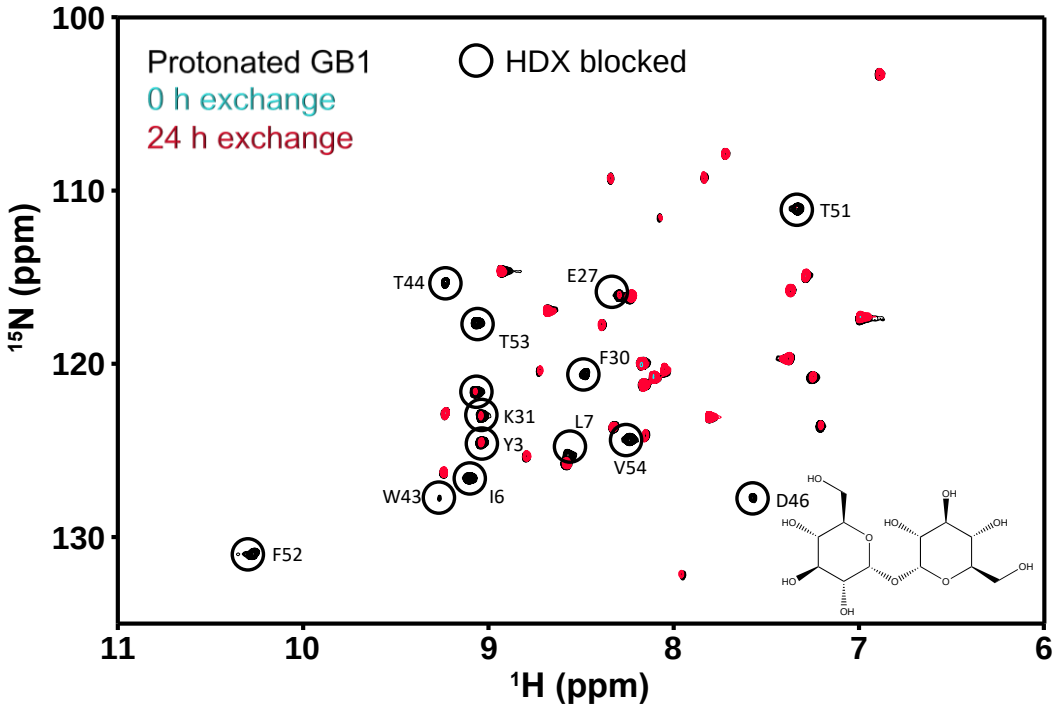


Figure 4.3. Trehalose inhibits hydrogen-deuterium exchange (HDX) of lyophilized GB1**.** ^{15}N-^{1}H HSQC spectra of protonated, lyophilized GB1 (black); deuterated GB1 lyophilized 1:1 by mass with trehalose after 0 h in a humidity chamber (cyan); and deuterated GB1 lyophilized 1:1 by mass with trehalose after 24 h in a humidity chamber (red). Residues protected from exchange are circled. Inset: structure of trehalose.

I also tested inhibition of exchange by a cytosolic abundant heat soluble (CAHS) protein. Tardigrades require CAHS proteins to survive desiccation,[10] and a CAHS

homolog fully protects the enzyme lipoprotein lipase during lyophilization.[14] We formulated GB1 with CAHS D at a 2.5:1 mass ratio and observed inhibition of exchange of multiple amide deuterons (Figure 4.4).

