Protein Denaturation in Complex Solvents: Using FTIR spectroscopy to understand cryopreservation

Thesis presented to

The University of Texas at Austin

Department of Chemistry

In partial fulfillment of the requirement for the Degree of

B.S. Chemistry, Honors

Dean's Scholars Honors Program

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Spring 2020

May 4, 2020

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May 3, 2020

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Abstract: Cryoprotectants are commonly used to stop ice formation in biological systems, allowing for long term preservation and storage. Dimethyl sulfoxide (DMSO) is a commonly used cryoprotectant, however; it can also be toxic to cells which limits the use of DMSO in organs. Modulating the toxicity of DMSO could aid in long term organ storage and cryopreservation, creating organ banks and dramatically reducing the size of the organ donation waitlist. Cryoprotectant toxicity neutralizers, including formamide, have been shown to aid in neutralizing the toxicity of cryoprotectants such as DMSO. However, organs are large and multifaceted which presents a host of experimental challenges. Simple proteins like lysozyme can be used to model complex organ systems. Here we use amide I infrared spectroscopy to measure thermal denaturation curves of lysozyme in ternary mixtures of DMSO, water, and formamide or dimethylformamide. We show that formamide increases the stability of lysozyme in DMSO/water mixtures, suggesting that it protects against DMSO denaturation. We also show the effects of other amides like N-methylacetamide and N-methylformamide on lysozyme. The structures of these co-solvents are shown in Figure 1. Based on their differing structures and hydrogen bonding capabilities, these amides modulate the toxicity of DMSO in different ways. This information can be used to create mixtures of co-solvents to mitigate DMSO toxicity and eventually cryopreserve biological systems.

Background: Is it not currently possible to preserve organs with conventional freezing methods due to ice formation, which leads to the rupturing of cells and subsequent tissue death. As the water in cells freezes, ice nucleation occurs and pierces the cell membranes. Therefore, organs have extremely brief ex-vivo lifetimes of 8-24 hours with current cooling methods, and the organ donation waitlist remains unrealistically lengthy. 22 Americans die every day waiting for an organ transplant, but more than 60 percent of the hearts and lungs donated every year have to be discarded. Depending on the type of organ, extremely high recovery rates are needed for the organ to be beneficial for the patient. Complex tissue banking would have an immense impact in science and medicine, enabling advances in public health, cancer treatment, trauma care, tissue engineering, and regenerative medicine.

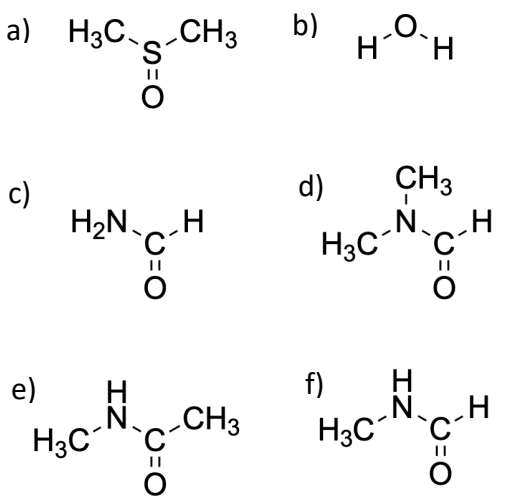

Figure 1. Chemicals of interest in cryopreservation studies (a) Dimethyl sulfoxide (DMSO) (b) Water (c) Formamide (FA) (d) Dimethylformamide (DMF) (e) N-methylacetamide (NMA) and (f) N-methylformamide (NMF)

