Temperature Dependences of Urea $m$-values of Globular Proteins

by

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A thesis submitted in conformity with the requirements
for the degree of Master of Science
Department of Pharmaceutical Sciences at the Leslie Dan Faculty of Pharmacy
University of Toronto

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Abstract

This thesis aims at understanding the molecular origins of urea-induced protein denaturation, in particular, concentrating on the $m$-value, a thermodynamic parameter describing the urea-dependence of protein stability within the framework of linear extrapolation model (LEM). We analyze the temperature and urea dependences of the $m$-value of globular proteins using a modified statistical thermodynamic model. Circular dichroism spectral measurements were performed to determine the urea-induced unfolding profiles of hen egg-white lysozyme and horse heart cytochrome c at various temperatures. Our combined experimental and computational results collectively paint a picture in which the relative independence of protein $m$-values of urea concentration originates from fortuitous compensatory effects of a progressive increase in the solvent accessible surface area of the unfolded state and a slightly higher urea binding constant of the unfolded state relative to the folded state. Other denaturing cosolvents which lack such a compensation make poor candidates for linear extrapolation model-based protein stability determination studies.
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# Table of Contents

Abstract .................................................................................................................................. ii
Acknowledgments ...................................................................................................................... iii
Table of Contents....................................................................................................................... iv
List of Tables .............................................................................................................................. vi
List of Figures ............................................................................................................................ vii
List of Abbreviations .................................................................................................................. x

## CHAPTER 1 ............................................................................................................................... 1

1.1 General Introduction ............................................................................................................ 1

1.2 Solute-Solvent Interactions ............................................................................................... 2

1.2.1 Protein-Water Interactions ......................................................................................... 2

1.2.2 Protein-Cosolvent Interactions .................................................................................. 3

1.3 Proposed Mechanisms of Urea-Induced Protein Unfolding .............................................. 7

1.4 Solubility Studies and Transfer Free Energy ..................................................................... 9

1.5 Linear Extrapolation Model & The m Value ..................................................................... 11

1.6 Scaled Particle Theory ..................................................................................................... 13

1.7 Research Aims .................................................................................................................. 13

1.7.1 Lysozyme .................................................................................................................... 14

1.7.2 Cytochrome C ............................................................................................................. 14

## CHAPTER 2: On urea and temperature dependences of m-values .......................................... 16

## CHAPTER 3: General Conclusions and Future Perspectives .................................................. 42

3.1 Conclusion .......................................................................................................................... 42
3.2 Future Directions .................................................................................................................. 43

3.2.1 Comparison of Pressure Perturbation vs. Chemical Denaturation .................. 43

REFERENCES ................................................................................................................................. 45

APPENDIX A: SUPPLEMENTARY MATERIAL FOR CHAPTER 2 ................................................. 61
List of Tables

Table 2.1. Densities of aqueous solutions of urea ................................. 22

Table 2.2. Molar concentrations of water in aqueous solutions of urea .............. 33

Table 2.3. Molar concentrations of urea in an aqueous solution .................................. 33

Table 2.4. The solvent accessible surface areas, \( S_{AD} = 5755(1.8 + 0.05a_3) \), spherocylindrical curvatures, \( a \), and cylindrical lengths, \( l \), of a spherocylinder with a molecular volume, \( V_M \), of 33,510 Å\(^3 \) .............................................................................................................................. 38
List of Figures

**Figure 2.1.** A representative urea-induced denaturation profile of cytochrome c at 25 °C as measured by the molar ellipticity at 222 nm. Experimental data were fitted by Eq. (5) (solid line) (see in text) ................................................................. 24

**Figure 2.2.** Temperature dependences of the stabilities of lysozyme [panel (a)] and cytochrome c [panel (b)]. Solid lines represent temperature dependences of the unfolding free energy simulated with Eq. (6) (see in text) ................................................................. 26

**Figure 2.3.** Temperature dependences of the urea m-values of lysozyme [panel (a)] and cytochrome c [panel (b)] .................................................................................................................... 28

**Figure 2.4.** Urea dependences of changes in free energy of cavity formation, ΔΔGC, accompanying an unfolding of a spherical protein with a radius of 20 Å (an S_A of 5755 Å² and a V_M of 33,510 Å³) into a spherocylinder with an S_A of 11,510 Å² and a V_M of 33,510 Å³ at 18 (black), 25 (red), 35 (blue), and 45 (magenta) °C .................................................................................................................. 32

**Figure 2.5.** Urea dependences of the m-values for an unfolding of a spherical protein with a radius of 20 Å (with an S_A of 5755 Å² and a V_M of 33,510 Å³) into a spherocylinder with an S_A of 11,510 Å² and a V_M of 33,510 Å³ at 18 (black), 25 (red), 35 (blue), and 45 (magenta) °C .......................................................................................................................... 35
**Figure 2.6.** Urea dependences of changes in free energy of cavity formation, \( \Delta \Delta G_C \), accompanying an unfolding of a spherical protein with a radius of 20 Å (an \( S_A \) of 5755 Å\(^2\) and a \( V_M \) of 33,510 Å\(^3\)) into a spherocylinder with \( S_A = 5755(1.8 + 0.05a_3) \) and a \( V_M \) of 33,510 Å\(^3\) at 18 (black), 25 (red), 35 (blue), and 45 (magenta) °C ................................................................. 38

**Figure 2.7.** Urea dependences of the m-values for an unfolding of a spherical protein with a radius of 20 Å (with an \( S_A \) of 5755 Å\(^2\) and a \( V_M \) of 33,510 Å\(^3\)) into a spherocylinder with \( S_A = 5755(1.8 + 0.05a_3) \) and a \( V_M \) of 33,510 Å\(^3\) at 18 (black), 25 (red), 35 (blue), and 45 (magenta) °C ........................................................................................................................................................................... 39
List of Figures: Appendix

Figure S2.1a. Urea-induced denaturation profile of lysozyme at 20 °C ........................................ 61

Figure S2.1b. Urea-induced denaturation profile of lysozyme at 25 °C ........................................ 61

Figure S2.1c. Urea-induced denaturation profile of lysozyme at 30 °C ........................................ 62

Figure S2.1d. Urea-induced denaturation profile of lysozyme at 35 °C ........................................ 62

Figure S2.1e. Urea-induced denaturation profile of lysozyme at 40 °C ........................................ 63

Figure S2.1f. Urea-induced denaturation profile of lysozyme at 45 °C ........................................ 63

Figure S2.2a. Urea-induced denaturation profile of cytochrome c at 20 °C ................................. 64

Figure S2.2b. Urea-induced denaturation profile of cytochrome c at 25 °C ................................. 64

Figure S2.2c. Urea-induced denaturation profile of cytochrome c at 30 °C ................................. 65

Figure S2.2d. Urea-induced denaturation profile of cytochrome c at 35 °C ................................. 65

Figure S2.2e. Urea-induced denaturation profile of cytochrome c at 40 °C ................................. 66

Figure S2.2f. Urea-induced denaturation profile of cytochrome c at 45 °C ................................. 66

Figure S2.3a. UV melting profile of lysozyme at 280nm in neat buffers ................................. 67

Figure S2.3b. UV melting profile of cytochrome c at 290nm in neat buffers ................................. 67
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>CP</td>
<td>Heat capacity</td>
</tr>
<tr>
<td>HM</td>
<td>Enthalpy at ( T_M )</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared spectroscopy</td>
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<tr>
<td>k</td>
<td>Binding Constant</td>
</tr>
<tr>
<td>LEM</td>
<td>Linear extrapolation model</td>
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<tr>
<td>MD</td>
<td>Molecular Dynamics</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance spectroscopy</td>
</tr>
<tr>
<td>S_A</td>
<td>Surface Area</td>
</tr>
<tr>
<td>SAS</td>
<td>Solvent Accessible Surface area</td>
</tr>
<tr>
<td>SEM</td>
<td>Solvent Exchange Model</td>
</tr>
<tr>
<td>SPT</td>
<td>Scaled Particle Theory</td>
</tr>
<tr>
<td>T_M</td>
<td>Transition temperature</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V_M</td>
<td>Molecular volume</td>
</tr>
</tbody>
</table>
CHAPTER 1

1.1 General Introduction

Proteins are biological macromolecules essential for cellular function. To be biologically active, globular proteins have to fold into a unique native conformation (Kauzmann, W., 1959; Tanford, C., 1968; Tanford, C., 1970; Creighton, T.E., 1984; Kauzmann, W., 1987; Pfeil, W., 1998; Pfeil, W., 2001; Michael Gromiha, M., 2010; Thirumalai, D., et al., 2010; Canchi, D.R., & Garcia, A.E., 2013). The conformational stability of proteins is influenced by changes in environmental conditions, including temperature, pH, pressure, and presence of cosolvents (Canchi, D.R. et al., 2010; Canchi, D.R., & Garcia, A.E., 2013). Any change in the environmental conditions may cause a shift in the equilibrium between the native and denatured conformations.