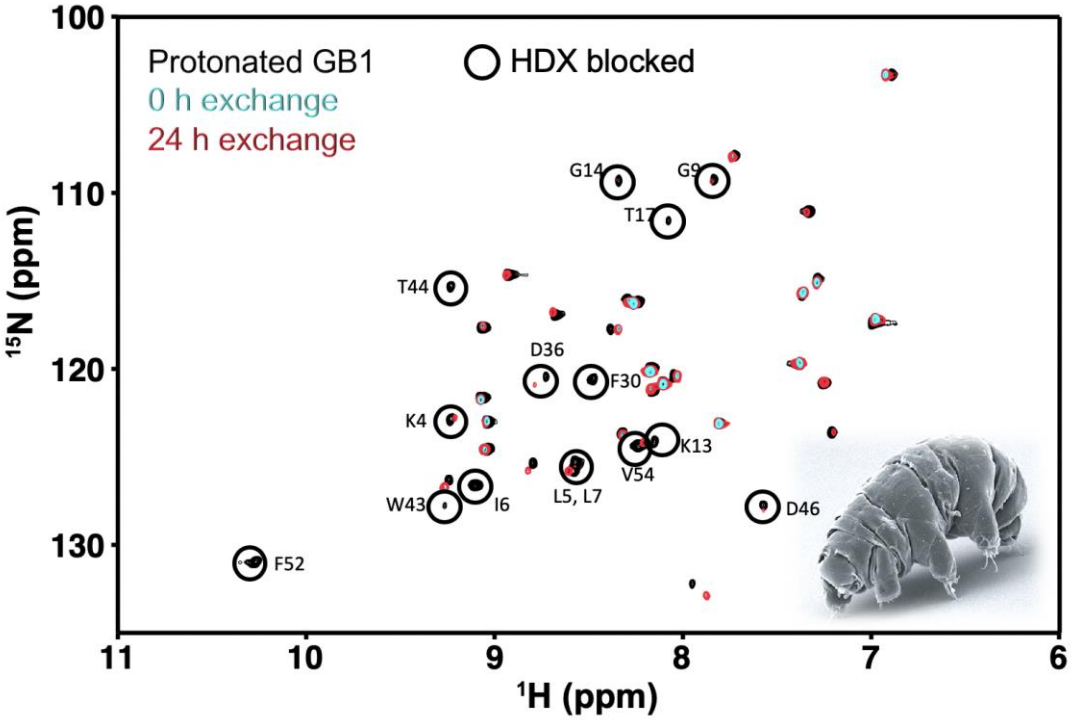


Figure 4.4. CAHS D inhibits hydrogen-deuterium exchange (HDX) of lyophilized GB1. ^{15}N-^{1}H HSQC spectra of protonated, lyophilized GB1 (black); deuterated GB1 lyophilized 2.5:1 by mass with CAHS D after 0 h in a humidity chamber (cyan); and deuterated GB1 lyophilized 2.5:1 by mass with CAHS D after 24 h in a humidity chamber (red). Residues protected from exchange are circled. Inset: a tardigrade.

Trehalose almost exclusively blocks hydrogen-deuterium exchange of global unfolders (Figure 4.5). Although CAHS D blocks exchange of many of the same amides as trehalose, it also blocks exchange of five more accessible residues (Figure 4.5). Better inhibition by CAHS D than trehalose may arise because I used a larger mass ratio of CAHS D. However, differences in formulation do not explain why trehalose more effectively blocks exchange of C-terminal residues than CAHS D. Importantly, we do not

know if trehalose and CAHS D inhibit unfolding of GB1 during lyophilization or if they simply shield unfolded GB1 from water vapor. Additional experiments are required to understand these results. Nevertheless, it appears that trehalose and CAHS D inhibit exchange via different mechanisms.