Cryopreservation is a method to stop ice formation during the freezing process of water. Cryopreservation is the formation of a glass state in a process known as vitrification, where the end result is a non-crystalline amorphous solid, without ice crystals. This glass transition state prevents ice formation and allows cell and tissue samples to be successfully preserved. Cryoprotectant agents (CPAs) prevent ice formation by forming hydrogen bonds with water, and dimethyl sulfoxide (DMSO) is one such cryoprotectant. DMSO is commonly used in many laboratories to preserve small tissue samples and cells, but it is known to be toxic at high concentrations and is a well-studied protein denaturant. Solvents called cryoprotectant toxicity neutralizers (CTNs) have been hypothesized to

interact with cryoprotectants and reduce their toxicity. Formamide is one such cryoprotectant toxicity neutralizer of interest and its effects have not been fully studied. For comparison, studies can also be done with similar molecules that do not have cryoprotectant toxicity neutralizing effects, such as dimethyl formamide (DMF) to act as control experiments. DMF is known to actually increase toxicity and act as a protein denaturant, in contrast to formamide. Other amides of interest include N-methylacetamide (NMA) and N-methylformamide (NMF). The ability to use CPAs and CTNs in conjunction for complex tissue preservation could lead to significant breakthroughs. Model systems with simple proteins can be used in order to model this complex problem. These insights can then be applied to larger systems like tissues and organs. There are many considerations to make when studying protein structure.

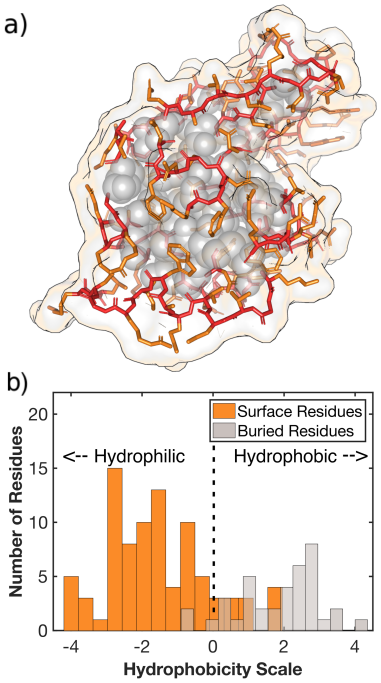

Figure 2. (a) Hen egg-white lysozyme (HEWL). Buried residues are shown as grey spheres and surface residues as red (backbone) and orange (sidechains) sticks (PDB: 1AKI). Surface residues are defined as having greater than 6Å^2 solvent-accessible surface area with a solvent radius of 1.4 Å. (b) Kyte and Doolittle Hydrophobicity histogram of surface and buried residues in lysozyme.

Protein structures are the result of a balance between charge, hydrophobicity, and solvent exposure[1–9]. Considerable efforts have been directed at understanding the effects of cosolvents like DMSO and FA on proteins, and while current models can predict some aspects of protein thermodynamics for common denaturants, the molecular aspects of these interactions are not well understood[10,11]. We now know that direct electrostatic interactions with denaturants disrupt hydrogen-bond contacts within the protein backbone[12–14], free energies are affected by solvent polarity and hydrogen-bonding[15,16], and crowding has a net enthalpic stabilization effect on proteins[17]. While these interactions are generally understood for common denaturants, the effects of mixed solvents are not well characterized. Understanding how cryoprotectant agents (CPAs) and cryoprotectant toxicity neutralizers (CTNs) affect protein structure and thermodynamics in solution is vital to elucidate how these chemicals can be used together to stop protein denaturation.

DMSO is a commonly used cosolute and cryoprotectant[18]. Its effects on protein stability are dominated by the strong H-bond-accepting S=O group, which disrupts the hydrogen donor/acceptor balance and “dehydrates” proteins. In addition, its methyl groups interact with hydrophobic residues[19,20]. Protein structure and stability have been extensively studied in DMSO mixtures[21]. Recent work suggests that low DMSO concentrations stabilize folded proteins and can even increase enzyme activity[22]. However, higher DMSO concentrations destabilize folded states[23].