Stabilizing or protecting cosolvents (i.e. trimethylamine N-oxide (TMAO), glycine, and betaine) are known to stabilize the folded protein structure, whilst those that favor the unfolded state are known as denaturing or destabilizing cosolvents (i.e. urea and guanidium chloride) (Yancey, P.H. et al., 1982; David-Searles, PR. et al., 2001; Das, A., & Mukhopadhyay, C., 2009). Interactions between a cosolvent and protein groups can have a profound effect on the folding thermodynamics of a protein in aqueous solutions thereby making cosolvents a common tool in protein folding studies (Arakawa, T., & Timasheff, S.N. 1985; Bennion, B.J., & Daggett, V., 2003; Auton, M. et al., 2007). In this thesis, we aim to characterize the molecular basis of the destabilizing effect of urea on globular proteins.
1.2 Solute-Solvent Interactions

1.2.1 Protein-Water Interactions


Water that interacts with a protein molecule exhibits thermodynamic and kinetic properties that differ significantly from those exhibited by bulk water (Oobatake, M., & Ooi, T., 1993; Xie, G., & Timasheff, S.N., 1997; Timasheff, S.N., 1998). In general, solute-solvent interactions cause a decrease in vapor pressure and chemical potential of the water and may be accompanied by a decrease in entropy, enthalpy, and volume of the system (Oobatake, M., & Ooi, T., 1993; Makhatadze, G.I. & Privalov, P.L., 1995). Solute-solvent interactions are influenced by the chemical nature of solvent-exposed solute groups on the surface of the protein. In an aqueous environment, the hydrophobic groups of a protein tend to collapse
and form a tightly packed core composing ~80% nonpolar side chains (Tanford, C., 1962). At room temperature, the hydrophobic collapse is driven by an unfavorable decrease in entropy caused by water interacting with nonpolar groups over a large surface area, a phenomenon known as the hydrophobic effect (Tanford, C., 1978; Tanford, C., 1980; Levy, Y., & Onuchic, J., 2006). In a strictly thermodynamic sense, hydration refers to all water molecules whose thermodynamic properties are distinct from bulk water regardless of their physical proximity to the protein surface (Timasheff, S.N., 2002).

The importance of water has been well recognized with the hydrophobic effect proposed as being one of the main driving forces in protein folding (Dill, K.A., 1990; Bogan, A.A. & Thorn, K.S., 1998; Pratt, L.R., & Pohorille, A., 2002). Studies have shown that increasing hydration typically enhances enzymatic activity (Klibanov, A.M., 2001; Dunn, R.V., & Daniel, R.M., 2004). Dehydration studies show that, at least, a monolayer of water molecules is required for proteins to be fully functional (Careri, G., 1999). It becomes increasingly clear that, indeed, water is a key factor for understanding protein structure and function.

1.2.2 Protein-Cosolvent Interactions

Although water is the primary solvent, a variety of other water miscible compounds are found in biological systems. They are small molecules that may occupy a significant portion of the solution volume. Such cosolvents, often called osmolytes, can be classified as protein denaturants (e.g., urea, guanidine hydrochloride) or stabilizers (e.g., glycine betaine, trimethylamine N-oxide, proline, trehalose), which help unfold and refold proteins,
respectively (Yancey, P.H., et al., 1982; Davis-Searles, P.R., et al., 2001; Canchi, D.R., & Garcia, A.E., 2013).

Early research pioneered by Timasheff focused on the interactions between protein and osmolyte by measuring the preferential interaction parameter which reflects the mutual perturbation of the chemical potentials of protein and cosolvent when the protein is added to the binary solvent (Timasheff, S.N., 1998). In a water-cosolvent solution, the composition of the solvent in the immediate vicinity of the protein differs from that in the bulk. As such, the preferential binding parameter, $\Gamma_{23}$, is a measure of the excess of osmolyte in the immediate vicinity of the protein relative to the bulk (Xie, G., & Timasheff, S.N., 1997; Timasheff, S.N., 1998). It can be expressed as:

$$
\Gamma_{23} = \left( \frac{\partial m_3}{\partial m_2} \right)_{T,P,\mu_1,\mu_3} - \left( \frac{\partial \mu_2}{\partial m_3} \right)_{T,P,\mu_1,m_2}
$$

(1.1)

where subscripts 1, 2, and 3 denote water, protein, and cosolvent, respectively; $\mu_i$ is the chemical potential of component $i$; and $m_i$ is the molal concentration of component $i$. Experimentally, the preferential binding parameter is determined from equilibrium dialysis and high precision densimetry measurements (Xie, G., & Timasheff, S.N., 1997; Timasheff, S.N., 1998).

A negative preferential binding parameter indicates exclusion of osmolytes from the vicinity of the protein, while a positive $\Gamma_{23}$ signifies accumulation of the osmolyte near the protein surface relative to the bulk (Timasheff, S.N., 1993; Timasheff, S.N., 1998). The ‘immediate vicinity’ refers to the domain whereby the protein exerts a thermodynamic influence on the osmolyte and water molecules. While the cosolvent and water molecules within the local domain are chemically identical to their counterparts in the bulk, the
composition and density of the solvent components in the vicinity of a protein are distinct from those at the bulk solvent (Xie, G., & Timasheff, S.N., 1997).

Various research groups have measured the preferential binding parameter of many water-soluble proteins in various stabilizing and destabilizing osmolytes (Lee, J.C., & Timasheff, S.N., 1974; Prakash, V. et al., 1981; Arakawa, T., & Timasheff, S.N, 1983; Arakawa, T., & Timasheff, S.N, 1985; Timasheff, S.N., & Xie, G., 2003). Lee and Timasheff observed that, in the presence of guanidine hydrochloride, several proteins exhibit positive $\Gamma_{23}$ values, ranging from 0 to 55 (Lee, J.C., & Timasheff, S.N., 1974). Prakash et al. also observed positive $\Gamma_{23}$ values ranging from 2 to 96 for nine proteins in 8M urea (Prakash, V. et al., 1981). Such experiments suggest that a positive sign in preferential binding parameter translates into preferential exclusion of water from the protein surface, meaning the affinity of a protein for the destabilizing cosolvents is greater than that for water.

On the other hand, the preferential binding parameters of proteins in solutions of stabilizing cosolvents are negative. The preferential binding parameter of lysozyme decreases from $-6.45$ to $-13.5$ as the concentration of glycine betaine increases from 0.7 to 2.0 M, while the preferential binding parameter of bovine serum albumin (BSA) decreases from $-26.9$ to $-72.5$ over the same range of glycine betaine concentrations (Arakawa, T., & Timasheff, S.N, 1983). In the aggregate, the reported results indicate that destabilizing cosolvents (i.e. urea and guanidium chloride) exhibit positive values of $\Gamma_{23}$, suggesting that they interact preferentially with the protein, whereas stabilizing cosolvents (i.e. trimethylamine N-oxide (TMAO), glycine, and betaine) exhibit negative $\Gamma_{23}$, implying preferential exclusion from the vicinity of the protein (Arakawa, T., & Timasheff, S.N, 1985; Timasheff, S.N., 1998).
Although equilibrium dialysis experiments offer a great deal of insights into the distribution of water and osmolytes around proteins, the preferential binding parameter describes the differential interactions of proteins with water and cosolvents over the entire protein surface (Timasheff, S.N., 2002). It does not specify the locality of protein-cosolvent interactions. Furthermore, this technique is limited to proteins as it cannot be applied to studying low molecular weight model compounds mimicking protein groups. Thus, equilibrium dialysis cannot be used for investigating specific interactions between individual protein groups and osmolytes (Timasheff, S.N., 2002).

The Kirkwood-Buff (KB) theory has been applied to investigating solvation of a solute in binary solvents consisting of water and cosolvent (Shimizu, S., & Smith, D.J., 2004; Shimizu, S., 2004; Smith, P.E., 2006; Rosgen, J. et al., 2007). This theory relates the thermodynamic properties (i.e. partial molar volume) of various components in a solution to their radial distribution functions, $g_{ij}(r)$ (Hall, D.G., 1971; Ben-Naim, A., 2006). Such properties are expressed by the KB integrals:

$$G_{ij} = G_{ji} = 4\pi \int_0^\infty [g_{ij}^{VT}(r) - 1] r^2 dr,$$

which can be calculated if the radial distribution functions are known. The term $g_{ij}$ is the corresponding center of mass based radial distribution function (rdf), which measures the deviation in the distribution of $j$ molecules around a central molecule, $i$, in a random distribution in an equivalent volume of the bulk solution. The KB integral can be expressed as $G_{ij} = -V_{ij} + I_{ij}$, where $V_{ij}$ represents the excluded volume effect of the solute on the solvent or cosolvent, and $I_{ij}$ depicts the volume effect of the solute-solvent and solute-cosolvent interactions.
interactions (Ben-Naim, A., 1977). A negative $I_{ij}$ signifies preferential exclusion, while a positive term means preferential binding.

Many experimental and theoretical research groups have studied the thermodynamics of solute-cosolvent interactions (Timasheff, S.N., 1993; Shimizu, S., 2004; Shimizu, S. & Smith, D.J., 2004). Such efforts have revealed important insights into the molecular origins of the stabilizing/destabilizing action of various osmolytes. The most extensively studied cosolvent with respect to protein stability and folding is urea.

1.3 Proposed Mechanisms of Urea-Induced Protein Unfolding

A change in solution properties affects the equilibrium between the folded and unfolded states. Shifting the equilibrium towards the unfolded state is known as denaturation (Canči, D.R., & Garcia, A.E., 2013). In denaturant-induced protein unfolding, solvent-protein interactions become thermodynamically more favorable than protein-protein interactions. Denaturation studies provide insights into the thermodynamic parameters, such as changes in enthalpy ($\Delta H$), entropy ($\Delta S$), and free energy ($\Delta G$), which are needed to characterize mechanisms of protein folding and stability. Therefore, denaturation studies are the primary method of characterizing the conformational stability of proteins and measuring changes in thermodynamic parameters accompanying their folding or unfolding transitions (Makhatadze, G.I. & Privalov, P.L., 1992; Timasheff, S.N., 1998; Bennion, B.J., & Daggett, V., 2003; O'Brien, E.P. et al., 2009; Canči, D.R., & Garcia, A.E., 2013).