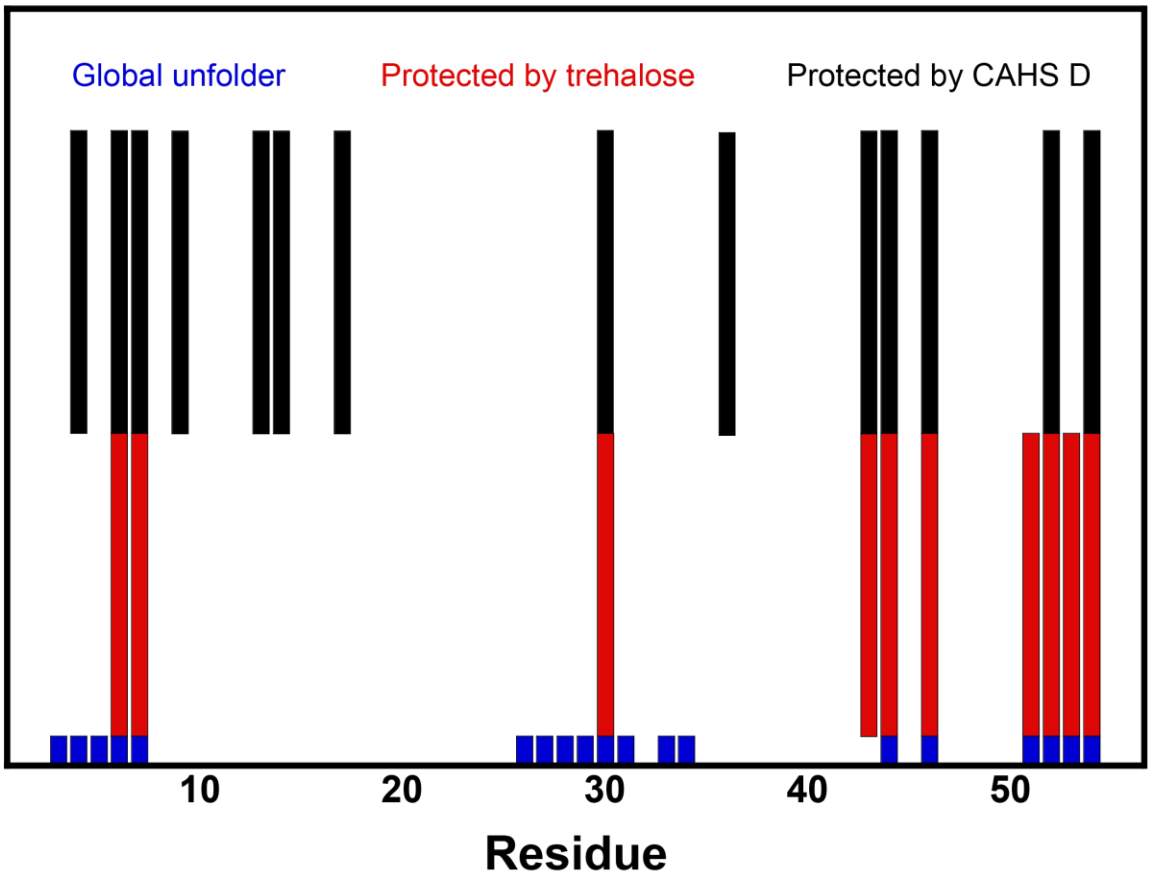


Figure 4.5. Comparison of GB1 residues protected from hydrogen-deuterium exchange by trehalose and CAHS D with the global unfolders of GB1.

Materials and Methods

Protein Purification

CAHS D was expressed and purified as described in Chapter 2. ^{15}N-enriched GB1 was expressed[103] and purified[107] as described.

NMR

GB1, CAHS, trehalose, and mannitol were fully exchanged in D_2O before use. Aliquots containing 2 mg of GB1 and 1 mL of D_2O were heated to 95 °C for 10 min, and cooled to room temperature. CAHS D (~60 mg) was resuspended in 10 mL of D_2O, heated to 95°C for 10 min and cooled to room temperature. Trehalose (20 mg) and mannitol (20 mg) were dissolved in 1 mL of D_2O and left at room temperature for a few hours. After deuteration, samples were flash frozen and lyophilized.

The deuterated additives were resuspended at the desired concentration in unbuffered D_2O. To fully dissolve CAHS D, the solution was heat shocked at 95 °C. Its final concentration was determined by a Bradford assay.[123] Trehalose and mannitol concentrations were based on mass. GB1 (2 mg) was resuspended in 1 mL additive solution to the desired mass ratio. Samples were flash frozen and lyophilized for ~16 h. Immediately after removal from the lyophilizer, a 0-h time point sample was resuspended in 650 μL of quench buffer (20 mM sodium citrate, 100 mM NaCl, uncorrected pH 3.5 in D_2O). For every 0 h time point, an identical sample was placed in a sealed chamber over a saturated aqueous KI solution at room temperature (70% relative humidity).[174] Exchange with atmospheric water was halted with quench buffer after 24 h.

Protonated controls were prepared in parallel. Two 2 mg samples of GB1 were suspended in 1 mL of H_2O, heated to 95°C for 10 min, and cooled to room temperature before flash freezing and lyophilization. One sample was resuspended in 650 μL of deuterated quench buffer and the other in protonated quench buffer (20 mM sodium citrate, 100 mM NaCl, pH 3.9 in H_2O) immediately after removal from the lyophilizer.

The sample in protonated quench buffer was used to assign peaks based on published assignments.[103,175] These data also confirm that deuteration via heat shock does not affect GB1 structure. The sample in deuterated quench buffer represents the maximum possible signal from fully protonated residues.

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