DMSO is routinely used as a cryoprotectant to preserve cells and tissues at low temperatures since it prevents ice crystal formation[18]. However, DMSO is a mild denaturant, and is toxic to cells at

high concentrations[24,25]. Certain amides increase cell viability, for example, DMSO-formamide (FA) mixtures increase rabbit kidney cell viability by as much as 50% compared to DMSO-only, while dimethylformamide (DMF) has the opposite effect, reducing cell viability[26]. Toxicity mechanisms are likely a result of complex interactions, but we hypothesize that protein stability is an important factor since unfolded proteins cannot perform their typical functions in cells. Evidence for protein destabilization has been observed in animal models, where expression levels of heat-shock proteins increase upon chemical stress induced by exposure to DMSO[27,28]. In this work, we quantify the effects of aqueous DMSO with two amides and show that DMSO together with FA stabilizes native states compared to DMSO alone, while the addition of DMF has the opposite effect. We also show the effect of NMA and NMF on the stability of lysozyme in DMSO mixtures. While these effects need to be studied further, these results are a starting point.
The model protein used here is hen egg-white lysozyme, which is one of the most common models since it undergoes reversible two-state transitions and is not prone to aggregation at moderate concentrations (Figure 2)[29,30]. Lysozyme has a +7.9 charge at neutral pH and a well-understood structure featuring solvent-exposed hydrophilic residues surrounding a primarily hydrophobic core. Protein structure is routinely studied with methods such as light scattering, x-ray crystallography, calorimetry, circular dichroism, fluorescence correlation, and NMR spectroscopy[31–36]. FTIR spectroscopy is used to quantify secondary structure, and is a well-known technique used in many protein studies[37–40]. Backbone C=O absorptions, known as Amide I absorption modes, appear in the 1600-1700 cm^{-1} region and are sensitive to secondary structure. The amide I band is well-separated from any DMSO absorptions, allowing for measurements at high DMSO concentrations. On the other hand, amides (such as FA and DMF) produce overlapping absorptions due to their amide moieties but can be background subtracted at low concentrations. Using FTIR spectroscopy on lysozyme in mixtures of DMSO, FA, and DMF, allows us to extract thermodynamic parameters associated with protein unfolding and understand protein stability in these different mixtures.

Methods: Dimethyl sulfoxide (DMSO, 99.9%), formamide (FA, 99.9%), N,N-dimethylformamide (DMF, 99.8%), deuterium oxide (D2O, 99.9%) and twice lyophilized hen egg white lysozyme (HEWL, 90%) were purchased from Sigma-Aldrich Corp. (St. Louis, MO) and used without further purification. Lysozyme was dissolved in D_2O and allowed to equilibrate for 24 hours at room temperature to allow for full deuteration of the amide groups. This was performed by dissolving a few milligrams of lysozyme in D_2O in a microcentrifuge tube. Spectra before and after were compared to check for reduction in in the intensity of the O-H band. Following the H/D exchange, the protein was lyophilized for 24 hours. A needle was used to poke a hole in the tip of the microcapillary tube and a piece of Kimwipe was placed underneath the cap before closing the tube. This tub was submerged in liquid nitrogen until frozen and placed in the lyophilizer.

After lyophilization, the lysozyme samples with various concentrations of DMSO, FA, and DMF were made. The molar mass and density of these cosolvents were used to calculate the required volumes to make the required concentrations, specified below. A concentration of 30 mg/mL of lysozyme was used. This is low enough to avoid aggregation, but high enough for the lysozyme amide absorption to be greater than contributions from FA and DMF. Dilute NaOD and DCl were used to adjust the pH to 7-7.5 (uncorrected for deuterium effects)[41].

Infrared absorption spectra were measured using a Bruker VERTEX 70 spectrometer purged with dry air. Samples were held in between pair of CaF_2 windows without a spacer to achieve thin path lengths due to the strong absorbance of formamide and DMF. Denaturation curves were measured using a house-built temperature-controlled sample cell connected to a recirculating chiller. Temperature-dependent FTIR spectra were measured from 5 to 90 °C in 5 °C intervals with a 10-minute delay at each temperature to ensure proper equilibration. Spectra were measured repeatedly with different samples and independently analyzed thermodynamic fits were shown to vary less than 5 kcal/mol for repeated measurements. Temperature series were taken at the following DMSO concentrations [0, 10, 20, 30] mol%, as well as [0, 10, 20, 30] mol% with 5 w/v% formamide and 5 w/v% dimethylformamide.