Urea is a widely used cosolvent in denaturation studies. It is preferred over guanidium chloride, since urea exhibits a more linear $\Delta G$-versus-denaturant profile
(Makhatadze, G.I., 1999). However, the precise mode of urea interaction with protein groups is not yet well understood (Myers, J.K. et al., 1995; Lindgren, M., 2010). Two mechanisms of urea-induced protein unfolding have been proposed (Bennion, B.J., & Daggett, V., 2003; Rossky, P.J., 2008). In the indirect mechanism, urea disrupts the structure of water thereby weakening the hydrophobic effect (Frank, H.S., & Franks, F., 1968). This view emerged from solute transfer experiments showing that hydrophobic compounds are more soluble in aqueous urea than in water (Frank, H.S., & Franks, F., 1968). However, studies have shown that urea can be incorporated into the hydrogen-bonded network of water without perturbing the spatial distribution of water molecules around urea (Wallqvist, A. et al., 1998; Mountain, R.D., & Thirumalai, D., 2004). A neutron scattering study showed that the radial distribution function of urea around water in a 1:4 solution resembles that of water around water (Soper, A.K. et al., 2003). MD simulations have also shown that urea does not disrupt the structure of water even at high concentrations (Wallqvist, A. et al., 1998; Mountain, R.D., & Thirumalai, D., 2004). As such, the indirect mechanism lacks conclusive experimental evidence to justify it.

The direct mechanism involves favourable interactions between urea and various protein groups, including the peptide backbone and all amino acid side chains. Given a protein’s heterogenous surface, many researchers have investigated the nature and strength of such interactions (Bennion, B.J. et al., 2003; Auton, M., & Bolen, D.W, 2005; Stumpe, M. C., & Grubmüller, H, 2008; Auton, M. et al., 2011). MD simulations have revealed that the formation of hydrogen bonds between urea and the peptide backbone and urea with charged side chain groups is the driving force of urea-induced protein unfolding (Thirumalai, D. et al., 2010). Results of another MD simulation suggest that water molecules solvating non-polar groups of a protein are displaced by accumulating molecules of urea and that urea
tends to interact with less polar side chains and the backbone (Stumpe, M. C., & Grubmüller, H, 2008). MD simulations of individual amino acids in urea have revealed that urea interacts favourably with almost all amino acids leading to its preferential binding over water (Horinek, D., & Netz, R.R, 2011).

Lee et al. measured the adiabatic compressibilities and partial molar volume contribution of the peptide backbone and the amino acid side chains at urea concentrations ranging from 0 to 8 M. Such volumetric measurements have been analyzed based on the solvent exchange model to determine the effective equilibrium constants, $k$, for the binding of urea to protein groups with replacement of two waters of hydration. The authors found that the equilibrium constant ranges from 0.04 to 0.39 M indicative of favourable interactions of the protein groups with urea (Lee, S. et al., 2010). Furthermore, the equilibrium constants were used to determine the differential free energy of solute-solvent interactions, $\Delta \Delta G$, in urea and water which ranged from highly favourable to slightly unfavourable. Collectively, this data supports the direct interaction mechanism in which urea denatures proteins by a concerted action via favourable interactions with a wide range of protein groups (Lee, S. et al., 2010). Despite the fact that many studies favor the direct mechanism, the precise molecular basis of protein unfolding via urea is still unknown.

### 1.4 Solubility Studies and Transfer Free Energy

Solubility studies examine the contribution of protein-cosolvent interactions to protein stability via determining the free energy associated with the transfer of amino acid side chains and the peptide backbone from water to a binary solvent containing osmolyte
and water. The free energy associated with transferring a protein group from water to a water-solvent solution, $\Delta \mu_{tr}$, represents the difference in chemical potential of the solute in water, $\mu_{21}$, and in the water-solvent mixture, $\mu_{23}$. It is derived from the measurements of the differential solubility of the indicated compound in water, $s_{21}$, and in the binary solvent, $s_{23}$ (McMeekin, et al., 1935; McMeekin, et al., 1936; Auton, M., & Bolen, D.W., 2005; Auton, M. et al., 2007):

$$\Delta \mu_{tr} = \mu^0_{23} - \mu^0_{21} = RT \ln \left( \frac{a_{21}}{a_{23}} \right) = RT \ln \left( \frac{s_{21}}{s_{23}} \right) + RT \ln \left( \frac{\gamma_{21}}{\gamma_{23}} \right)$$

(1.3)

where $T$ is the temperature; $R$ is the universal gas constant; and $a_{21} = \gamma_{21}s_{21}$ and $a_{23} = \gamma_{23}s_{23}$ are the activities of the solute in water and water-cosolvent mixture, respectively. Since the activity coefficients terms ($\gamma_{21}$ and $\gamma_{23}$) are considered to be equal to one (Auton, M., & Bolen, D.W., 2005; Auton, M. et al., 2007), the equation above can be simplified to the apparent transfer free energy, $\Delta \mu'_{tr}$:

$$\Delta \mu'_{tr} \approx RT \ln \left( \frac{s_{21}}{s_{23}} \right)$$

(1.4)

The transfer free energies, $\Delta \mu'_{tr}$, for amino acid side chains and the peptide backbone were measured by Bolen’s group in order to predict the m-values for stabilizing and destabilizing cosolvents (Auton, M., & Bolen, D.W., 2005; Auton, M. et al., 2007). It was concluded that the peptide backbone contribution to the free energy of unfolding prevails over the sum of side chain contributions (Auton, M., & Bolen, D.W., 2005; Auton, M. et al., 2007; Bolen, D.W., & Rose, G.D., 2008). This notion suggests that it is predominantly the backbone that controls the folding/unfolding transitions of the protein. The impact of the peptide backbone to the estimated m-values is larger than the contribution of side chains,
even though the peptide backbone represents only a fraction of the total surface area (Auton, M. et al., 2007). In 1 M urea, the peptide backbone has a predominantly negative contribution to $\mu'_r$ in contrast to the positive contribution of side chains (Auton, M. et al., 2007; Auton, M. et al., 2011). Stabilizing osmolytes, such as TMAO and proline, interact favourably with most of the side chains, while interacting unfavourably with the peptide backbone, preventing and reversing protein unfolding (Auton, M., & Bolen, D.W., 2005).

### 1.5 Linear Extrapolation Model & The m Value

To relate the thermodynamic stability of a protein to urea denaturation, we can calculate the m-value, which is the slope of the free energy of unfolding, $\Delta G^\circ$, as a function of the denaturant concentration within the linear extrapolation model (LEM) (Pace, N.C., & Shaw, K.L., 2000). The free energy of unfolding has been shown to vary nearly linearly with the molar concentration of denaturant, typically at moderate to high denaturant concentrations where the unfolding transition takes place (Pace, N.C., & Shaw, K.L., 2000). The following equation was proposed for the linear extrapolation model:

$$\Delta G^\circ = \Delta G^\circ_0 - mC_3$$

where $\Delta G^\circ_0$ is the free energy of unfolding at zero denaturant concentration, $C_3$, and the m represents the response of protein stability to the addition of cosolvents. The LEM is often the method of choice to analyse urea denaturation curves because $\Delta G^\circ$ depends solely on an empirical parameter, $m$, thereby making it a convenient method for determining protein

The m-value has been shown to be proportional to changes in solvent accessible surface area, $\Delta S_A$, accompanying urea-induced protein denaturation (Myers, J.K. et al., 1995; Auton, M., & Bolen, D.W., 2005; Auton, M. et al., 2007). In these studies, the $\Delta S_A$ is computed as the difference in solvent accessible surface area between the native and fully extended protein conformations. However, it is known that proteins do not adapt a fully extended conformation, even at the harshest denaturing conditions. Therefore, more realistic correlations between the m values and the $\Delta S_A$ are needed for such evaluations to be deemed accurate. The volumetric approach used by Son et al. provides realistic $\Delta S_A$ values for specific protein denaturation events that, in conjunction with their respective m-values, may produce a better parametrization with subsequent applications to m-value (Son, I. et al., 2014). The partial molar volumes and adiabatic compressibilities were measured for various proteins at urea concentrations ranging from 0 to 8 M (Son, I. et al., 2014). Analysis of the volumetric properties of the unfolded protein apocytochrome c suggests that, in the absence of urea, the protein exhibits a solvent-accessible surface area, $S_A$, comparable to that of folded holocytochrome c. An increase in urea concentration from 0 to 8 M causes an additional ~20% increase in $S_A$. It was also shown that ribonuclease A and $\alpha$-chymotrypsinogen A undergo urea-induced unfolding transitions, and that the denatured states of ribonuclease A and $\alpha$-chymotrypsinogen A exhibit solvent-accessible surface areas which are roughly twice as large as those of their respective native states (Son, I. et al., 2014).
1.6 Scaled Particle Theory

Chemical equilibria in solutions can be analyzed in terms of the free energy differences between the states involved. Scaled particle theory (SPT) is a commonly used to calculate the “free energy of cavity formation” (i.e., excluded volume effect) (Pierotti, R.A., 1976). The excluded volume effect refers to the idea that solvent molecules are excluded from the space occupied by other solute molecules in solution (Lee, S., & Chalikian, T.V., 2009; Lee, S. et al., 2010; Son, I. et al., 2014, Son, I., & Chalikian, T.V., 2016). SPT describes the work required to introduce a hard sphere spherical cavity of the size of the solute molecule into a hard sphere solvent of a given molecular diameter, \(a\), by fitting a third-degree polynomial to the exact solution available for small cavity sizes (Tang, K., & Bloomfield, V., 2000). It assumes that the solute and solvent can be adequately described by hard spheres of a given radius and that the properties of the solvent are inherent in the density, \(\rho\), of the pure liquid. The van der Waals repulsions rise very steeply. It is advantageous in that it does not require information about the unique structures of the solvent; the only parameters needed are the solute radius and the concentration and radii of the solvent (Tang, K., & Bloomfield, V., 2000; Heying, M., & Corti, S.C., 2004; Ashbaugh, H.S., & Pratt, L.R., 2005).