All background spectra were measured under identical conditions as the corresponding protein samples. Temperature series were corrected for water vapor absorption and normalized to the maximum intensity of the amide I band. For background subtraction of the FA and DMF series, sample and background spectra were separately baseline-corrected and normalized by area in Matlab. Background spectra were multiplied by the ratio of FA concentration to the total amide carbonyl concentration, which includes Lysozyme and FA or DMF before subtraction. For example, in 30 mol% DMSO with 5 w/v% FA, the background spectra would be multiplied by a factor of approximately 0.6 before subtraction. Background-subtracted sample spectra were analyzed using singular value decomposition (SVD) to extract temperature-dependent line shape changes. SVD decomposes a matrix into U, S, and V components. It is not possible to analyze the entire amide I line shape all at once, so performing SVD allows us to look at a single point. By plotting these points as a function of temperature, we gain insight into the melting temperature of lysozyme based on the line shape changes. In Matlab, the svd function was used on the normalized sample spectra and the second column of the U matrix was plotted as a function of temperature. This curve, (the normalized second component SVD) was then baseline corrected and fit to a sigmoidal function (Equation 1) in Matlab to extract the folded and denatured populations.

$$S_{SVD} = \frac{a}{1+\exp\left[s\left(T-T_m\right)\right]} + b \qquad (1)$$

Coefficients a and b account for the amplitude and offset of the second component SVD signal (S_{SVD}), respectively, s is the stretching factor, and T_m is the melting temperature. Root-mean-squared residuals were below 2% for all fits. The thermodynamic stability is the difference between the free energy of the protein in its folded and unfolded ($G_{f\rightarrow u}$) state at a specified temperature as shown in Equation 2. P_f is the population of the folded protein and thus 1-P_f represents the population of the unfolded protein. These populations are used to obtain the free energies:

$$\Delta G_{f\rightarrow u} = -RTln\left(\frac{P_f}{1-P_f}\right) = -RTln\left(\frac{1}{1+\exp[s(T-T_m)]}\right) \qquad (2)$$

The denaturation reaction of lysozyme can be regarded as a two-state transition, and ΔG, ΔS°, and ΔH° can be experimentally defined. After converting the populations into free energies using the Boltzmann equation, thermodynamic parameters were extracted from the following model[42]:

$$\Delta G = \Delta H^{\circ} - T\Delta S^{\circ} + \Delta C_p\left[T - T_m - T\ln\left(T/T_m\right)\right] \quad (3)$$

Where T_m is the melting temperature, ΔH° and ΔS° are the denaturation enthalpies and entropies, and ΔC_p is the change in heat capacity. This fitting procedure was performed in Matlab. At the melting temperature (T_m), the ΔG is 0, since the population of the folded state equals the population of the unfolded state[42]. Free energies of denaturation are calculated in reference to ΔG of lysozyme in water at 298 K. Therefore, at room temperature, free energies of denaturation are lower, and in general lower melting temperatures correlate to lower ΔG values. The thermodynamic errors were estimated by repeating the free energy fit 10 times while randomly sampling initial values based on the 95% confidence intervals of the coefficients in the aforementioned sigmoidal fits.

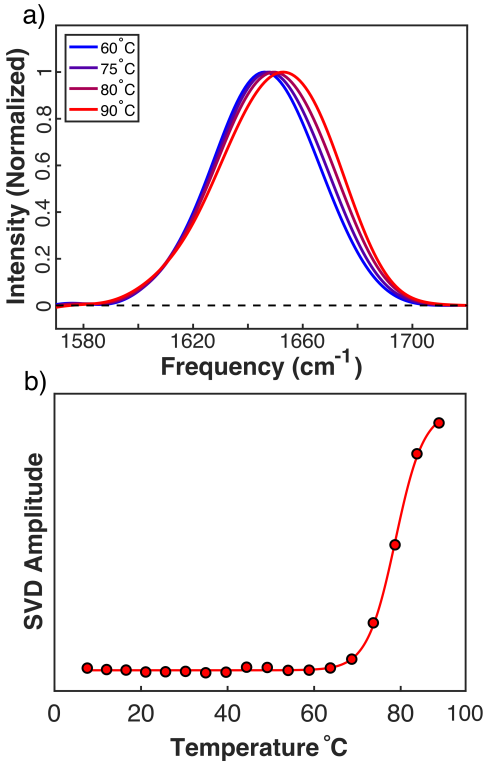

Figure 3. (a) Spectra of Amide I band of lysozyme at four representative temperatures. Line shapes broaden and shift to higher frequencies as protein unfolds. (b) Second component singular value decomposition of FTIR spectra as a function of temperature. Temperature series in 5 °C intervals are shown in Figure S1. The solid line represents the fit to the sigmoidal function shown in Equation 1.