1.7 Research Aims

Previously conducted studies have dealt with protein-cosolvent interactions and characterized the energetic contributions of the excluded volume effect, \(\Delta G_c\), and direct urea-protein interactions, \(\Delta G_i\) (Lee, S., & Chalikian, T.V., 2009; Lee, S. et al., 2010; Lee, S. et al., 2010;
In this work, we aim to use previous results from our lab, along with computational data, to investigate the urea and temperature dependence of the m-value of globular proteins, specifically lysozyme and cytochrome c. We use a modified statistical model to analyze m-values to further understand the molecular mechanism underlying urea-induced protein denaturation.

1.7.1 Lysozyme

Lysozyme has been exhaustively studied; its structure, stability, and folding behavior are well known (Redfield, C. & Dobson, C.M., 1990; Hooke, S.D., et al., 1994; Kelly, J.W., 1996; Wetzel, R., 1996; Frare, E. et al., 2006). It causes a damage to bacterial cell walls by catalyzing hydrolysis of a 1,4-β-linkages between the C-1 of N-acetylmuramic acid and the C-4 of N-acetylglucosamine in the peptidoglycan layer of the bacterial cell wall (Koo, E. H. et al., 1999; Frare, E. et al., 2006). Further knowledge into its thermal stability and folding intermediates may be medically relevant as the protein in humans has been shown to be involved in the formation of amyloid fibers associated with amyloidosis, leading to severe pathogenic consequences (Merlini, G. & Bellotti, V., 2003; Stefani, M. & Dobson, C.M., 2003; Frare, E. et al., 2006).

1.7.2 Cytochrome c

Cytochrome c is a heme protein that is localized in the compartment between the inner and outer mitochondrial membranes where it functions to transfer electrons between complex III and complex IV of the respiratory chain (Moore, G.W. & Pettigrew, G.W., 1990;
Berghuis, A.M., & Brayer, G.D., 1992; Schwartz-Stenner, R. et al., 2009). It can also bind the mitochondrion-specific phospholipid cardiolipin inducing a conformational change in the protein (Kagan, V.E. et al., 2005; Belikova, N.A. et al., 2006; Kapralov, A.A. et al., 2007; Schwartz-Stenner, R. et al., 2009). This enables it to act as a peroxidase catalyzing the oxidation of cardiolipin thus instigating a chain of events ultimately leading to apoptosis (Green, D.R. & Kroemer, G., 2004; Jiang, X. & Wang, X., 2004; Schwartz-Stenner, R. et al., 2009).
CHAPTER 2

On Urea and Temperature Dependences of m-Values


Contributions: Experiments were designed by Alah Amsdr and Tigran V. Chalikian. The experiments were conducted by Alah Amsdr with technical assistance from Negar Dehghan Noudeh and Lutan Liu. Data collection and analysis were performed by Alah Amsdr with computational analysis by Tigran V. Chalikian. Alah Amsdr participated in the preparation of the manuscript.

2.1 Abstract

The denaturing or stabilizing influence of a cosolvent on a protein structure is governed by a fine balance of the energetics of the excluded volume effect and the energetics of direct protein-cosolvent interactions. We have previously characterized the energetic contributions of excluded volume and direct interactions with urea for proteins and protein groups. In this work, we examine the molecular origins underlying the relatively weak temperature and urea dependences of the m-values of globular proteins. Our combined experimental and computational results collectively paint a picture in which the relative independence of protein m-values of urea concentration originates from fortuitous
compensatory effects of a progressive increase in the solvent accessible surface area of the unfolded state and a slightly higher urea binding constant of the unfolded state relative to the folded state. Other denaturing cosolvents which lack such a compensation make poor candidates for linear extrapolation model-based protein stability determination studies. The observed diminution in m-values with increasing temperature reflects, in addition to the aforementioned compensatory effects, a decrease in protein-urea binding constants with temperature in accordance with the negative sign of the binding enthalpy.

2.2 Introduction

The m-value is a fundamental thermodynamic property describing the stability of a protein with respect to cosolvent-induced denaturation within the framework of the linear extrapolation model (LEM) (Makhatadze, G.I., 1999; Pace, C.N. & Shaw, K.L., 2000; Scholtz, J.M. et al., 2009). LEM is a simple and, perhaps, the most widely used approach for determining the protein stability in biochemical and biophysical studies (Pace, C.N. & Shaw, K.L., 2000; Scholtz, J.M. et al., 2009). In LEM, the protein stability, $\Delta G^\circ$, is a linear function of the denaturant concentration, $C_3$:

$$\Delta G^\circ = \Delta G^\circ_0 - mC_3$$  \hspace{1cm} (2.1)

where $\Delta G^\circ_0$ is the protein stability in the absence of the denaturant; and $m$ is the m-value (Scholtz, J.M. et al., 2009).

For LEM to be valid, the m-value in Eq. (2.1) needs to be independent of cosolvent concentration. Despite being a popular way of evaluating protein stability, the thermodynamic foundation of LEM and the molecular origins of the constancy of m-values
are not fully understood (Makhatadze, G.I., 1999; O’Brien, E.P. et al., 2009). The molecular interpretation of the m-value relies upon the relationship \( m = RT\Delta \Gamma_{23}/C_3 \), where \( \Delta \Gamma_{23} \) is the differential preferential interaction (or binding) parameter of the unfolded and folded states of the protein (Canchi, D.R., & Garcia, A.E., 2013). Thus, the m-value is positive (indicating that the cosolvent is a denaturant) if \( \Delta \Gamma_{23} \) is positive, i.e., when the unfolded state of a protein accumulates in its vicinity a higher number of cosolvent molecules than the folded state (Timasheff, S.N., 1993; Timasheff, S.N., 1998). Otherwise, if \( \Delta \Gamma_{23} \) is negative, the m-value is also negative signifying the stabilizing nature of the cosolvent.

Urea and guanidinium chloride are two denaturants most frequently used in LEM-based protein stability characterizations (Tanford, C., 1968; Tanford, C., 1970; Makhatadze, G.I., 1999). Compared to guanidinium chloride, urea exhibits a more linear \( \Delta G^\circ \text{-vs-} C_3 \) dependence (the m-value is essentially constant) and, therefore, is a more appropriate choice as a denaturant for LEM-based characterizations (Makhatadze, G.I., 1999). A large body of experimental and theoretical investigations has sought to elucidate the mechanisms of the destabilizing effect of urea on a protein structure (Davis-Searles, P.R. et al., 2001; Stumpe, M.C., & Grubmuller, H., 2007; Rossky, P.J., 2008; Chalikian, T.V., 2011; England, J.L., & Haran, G., 2011; Guinn, E.J. et al., 2011; Canchi, D.R., & Garcia, A.E., 2013; Moeser, B., & Horinek, D., 2014). The prevailing view is that urea shifts the folded/unfolded equilibrium toward the unfolded state via the “direct” mechanism which involves a concerted action of a multitude of favorable solute-cosolvent interactions with a wide range of protein groups (Hua, L. et al., 2008; Almarza, J. et al., 2009; Canchi, D.R., & Garcia, A.E., 2013).

In several recent publications from our lab, we have applied a statistical thermodynamic approach to modeling and characterizing protein-urea interactions and the
denaturing action of urea in general (Lee, S., & Chalikian, T.V., 2009; Lee, S. et al., 2010; Chalikian, T.V., 2011; Son, I. et al., 2014; Son, I., & Chalikian, T.V., 2016). Our results collectively support the direct mechanism of urea action and suggest that, in general, the mode of action of an individual cosolvent is determined by a fine balance between the energetics of direct protein-cosolvent interactions and the energetics of cavity formation (Chalikian, T.V., 2014; Chalikian, T.V., 2016). The following expressions have been derived for the cosolvent dependence of protein stability, $\Delta G^o$, and m-value (Chalikian, T.V., 2014; Chalikian, T.V., 2016):

\[
\Delta G^o = \Delta \Delta G_C + \frac{RT\Delta n}{r} \ln(k_1 a_1^r + k_3 a_3) \tag{2.2}
\]

\[
m \approx -\left(\frac{\partial \Delta G^o}{\partial a_3}\right)_{T,P} = -\left(\frac{\partial \Delta \Delta G_C}{\partial a_3}\right)_{T,P} - RT\Delta n \left(\frac{N_3}{N_1}\right) \frac{a_1^r/a_3}{a_1^r+ka_3} + RT\left(\frac{\Delta n}{r}\right) \frac{k}{a_1^r+ka_3} \tag{2.3}
\]

where $a_1$ is the mole fraction activity of water (the principal cosolvent); $a_3$ is the molar activity of a cosolvent; $N_1$ and $N_3$ are, respectively, the numbers of moles of water and cosolvent in the solution; $r$ is the number of water molecules replaced by a cosolvent molecule upon its association with a binding site on the surface of a protein (for urea, $r$ equals 2); $k_1$ and $k_3$ are the equilibrium constants for elementary reactions of protein binding with water and cosolvent, respectively; $k = k_3/k_1$ is the equilibrium constant for an elementary reaction in which a cosolvent binds to a binding site replacing $r$ waters of hydration; $\Delta n$ is the differential number of water molecules hydrating the unfolded and folded protein conformations in the absence of a cosolvent; and $\Delta \Delta G_C$ is the differential free energy of cavity formation for the unfolded and folded protein states.
In Eq. (2.3) and below in the text, the activity and molar concentration of a cosolvent are treated as equal. Such a treatment is justified for urea for which the molar coefficient of activity does not differ significantly from unity up to the solubility limit (Felitsky, D.J., & Record, M.T., 2004). Eq. (2.3) can be rearranged to

\[ m = -\left(\frac{\partial \Delta G_C}{\partial a_3}\right)_{T,P} - \frac{RT \Delta n}{a_1^r + ka_3} \left(\frac{N_3 a_1^r}{N_1 a_3} - \frac{k}{r}\right) \]

and, taking into account that \( \frac{N_3}{N_1 a_3} \approx \frac{V}{N_1} = \frac{1}{C_1} \) (where, \( V \) is the volume of solution; and \( C_1 \) is the molar concentration of water), it simplifies to the relationship:

\[ m = -\left(\frac{\partial \Delta G_C}{\partial a_3}\right)_{T,P} - \frac{RT \Delta n}{a_1^r + ka_3} \left(\frac{a_1^r}{C_1} - \frac{k}{r}\right) \]  

(2.4)

Note that under simplifying assumptions of \( a_1 = 1 \) and \( r = 1 \), Eqs. (2.2) – (2.4) convert into the well-known set of equations describing the original solvent-exchange model developed by Schellman (Schellman, J.A., 1978; Schellman, J.A., 1987; Timasheff, S.N., 1993; Schellman, J.A., 1994; Schellman, J.A., 2003). We have previously studied the urea-binding properties of amino acid side chains and the peptide group at 25 °C (Lee, S. et al., 2010; Chalikian, T.V., 2011). These functionalities exhibit a great deal of diversity with respect to the magnitude of the equilibrium constant, \( k \). We have subsequently characterized the “average” or effective urea-binding properties of a folded protein, lysozyme, at temperatures ranging from 18 to 45 °C (Son, I., & Chalikian, T.V., 2016). The binding constant, \( k \), decreases from 0.15 ± 0.01M at 18 °C to 0.05 ± 0.01M at 45 °C corresponding to a binding enthalpy of −8.7 ± 0.4 kcal mol−1 (Son, I., & Chalikian, T.V., 2016). While a strong temperature-induced decrease in \( k \) is consistent with a steep decrease in m-values with temperature, the available
experimental data suggest a modest or no change in m-values with increasing temperature (Zweifel, M.E., & Barrick, D., 2002).

In the current work, we examine the molecular origins underlying the relative constancy of the m-values with respect to the urea concentration and their temperature dependences. To this end, we scrutinize ours and the reported experimental results on the temperature dependences of the m-values of globular proteins within the framework of a statistical thermodynamic model to glean insights into the nature of urea-induced unfolding transitions of proteins and the thermodynamic foundation underlying LEM. Our results are consistent with the picture in which urea binds more strongly to the unfolded state relative to the folded state with the former state becoming progressively more solvent-exposed as the concentration of urea increases. The combination of the two events (the stronger urea binding and an increase in solvent accessibility of the unfolded state) results in relative urea-independence of m-values which slightly decrease with increasing temperature.

2.3 Materials and Methods

Hen egg white lysozyme and horse heart cytochrome c were purchased from Sigma-Aldrich Canada, Ltd. (Oakville, ON). The proteins were exhaustively dialyzed against distilled water and lyophilized. The conformational studies of lysozyme reported here were performed in a pH 2.5 buffer containing 10 mM glycine and 10 mM NaCl. Cytochrome c was studied in a pH 5.0 buffer containing 10 mM sodium acetate and 10 mM NaCl. The concentrations of lysozyme and cytochrome c were measured spectrophotometrically using extinction coefficients $\varepsilon_{280} = 37,900$ M$^{-1}$ cm$^{-1}$ (Mach, H. et al., 1992) and $\varepsilon_{409} = 106,000$ M$^{-1}$ cm$^{-1}$, (Robinson, M.N. et al., 1983) respectively.
Solutions of urea with concentrations of 0, 1, 2, 3, 4, 5, 6, 7, and 8M were prepared by weighing 10–50 g of urea and adding predetermined amounts of water, glycine buffer, or acetate buffer to achieve the desired molalities, \( m \). The molar concentration, \( C \), of urea was evaluated from the molal value, \( m \), from 
\[
C = \left[ \frac{1}{(m \rho_b)} + \frac{\phi V}{1000} \right]^{-1},
\]
where \( \rho_b \) is the density of the pure buffer, and \( \phi V = 44.1 \, \text{cm}^3 \, \text{mol}^{-1} \) is the apparent molar volume of urea (Lee, S., & Chalikian, T.V. 2009). Urea solutions in a glycine or acetate buffer were subsequently used as solvents for preparing the protein solution.

Urea solutions in water were used for density measurements. The densities of aqueous urea solutions were measured at 18, 25, 35, and 45 °C with a precision of \( \pm 1.5\times10^{-4} \) % using a vibrating tube densimeter (DMA-5000, Anton Paar, Gratz, Austria). Table 2.1 presents the results of densimetric measurements.

<table>
<thead>
<tr>
<th>[urea], M</th>
<th>18 °C</th>
<th>25 °C</th>
<th>35 °C</th>
<th>45 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.998564</td>
<td>0.997047</td>
<td>0.994035</td>
<td>0.990216</td>
</tr>
<tr>
<td>1</td>
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<td>1.013104</td>
<td>1.009342</td>
<td>1.006174</td>
</tr>
<tr>
<td>2</td>
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<td>1.028398</td>
<td>1.024466</td>
<td>1.019929</td>
</tr>
<tr>
<td>3</td>
<td>1.046351</td>
<td>1.043789</td>
<td>1.039392</td>
<td>1.034506</td>
</tr>
<tr>
<td>4</td>
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<td>1.058878</td>
<td>1.054168</td>
<td>1.048967</td>
</tr>
<tr>
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<td>1.076789</td>
<td>1.073753</td>
<td>1.069664</td>
<td>1.063293</td>
</tr>
<tr>
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<td>1.088348</td>
<td>1.083040</td>
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</tr>
<tr>
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<td>1.102861</td>
<td>1.097264</td>
<td>1.091347</td>
</tr>
<tr>
<td>8</td>
<td>1.120786</td>
<td>1.117137</td>
<td>1.111286</td>
<td>1.105126</td>
</tr>
</tbody>
</table>
UV light absorbance spectra required for protein concentration determinations were measured at 25 °C with a Cary 300 Bio spectrophotometer (Varian Canada, Inc., Mississauga, ON, Canada). The instrument was also used for measuring the heat-induced denaturation (UV melting) profiles of cytochrome c (at 290 nm) and lysozyme (at 280 nm) in the absence of urea (in the neat buffers). The denaturation profiles were treated as two-state events to determine the transition temperatures, $T_M$, and enthalpies, $\Delta H_M$ (Marky, L.A., & Breslauer, K.J., 1987).

Circular dichroism (CD) spectra were recorded with a JASCO J-1100 circular dichroism spectrophotometer (JASCO, Easton, MD). All urea-dependent CD spectroscopic measurements were performed in a 1 mm path-length cuvette at 20, 25, 30, 35, 40, and 45 °C. The urea-induced unfolding profiles of lysozyme and cytochrome c were measured by recording the molar ellipticity at 222 nm.
2.4 Results

Figure 2.1 shows a representative urea-induced unfolding profile of cytochrome c measured at 25 °C. The unfolding profile in Figure 2.1 was treated as a two-state transition within the framework of LEM,

\[
\alpha = \left[ 1 + \exp \left( \frac{\Delta G^o - mC_3}{RT} \right) \right]^{-1}
\]  

(2.5)

where \(\alpha\) is the fraction unfolded.

Figure 2.1. A representative urea-induced denaturation profile of cytochrome c at 25 °C as measured by the molar ellipticity at 222 nm. Experimental data were fitted by Eq. (2.5) (solid line) (see in text).
We measured similar profiles for lysozyme and cytochrome c at 20, 25, 30, 35, 40, and 45 °C [Figs. S2.1(a)–S2.1(f) and S2.2(a)–S2.2(f)], from which the unfolding free energies, \( \Delta G^0 \), and m-values were calculated with Eq. (2.5). Figure 2.2 presents the temperature dependences of the unfolding free energy, \( \Delta G^0 \), for lysozyme [panel (a)] and cytochrome c [panel (b)] in the absence of urea. Comparison with published works reveals a good agreement between ours and the reported \( \Delta G^0 \)-vs-T dependences (Privalov, P.L., & Khechinashviki, N.N., 1974; Privalov, P.L., 1979). Figures 2.2(a) and 2.2(b) also show the temperature dependences of the unfolding free energy of lysozyme and cytochrome c simulated with the equation:

\[
\Delta G^0(T) = \Delta H_M \left(1 - \frac{T}{T_M}\right) + \Delta C_P \left[T - T_M - T \ln \left(\frac{T}{T_M}\right)\right]
\]

where \( T_M \) is the transition temperature; and \( \Delta H_M \) and \( \Delta C_P \) are the changes in enthalpy (at the transition temperature) and heat capacity, respectively. For lysozyme (Figure 2.2(a)), we used the values of \( T_M \), \( \Delta H_M \), and \( \Delta C_P \) of 62 °C, 97 kcal mol\(^{-1}\), and 1.9 kcal mol\(^{-1}\)K\(^{-1}\), respectively. For cytochrome c (Figure 2.2(b)), we used \( T_M \), \( \Delta H_M \), and \( \Delta C_P \) of 82 °C, 96 kcal mol\(^{-1}\), and 1.2 kcal mol\(^{-1}\)K\(^{-1}\), respectively. The values of \( T_M \) and \( \Delta H_M \) for the two proteins were determined from UV melting profiles (Figures S2.3(a) and S2.3(b)), while the values of \( \Delta C_P \) were taken from literature (Privalov, P.L., & Khechinashviki, N.N., 1974; Sturtevant, J.M., 1977; Robertson, A.D., & Murphy, K.P., 1997). For both proteins, the UV melting profiles were reversible within ±5%. Inspection of Figures 2.2(a) and 2.2(b) reveals that the experimental points are in excellent agreement with the simulated data.
Figure 2.2. Temperature dependences of the stabilities of lysozyme (panel A) and cytochrome c (panel B). Solid lines represent temperature dependences of the unfolding free energy simulated with Eq. (2.6) (see in text).
Figure 2.3 presents the temperature dependences of the m-values for lysozyme [panel (a)] and cytochrome c [panel (b)]. The m-values for the two proteins at 25 °C agree with the reported data (Myers, J.K. et al., 1995). Inspection of Figures 2.3(a) and 2.3(b) reveals that an increase in temperature causes slight decreases in m-values for both lysozyme and cytochrome c. This observation parallels published results on other proteins which all suggest that m-values either do not change or slightly decrease as the temperature increases (DeKoster, G.T., & Robertson, A.D., 1997; Zweifel, M.E., & Barrick, D., 2002). It should be noted, however, that all-atom replica exchange MD simulations of the Trp-cage miniprotein have revealed that, for this protein, the m-value for the urea-induced denaturation increases with temperature (Canchi, D.R. et al., 2010).
Figure 2.3. Temperature dependences of the urea m-values of lysozyme (panel A) and cytochrome c (panel B).
2.5 Discussion