Results: Figure 3a shows representative amide I spectra at four temperatures. In general, the Amide I band blue-shifts (moves to higher frequencies) at higher temperatures due to weakening of protein-protein and protein-water hydrogen-bonds. The band also broadens in width as the protein loses its primarily helical secondary structure. The SVD decomposition for the temperature series is performed as described in the methods section. Figure 3b shows a sigmoidal fit to the denaturation curve, as described in Equation 1 in the methods section.

Figure 4 shows that increasing the DMSO concentration shifts the thermal denaturation curves towards lower temperatures. In other words, the melting temperature decreases with increasing DMSO concentration. Consistent with previous literature, lysozyme melts at 78.8 °C in pure water; the addition of 30% DMSO lowers the melting temperature to 49.3 °C[40,48,49]. In concentrations up to 30 mol%, the two-state transition is maintained alongside native secondary structure. Addition of 5 w/v% formamide to the 30 mol% DMSO/water mixtures resulted in an increase in melting temperature compared to the respective binary mixtures, indicating that FA stabilizes the protein in higher DMSO concentrations (Figure 4). Interestingly, addition

of 5 w/v% formamide in the absence of DMSO does not significantly affect the melting temperature. In contrast, addition of 5 mol% DMF decreased the melting temperature across the entire DMSO concentration range. These results suggest that DMF also acts as a mild denaturant. Spectra were also taken at DMSO concentrations greater than 30 mol%, but no two-state transition was observed. This is likely because the protein was already denatured before heating due to the high DMSO concentration.

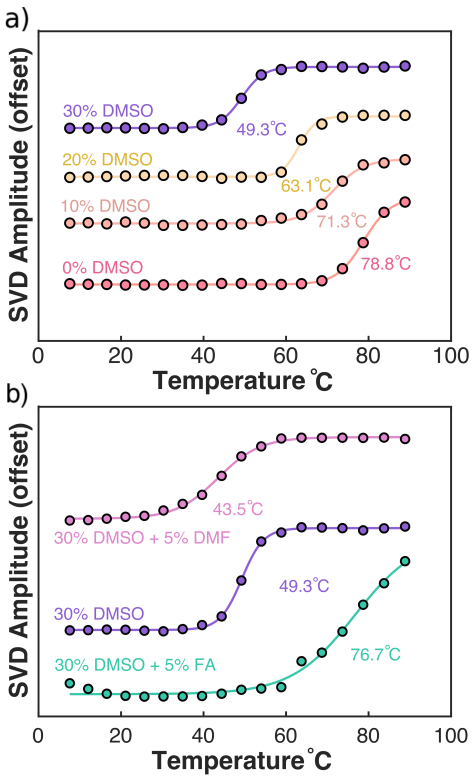

Figure 4. Thermal denaturation curves of HEWL fit to second component SVD. The circles are the SVD points and solid curves represent the two-state sigmoidal fits. (a) Solutions containing DMSO only: 0-30 mol% DMSO. (b) 30 mol% DMSO solution with addition of 5 w/v% FA or 5 w/v% DMF.

Figure 5 shows the enthalpic and entropic components of folding with increasing DMSO concentrations and with addition of FA or DMF. In the DMSO-only mixtures, we observe a decrease in enthalpy and increase in entropy. The values for 0 mol% and 10 mol% DMSO are similar, and differences become more pronounced in the 20-30 mol% solutions. Below 20 mol%, the $\Delta H°$ and $\Delta S°$ values show no significant differences with the addition of FA or DMF, consistent with the minor changes in melting temperatures. However, at 20 and 30 mol% DMSO + 5 w/v% FA, there is a significant increase in $\Delta H°$ accompanied by a decrease in $\Delta S°$. Figure 4 shows the calculated denaturation free energy at 25 °C for the mixtures. In general, a monotonic decrease in stability is observed with increasing DMSO concentration. DMSO lowers the free energy by approximately 0.87 kcal/mol for every 1 mol% increase in concentration. DMF shows a similar slope (1.02 [kcal/mol]/°C), but shifted by 4.5 kcal/mol, showing that the denaturation effects are additive for DMF. On the other hand, addition of 5 w/v% FA to 10 mol% DMSO only stabilizes the protein by 1.2 kcal/mol, whereas addition of 5 w/v% FA to 30 mol% DMSO results in a stabilization of 14.2 kcal/mol, showing that the effects are non-additive for FA. These large differences result in considerable stability shifts at higher DMSO concentrations as shown in Figure 3.

Discussion: The results show that the addition of FA increases the denaturation free energy of lysozyme in the presence of DMSO (Figure 5). These effects are most clearly observed in the high-DMSO mixtures, where the free energy is increased by 18 kcal/mol at room temperature. Consequently, the addition of 5w/v% FA to 30 mol% DMSO raises the melting temperature by 27 °C, almost to the temperature measured in pure water. These trends can begin to be explained by considering the polarity of the mixtures. Since DMSO's polarity is approximately half that of water, increasing the DMSO concentration leads to less polar binary mixtures. For example, the static dielectric constant of a 30 mol% solution is 69.0 compared to 78.8 for pure water[50]. The lower solvent polarity results in a reduced free energy cost of exposing hydrophobic side chains,

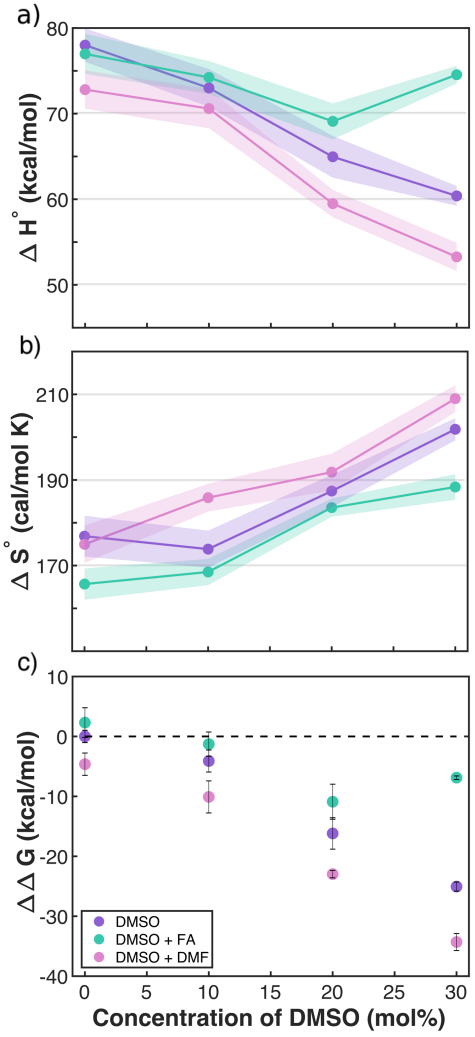

Figure 5. Thermodynamic parameters extracted from SVD analysis using Equation 2. (a) Enthalpic contributions (b) Entropic contributions and (c) Free energies calculated at room temperature. The shaded areas represent the 95% confidence intervals respectively and correspond to errors in Table 1. Errors are taken from iterations of 10 thermodynamic fits to Equation 3. The free energy error bars are calculated from the individual contributions.