Ours and the reported data on m-values represent an experimental framework against which theoretical models can be tested and the underlying ideas can be verified. To this end, we use Eq. (2.4) to model the urea and temperature dependences of the m-value for a hypothetical spherical protein with a molecular volume, \( V_M \) and solvent accessible surface area, \( S_A \), that unfolds into a spherocylinder with the same \( V_M \) but a higher \( S_A \) (Graziano, G., 2011; Graziano, G., 2011). The urea dependences of the differential free energies of cavity formation (the excluded volume effect), \( \Delta \Delta G_C \), for the native (spherical) and unfolded (spherocylindrical) conformations needed for calculations with Eq. (2.4) can be computed within the framework of scaled particle theory (SPT) (Pierotti, R.A., 1976; Graziano, G., 2011; Graziano, G., 2011). Specifically, the free energies of cavity formation for the native, \( \Delta G_{CN} \), and unfolded (denatured), \( \Delta G_{CD} \), states are given by the equations (Pierotti, R.A., 1976; Graziano, G., 2011; Graziano, G., 2011):

\[
\Delta G_{CN} = RT \left[ - \ln(1 - \xi_3) + \left( \frac{6\xi_2}{1-\xi_3} \right) r_N + \frac{12\xi_1}{1-\xi_3} + \frac{18\xi_2^2}{(1-\xi_3)^2} \right] \quad (2.7)
\]

\[
\Delta G_{CD} = RT \left[ - \ln(1 - \xi_3) + \frac{6a\xi_2}{1-\xi_3} + \frac{12a^2\xi_1}{1-\xi_3} + \frac{18a^2\xi_2^2}{(1-\xi_3)^2} + \frac{3il\xi_2}{2(1-\xi_3)} + \frac{6a\xi_1}{1-\xi_3} + \frac{9a^2\xi_2^2}{(1-\xi_3)^2} \right] \quad (2.8)
\]

where \( r_N \) is the radius of the sphere modeling the native state, \( a \) and \( l \) are, respectively, the spherocylindrical curvature and the cylindrical length of the spherocylinder modeling the ensemble of unfolded states, \( \xi_i = \frac{\sum_j N_M G_j \sigma_j}{N} \), \( N_0 \) is Avogadro's number, \( C_j \) is the molar concentration of solution component \( j \), and \( \sigma_j \) is the hard-sphere diameter of solution
component \( j \). The values of \( \sigma_j \) are equal to 2.74 and 4.41 Å for water and urea, respectively (Chalikian, T.V., 2011; Chalikian, T.V., 2014).

Given the polymeric, random coil-like nature of an unfolded protein, (Tanford, C. et al., 1966; Fitzkee, N.C., & Rose, G.D., 2004) the spherocylindrical representation is, clearly, an oversimplification. However, in this context, it is not unwarranted. A spherocylinder is an elongated shape for which SPT-based analytical equations for calculating the free energy of cavity formation, \( \Delta G_C \), have been derived (Graziano, G., 2011; Graziano, G., 2011). A spherocylinder should be viewed as an analytical approximation rather than a geometric approximation of the ensemble of unfolded conformations with respect to volume and solvent accessibility. Significantly, since \( \Delta G_C \) correlates linearly with solvent accessibility of the solute weakly depending on its specific geometry, (Graziano, G., 2009; Chalikian, T.V., 2016) computations performed with Eqs. (2.7) and (2.8) should also provide a good estimate for the energetics of the excluded volume effect for flexible conformers of the spherocylinder exhibiting the same solvent accessible surface area.

The molecular volumes of the native and unfolded protein states are given by \( V_{MN} = \frac{4\pi r_N^3}{3} \) and \( V_{MD} = \frac{\pi a^2}{3} \left( \frac{4a}{3} + l \right) \), respectively. Their solvent accessible surface areas are given by \( S_{AN} = 4\pi(r_N + r_W)^2 \) and \( S_{AD} = 2\pi(a + r_W)[2(a + r_W) + l] \), respectively, where \( r_W = 1.4 \) Å is the radius of a water molecule. Taking into account that \( V_{MN} = V_{MD} = V_M \), one derives the relationship \( \frac{2}{3} a^4 + \frac{8}{3} r_W a^3 + \left( 2r_W^2 - \frac{S_{AD}}{2\pi} \right) a^2 + \frac{V_M}{\pi}(a + r_W) = 0 \), which can be used to calculate the spherocylindrical curvature, \( a \), for any pair of \( V_M \) and \( S_{AD} \). The cylindrical length, \( l \), can be subsequently evaluated from \( l = \frac{V_M}{\pi a^2} - \frac{4}{3} a \).

A change in solvent-accessible surface area accompanying the unfolding transition equals \( \Delta S_A = S_{AD} - S_{AN} \) while the differential hydration number of the unfolded and folded
states in Eq. (2.4) is $\Delta n = \Delta S_A/S_W$ (where $S_W$ is the effective cross-sectional area of water of protein hydration). For proteins, an “average” water of hydration is characterized by a $S_W$ of $\sim 11$ Å$^2$ (Persson, F. et al., 2018).

Here, we model a hypothetical protein with a spherical radius of 20 Å in the native state corresponding to $S_A$ of 5755 Å$^2$ and $V_M$ of 33,510 Å$^3$. These values are on the order of the solvent accessible surface area, $S_A$, and intrinsic volume, $V_M$ of cytochrome c and lysozyme. Specifically, the solvent accessible surface areas, $S_A$, of cytochrome c and lysozyme are 6115 and 6685 Å$^2$, respectively, while their intrinsic volumes, $V_M$ equal 12,634 and 15,659 Å$^3$, respectively (Chalikian, T.V. et al., 1996). The native (spherical) protein undergoes an unfolding transition into a spherocylinder with the same intrinsic volume ($V_{MD} = V_{MN}$) and a solvent-accessible surface area double that of the native state ($S_{AD} = 2S_{AN}$). This is in line with the results of our previous work which have suggested that the urea-induced unfolding of globular proteins is accompanied by a two-fold increase in solvent accessible surface area (Son, I. et al., 2014). The spherocylindrical curvature, $a$, and the cylindrical length, $l$, of the unfolded spherocylinder with $V_M$ of 33,510 Å$^3$ and $S_A$ of 11,510 Å$^2$ are 7.2113 and 195.50 Å, respectively (see above).

Figure 2.4 shows the differential free energies of cavity formation, $\Delta G_C$, computed with Eqs. (2.7) and (2.8) as a function of urea concentration at 18, 25, 35, and 45 °C. In these computations, we used the concentrations of water, $C_1 = \frac{\rho - C_3 M_3}{M_1}$, and urea, $C_3(T) = C_3(25) \frac{\rho(T)}{\rho(25)}$, calculated from the densities of urea solutions, $\rho$, presented in Table 1 [where $M_1 = 18.02$ Da and $M_3 = 60.06$ Da are the molecular masses of water and urea, respectively; $\rho(T)$ is the density of the urea solution at temperature $T$]. Concentrations $C_1$ and $C_3$ are tabulated in Tables 2.2 and 2.3.
Figure 2.4. Urea dependences of changes in free energy of cavity formation, $\Delta \Delta G_C$, accompanying an unfolding of a spherical protein with a radius of 20 Å ($S_A$ of 5755 Å$^2$ and $V_M$ of 33,510 Å$^3$) into a spherocylinder with $S_A$ of 11,510 Å$^2$ and $V_M$ of 33,510 Å$^3$ at 18 (black), 25 (red), 35 (blue) and 45 (magenta) °C.
Table 2.2. The molar concentrations of water in aqueous solutions of urea.

<table>
<thead>
<tr>
<th>[urea], M</th>
<th>18 °C</th>
<th>25 °C</th>
<th>35 °C</th>
<th>45 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>55.430</td>
<td>55.345</td>
<td>55.178</td>
<td>54.966</td>
</tr>
<tr>
<td>1</td>
<td>52.991</td>
<td>52.902</td>
<td>52.706</td>
<td>52.541</td>
</tr>
<tr>
<td>2</td>
<td>50.530</td>
<td>50.418</td>
<td>50.225</td>
<td>50.003</td>
</tr>
<tr>
<td>3</td>
<td>48.056</td>
<td>47.938</td>
<td>47.736</td>
<td>47.512</td>
</tr>
<tr>
<td>4</td>
<td>45.563</td>
<td>45.442</td>
<td>45.240</td>
<td>45.017</td>
</tr>
<tr>
<td>5</td>
<td>43.055</td>
<td>42.934</td>
<td>42.770</td>
<td>42.516</td>
</tr>
<tr>
<td>6</td>
<td>40.533</td>
<td>40.410</td>
<td>40.213</td>
<td>40.020</td>
</tr>
<tr>
<td>7</td>
<td>38.006</td>
<td>37.882</td>
<td>37.689</td>
<td>37.486</td>
</tr>
<tr>
<td>8</td>
<td>35.456</td>
<td>35.340</td>
<td>35.155</td>
<td>34.960</td>
</tr>
</tbody>
</table>

Table 2.3. The molar concentrations of urea in an aqueous solution.

<table>
<thead>
<tr>
<th>18 °C</th>
<th>25 °C</th>
<th>35 °C</th>
<th>45 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.0017</td>
<td>1</td>
<td>0.9963</td>
<td>0.9932</td>
</tr>
<tr>
<td>2.0045</td>
<td>2</td>
<td>1.9924</td>
<td>1.9835</td>
</tr>
<tr>
<td>3.0074</td>
<td>3</td>
<td>2.9874</td>
<td>2.9733</td>
</tr>
<tr>
<td>4.0106</td>
<td>4</td>
<td>3.9822</td>
<td>3.9626</td>
</tr>
<tr>
<td>5.0141</td>
<td>5</td>
<td>4.9810</td>
<td>4.9513</td>
</tr>
<tr>
<td>6.0182</td>
<td>6</td>
<td>5.9707</td>
<td>5.9420</td>
</tr>
<tr>
<td>7.0230</td>
<td>7</td>
<td>6.9645</td>
<td>6.9269</td>
</tr>
<tr>
<td>8.0261</td>
<td>8</td>
<td>7.9581</td>
<td>7.9140</td>
</tr>
</tbody>
</table>

As it is seen from Figure 2.4, at all temperatures studied, the differential free energy of cavity formation, ΔGC, increases with urea concentration (activity) in an approximately linear manner. The slope \((\partial \Delta G_C / \partial a_3)_{T,P}\) slightly decreases as the temperature increases; \((\partial \Delta G_C / \partial a_3)_{T,P}\) equals 9.0 ± 0.1, 8.9 ± 0.1, 8.7 ± 0.1, and 8.5 ± 0.1 kcal mol⁻¹ M⁻¹ at 18, 25, 35,
and 45 °C, respectively. We use these results in conjunction with the previously determined urea binding constants, k (equal to 0.15, 0.11, 0.07, and 0.05M at 18, 25, 35, and 45 °C, respectively) (Son, I., & Chalikian, T.V., 2016), to compute the m-values with Eq. (2.4). In these computations, the mole fraction activity of water, $a_1$, was evaluated from the molar concentrations of water, $C_1$, and urea, $C_3$, shown in Tables 2.2 and 2.3 as $a_1 = \frac{C_1}{C_1 + C_3}$. In fact, $a_1$ and $a_3 (= C_3)$ are related via $a_1 = 1 - 0.0173 (\pm 0.0001) a_3 - 0.00072 (\pm 0.00001) a_3^2$ at all temperatures studied. Figure 2.5 graphically illustrates results of these computations. Note that the $(\partial \Delta G_c / \partial a_3)_{T,P}$ term in Eq. (2.4) is independent of urea concentration and weakly depends on temperature. Hence, the concentration- and temperature-dependent changes in m-values in Figure 2.5 originate from the second term of Eq. (2.4) which is strongly modulated by the binding constant, $k$. A decrease in $k$ would reduce the second (positive) term of Eq. (2.4), ultimately, resulting in negative m-values; i.e., a decrease in $k$ may lead to a change in the mode of action of the cosolvent switching it from a denaturant to a stabilizer.

Inspection of Figure 2.5 reveals that at 18 and 25 °C, the m-values, which are positive at low urea concentrations, become negative at higher urea concentrations. At 35 and 45 °C, the m-values are all negative, weakly depending on urea. These computational results clearly contradict the experimental observations not merely in quantitative (magnitude) but also in qualitative (sign) manner. Significantly, both the first and the second terms of Eq. (2.4) linearly change with the differential solvent accessible surface area, $\Delta S_A$, of the folded and unfolded states (Chalikian, T.V., 2016). A mere increase or decrease in $\Delta S_A$ in Eq. (2.4) will change the magnitude but not the sign of the computed m-values. We infer, therefore, that Eq. (2.4) poorly models the denaturing action of urea.
Figure 2.5. Urea dependences of the m-values for an unfolding of a spherical protein with a radius of 20 Å (with $S_A$ of 5755 Å$^2$ and $V_M$ of 33,510 Å$^3$) into a spherocylinder with $S_A$ of 11,510 Å$^2$ and $V_M$ of 33,510 Å$^3$ at 18 (black), 25 (red), 35 (blue) and 45 (magenta) °C.

To overcome the impasse, we modify the model by assuming that the unfolded state becomes “more unfolded” as the concentration of urea increases ($S_{AD}$ increases with urea) and that urea binds more strongly to the unfolded state compared to the folded state (the urea binding constant of the unfolded state, $k_D$, is larger than that of the native state, $k_N$). In fact, based on the results of detailed MD simulations, Stumpe and Grubmüller (Stumpe, M.C., & Grubmüller, H., 2009) have come to a conclusion that one of the driving forces of urea-induced protein denaturation is the stronger interaction of urea with the unfolded state relative to the folded state. On the other hand, experimental evidence suggests that the
solvent accessibility of the unfolded state, indeed, may increase with an increase in the denaturant concentration (Ziv, G., & Haran, G., 2009).

Thus, we assume that the hydration number, \( n_D = S_{AD}/S_W \), of the unfolded state linearly increases with the activity of the cosolvent, \( n_D = n_{D0}(1 + \gamma a_3) \); \( n_{D0} \) is the hydration number of the unfolded state in the absence of urea. With these modifications, Eq. (2.2) transforms into the following:

\[
\Delta G^o = \Delta \Delta G_C + \frac{RTn_N}{r} \ln (k_1 a_1^r + k_3 N a_3) - \frac{RTn_{D0}(1 + \gamma a_3)}{r} \ln (k_1 a_1^r + k_3 D a_3) \tag{2.9}
\]

Differentiating Eq. (2.9), one obtains the following relationship:

\[
\frac{\partial \Delta G^o}{\partial a_3} \bigg|_{T,P} = \frac{\partial \Delta \Delta G_C}{\partial a_3} \bigg|_{T,P} - \frac{RTn_N}{a_1^r + k_N a_3} \left( \frac{a_1^r}{C_1} - \frac{k_N}{r} \right) - \frac{\gamma n_{D0}RT}{r} \ln (a_1^r + k_D a_3) + \frac{\gamma RTn_{D0}}{r} \ln k_1 + \frac{RTn_{D0}(1 + \gamma a_3)}{a_1^r + k_D a_3} \left( \frac{a_1^r}{C_1} - \frac{k_D}{r} \right) \tag{2.10}
\]

Since, in the absence of cosolvent, the protein stability is given by \( \Delta G^o_0 = \Delta \Delta G_C^0 - \frac{RT(n_{D0} - n_N)}{r} \ln k_1 \) (where \( \Delta \Delta G_C^0 \) is the differential free energy of cavity formation in the absence of cosolvent), one obtains the following relationship for m-value:

\[
m \approx - \frac{\partial \Delta G^o}{\partial a_3} \bigg|_{T,P} = \frac{\partial \Delta \Delta G_C}{\partial a_3} \bigg|_{T,P} + \frac{RTn_N}{a_1^r + k_N a_3} \left( \frac{a_1^r}{C_1} - \frac{k_N}{r} \right) + \frac{\gamma n_{D0}RT}{r} \ln (a_1^r + k_D a_3) + \frac{\gamma n_{D0}}{n_{D0} - n_N} (\Delta \Delta G_{CW} - \Delta G^o_0) - \frac{RTn_{D0}(1 + \gamma a_3)}{a_1^r + k_D a_3} \left( \frac{a_1^r}{C_1} - \frac{k_D}{r} \right) \tag{2.11}
\]
We aim to test the conceptual validity of the modified model and its ability to qualitatively capture the main experimental trends (in this case, the relative urea-independence of m-values and their slight decrease with increasing temperature). As a reasonable assumption, we assign \( S_{AD} = S_{AN}(1.8 + 0.05a_3) \) (corresponding to an average two-fold increase in solvent accessible surface area in agreement with the experiment (Son, I., & Chalikian, T.V., 2016)) that results in \( n_D = n_{D0}(1 + \gamma a_3) = \frac{1.8S_{AN}}{11} (1 + \frac{0.05}{1.8}a_3) \). Table 2.4 shows the spherocylindrical curvature, \( a \) and cylindrical length, \( l \), calculated for the unfolded state at different urea concentrations. With these geometric parameters, we use Eqs. (2.7) and (2.8) to compute the differential free energy of cavity formation, \( \Delta \Delta G \), at urea concentrations between 0 and 8M and temperatures between 18 and 45 \( ^{\circ}\)C. Figure 2.6 graphically presents results of these computations. The slopes \( (\partial \Delta G / \partial a_3)_{T,P} \) are 29.7 ± 0.5, 29.7 ± 0.3, 30.4 ± 0.4, and 30.6 ± 0.4 kcal mol\(^{-1}\) M\(^{-1}\) at 18, 25, 35, and 45 \( ^{\circ}\)C, respectively. We use these data in conjunction with the binding constants \( k_N \) of 0.15, 0.11, 0.07, and 0.05M (Son, I., & Chalikian, T.V., 2016) and \( k_D \) of 0.15, 0.12, 0.096, and 0.08 at 18, 25, 35, and 45 \( ^{\circ}\)C, respectively, to calculate m-values from Eq. (2.11). The choice of the specific values of \( k_D \) was based on trial and error and aimed at qualitative reproduction of the experimental data on urea- and temperature dependences of m-values. Note that with the exemption of 18 \( ^{\circ}\)C, the values of \( k_D \) are slightly higher compared to \( k_N \). The values of \( \Delta G^0 \) in Eq. (2.11) are 8.5, 8.0, 7.0, and 6.0 kcal mol\(^{-1}\) at 18, 25, 35, and 45 \( ^{\circ}\)C, respectively [roughly corresponding to the average of the stabilities of lysozyme and cytochrome c in Figures 2.2(a) and 2.2(b)].
Table 2.4. The solvent accessible surface areas, \( S_{AD} = 5755(1.8 + 0.05a_3) \), spherocylindrical curvatures, \( a \), and cylindrical lengths, \( l \), of a spherocylinder with a molecular volume, \( V_M \), of 33510 Å³.