leading to overall lower denaturation free energies. This essentially means that the denatured state is stabilized while the native state is destabilized, since hydrophobic residues become solvent exposed upon denaturation. These trends are demonstrated in Figure 5 where denaturation free energy decreases with increasing DMSO concentration. The results show that that enthalpic contributions dominate, accounting for ~2/3 of the free energy changes between water and 30 mol% DMSO at room temperature. The same polarity argument applies to the DMF and FA mixtures: DMF has a lower polarity than DMSO and water, therefore the addition of DMF further decreases the bulk solvent polarity. On the other hand, FA is more polar than water or DMSO, therefore the bulk polarity in the DMSO/FA mixtures is higher compared to the binary DMSO/water environment. Although these qualitative arguments provide a simple, intuitive description of the trends, the concentrations of FA or DMF are very small compared to DMSO. For instance, 30 mol% DMSO and 5 w/v% FA translates to an ~8:1 DMSO:FA mole ratio. Bulk polarity may not accurately entirely account for the trends observed in the local environment surrounding the protein. hydrophilic residues exposed to the solvent, as shown in Figure 2. Molecular dynamics simulations on folded and unfolded lysozyme, while not discussed further in this work, can be used to understand these effects.

Together, these experiments suggest that protein solvent interactions are key to understanding the thermodynamic effects of these cosolvents. A qualitative analysis based on experiment is discussed here, but MD simulations are necessary to future elucidate these interactions and gain quantitative insight. Other amides can also be studies, as they have different structures and hydrogen bonding properties that will differently affect the thermodynamic stability of proteins.

Conclusions and Outlook: We showed that FA stabilizes lysozyme, a model protein, against the denaturing effects of DMSO, a common cryoprotectant. Surprisingly, the effects of amides are highly non-additive, and we observed that at 30 mol% DMSO, FA negates the effects of DMSO and increases the stability of lysozyme to approximately the same thermodynamic stability as

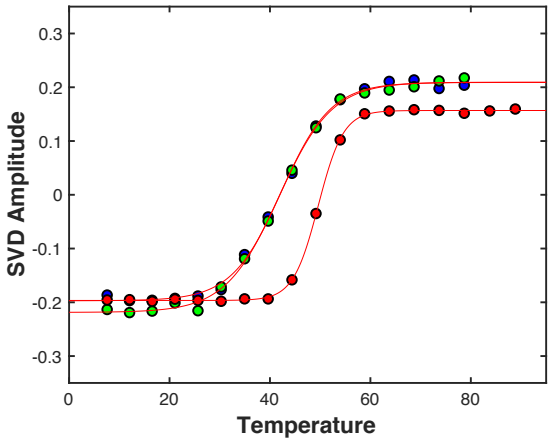

Figure 6. Thermal denaturation curves of HEWL fit to second component SVD in 30 mol% DMSO (red) 30 mol% DMSO + 5 w/v% NMA (blue) and 30 mol% DMSO + 5 w/v% NMF (green)

measured with 10% DMSO. Our results show that further studies are required to test complex protein–solvent interactions in multi-component mixtures.

Given the tissue toxicity of DMSO and its widespread use as a cryoprotectant, our results point towards the use of small amides as potential agents to increase cell viability. If protein stability is indeed an important contributor to toxicity, amides must be further explored as possible protecting osmolytes in DMSO mixtures. These results could be used to aid in study on more complex systems, such as tissues and organs, as we work to mediate ice formation during cold storage.

Future Work: There are many areas of further study on this subject in addition to molecular dynamics simulations. Other amides have also been shown to play a role in modulating protein stability, including NMA and NMF. Figure 6 shows thermal denaturation curves of lysozyme in 30 mol% with the addition of 5 w/v% NMA and 5 w/v% NMF. These solutions were prepared in the same manner as previously described. Preliminary results show that NMA and NMF both lower the melting temperature of lysozyme by 7-8 °C. It is interesting that these amides with different hydrogen bonding properties have similar effects of the stability of lysozyme, and more studies are needed to investigate these mechanisms further.

Acknowledgments: This is a Plan II SAWIAGOS Project. We gratefully acknowledge financial support from the College of Natural Sciences at the University of Texas at Austin, Welch Foundation (F-1891), and the National Science Foundation under grant CHE-1847199 to C.B. and CHE-1559839 for the Summer REU Program. Simulations were run at the Texas Advanced Computer Center (TACC).

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