<table>
<thead>
<tr>
<th>[urea], M</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>( S_{AD}, \text{Å}^2 )</td>
<td>10,359</td>
<td>10,647</td>
<td>10,935</td>
<td>11,222</td>
<td>11,510</td>
<td>11,798</td>
<td>12,086</td>
<td>12,373</td>
<td>12,661</td>
</tr>
<tr>
<td>( a, \text{Å} )</td>
<td>7.9740</td>
<td>7.7651</td>
<td>7.5690</td>
<td>7.3852</td>
<td>7.2113</td>
<td>7.0471</td>
<td>6.8917</td>
<td>6.7448</td>
<td>6.6048</td>
</tr>
<tr>
<td>( l, \text{Å} )</td>
<td>157.12</td>
<td>166.55</td>
<td>176.09</td>
<td>185.72</td>
<td>195.50</td>
<td>205.39</td>
<td>215.39</td>
<td>225.48</td>
<td>235.71</td>
</tr>
</tbody>
</table>

Figure 2.6. Urea dependences of changes in free energy of cavity formation, \( \Delta \Delta G_C \), accompanying an unfolding of a spherical protein with a radius of 20 Å (\( S_A \) of 5755 Å² and \( V_M \) of 33,510 Å³) into a spherocylinder with \( S_A = 5755(1.8 + 0.05a_3) \) and \( V_M \) of 33,510 Å³ at 18 (black), 25 (red), 35 (blue) and 45 (magenta) °C.
Figure 2.7 plots the resultant data generated with Eq. (2.11). Inspection of the urea dependences presented in Figure 2.7 reveals that the simulated m-values capture the main features of the experimental results. Specifically, the m-values decrease with increasing temperature and remain positive at all temperatures. A decrease in m-values at low to moderate urea concentrations is in agreement with experimental observations (Pace, C.N. et al., 1990; Yao, M., & Bolen, D.W., 1995; DeKoster, G.T., & Robertson, A.D., 1997). It is also in qualitative agreement with the results of coarse-grained MD simulations which suggest that the urea m-value of protein L depends on urea below 3M while becoming urea-independent at higher concentrations of the denaturant (O’Brien, E.P. et al., 2009).

**Figure 2.7.** Urea dependences of the m-values for an unfolding of a spherical protein with a radius of 20 Å (with $S_A$ of 5755 Å$^2$ and $V_M$ of 33,510 Å$^3$) into a spherocylinder with $S_A = 5755(1.8 + 0.05a_3)$ and $V_M$ of 33,510 Å$^3$ at 18 (black), 25 (red), 35 (blue) and 45 (magenta) °C.
The similarity of the experimental and simulated results is encouraging and lends credence to the validity of our model and the assumptions and the range of numerical parameters used in the simulations. It needs to be emphasized, however, that the computational results illustrated in Figure 2.7 should be taken at their qualitative rather than quantitative value. First, a sphere and a spherocylinder are poor geometric approximations of the folded and unfolded protein states. For example, based on the intrinsic volume, $V_m$, of cytochrome c, its spherical radius, $r_N$, is 14.5 Å, while based on the solvent accessible surface area, $S_A$, $r_N$ is 20.7 Å. Second, SPT with the spherical representation of solvent and cosolvent molecules is a simplified liquid theory. Third, the effective binding constants $k$ used in Eq. (2.11) ignore the heterogeneity of urea binding sites and represent “average” values common for the folded states and the ensemble of unfolded states. Nevertheless, the statistical thermodynamic model used to derive Eq. (2.11) is based on physically robust principles and provides a good qualitative description of cosolvent effects on protein stability. Thus, examination of the experimental data in light of the computational results enables one to glean insights into the nature of the unfolded state and the thermodynamic basis underlying the m-values and LEM in general.

Our results are consistent with the picture in which urea interacts slightly but significantly more strongly with the unfolded state which, in turn, becomes progressively more unfolded as the concentration of urea increases. In fact, an increase in solvent exposure of the unfolded state with an increase in urea concentration has been invoked to rationalize the relative independence of m-values of the concentration of urea (O’Brien, E.P. et al., 2009; Thirumalai, D. et al., 2010). On the other hand, our volumetric studies of urea interactions with amino acid side chains and peptide groups have revealed a wide range of urea binding constants, $k$ (Lee, S. et al., 2010; Chalikian, T.V., 2011; Chalikian, T.V., 2014). Our proposed
increase in the effective value of $k$ accompanying the folded-to-unfolded transition of the protein may reflect the associated change in the chemical nature and microenvironment of solvent exposed atomic groups of the unfolded polypeptide chain.

2.6 Conclusion

In conclusion, the relative independence of m-values of urea concentration originates from fortuitous compensatory effects of an increase in the solvent-accessible surface area of the unfolded state and a higher urea binding constant of the unfolded state relative to the folded state. Other denaturing cosolvents lacking such effects would make poor candidates for LEM-based protein stability determination studies. The observed temperature dependences of m-values reflect, in addition to the aforementioned compensatory effects, a diminution in the protein-urea binding constants with temperature consistent with the negative sign of the binding enthalpy.
CHAPTER 3: General Conclusion and Future Perspectives

3.1 Conclusion

Countless studies have dealt with solute-solvent and solute-cosolvent interactions to better understand protein stability leading to advancements in protein-based technologies.

In Chapter 2, we describe an experimental and computational study of the dependence of the m-value on urea concentration and temperature. We seek to understand, at the molecular level, the temperature dependences of urea m-values. Our combined experimental and computational results collectively suggest that urea interacts slightly but significantly more strongly with the unfolded state which, in turn, becomes progressively more unfolded as the concentration of urea increases. This relative independence of urea m-values originate from fortuitous compensatory effects of a progressive increase in the solvent accessible surface area of the unfolded state and a slightly higher urea binding constant of the unfolded state relative to the folded state. Other denaturing cosolvents which lack such a compensation make poor candidates for linear extrapolation model-based protein stability determination studies.
3.2 Future Directions

Herein, the interactions of the destabilizing cosolvent urea with globular proteins have been examined by combining the experimental and computational approaches. Below, I describe studies that will expand our general understanding of the role of protein–cosolvent interactions in protein stability further characterizing the influence of stabilizing and destabilizing cosolvents.

3.2.1 Comparison of Pressure Perturbation vs. Chemical Denaturation

Pressure effects on a protein reflect the differential packing and solvation of the folded and unfolded conformations (Gross, M., & Jaenicke, R., 1994; Sasahara, K. et al., 2001; Chalikian, T.V., & Macgregor, R.B. Jr., 2009; Nucci, N.V. et al., 2014). One contributing factor to the decrease in volume between the folded and the unfolded states is the presence of the intraglobular void volume in the folded state that is eliminated upon unfolding (Shortle, D., 1989; Roche, J., & Royer, C.A., 2018 and references therein). The mechanism of pressure-induced protein unfolding differs from that of denaturant-induced unfolding in the degree of population of intermediate states, the structural, energetic and dynamic properties of the unfolded state ensemble, and even, conceivably, the folding pathway(s) can differ between pressure and chemical denaturation (Meeker, A.K. et al., 1996; Shortle, D., 1989; Wang, J. et al., 1997; Whitten, S.T. & Garcia-Moreno, E.B., 2000; Isom, D.G. et al., 2008). We propose studying the effects of hydrostatic pressure on the m-value of urea-mediated protein denaturation via combination of pressure and temperature. Such experiments may involve studying lysozyme (as a well-characterized and well-behaving protein) at pressures of up to
100MPa in urea solutions ranging from 0 – 8 M at temperatures ranging from 10 to 90 °C. Comparison of the m-value results obtained from pressure perturbation with values obtained from our chemical denaturation experiments will provide insight and exploration into protein folding landscapes. Multiple approach studies tend to reveal more complexity into systems vs. single-approach studies.
References


structural transitions of cytochrome c bound to cardiolipin-containing membranes.

_Biochem., 45_, 4998–5009.


APPENDIX A: SUPPLEMENTARY MATERIAL FOR CHAPTER 2

Fig S1a. Urea-induced denaturation profile of lysozyme at 20 °C.

Fig S1b. Urea-induced denaturation profile of lysozyme at 25 °C.
Fig S1c. Urea-induced denaturation profile of lysozyme at 30 °C.

Fig S1d. Urea-induced denaturation profile of lysozyme at 35 °C.
**Fig S1e.** Urea-induced denaturation profile of lysozyme at 40 °C.

**Fig S1f.** Urea-induced denaturation profile of lysozyme at 45 °C.
**Fig S2a.** Urea-induced denaturation profile of cytochrome c at 20 °C.

**Fig S2b.** Urea-induced denaturation profile of cytochrome c at 25 °C.
Fig S2c. Urea-induced denaturation profile of cytochrome c at 30 °C.

Fig S2d. Urea-induced denaturation profile of cytochrome c at 35 °C.
Fig S2e. Urea-induced denaturation profile of cytochrome c at 40 °C.

Fig S2f. Urea-induced denaturation profile of cytochrome c at 45 °C.
Figure S3a. UV melting profile of lysozyme at 280nm in neat buffer.

Figure S3b. UV melting profile of cytochrome c at 290nm in neat buffer.