J|A|C|S
ARTICLES
Published on Web 01/23/2004

Impact of Protein Denaturants and Stabilizers on Water Structure

Joseph D. Batchelor,[†] Alina Olteanu,[†] Ashutosh Tripathy,[‡] and Gary J. Pielak*[,†,‡,§]

Contribution from the Departments of Chemistry, Biochemistry and Biophysics, and the Lineberger Cancer Research Center, University of North Carolina, Chapel Hill, North Carolina 27599

Received October 30, 2003; E-mail: gary_pielak@unc.edu

Abstract: It is of great interest to determine how solutes such as urea, sugars, guanidinium salts, and trimethylamine *N*-oxide affect the stability, solubility, and solvation of globular proteins. A key hypothesis in this field states that solutes affect protein stability indirectly by making or breaking water structure. We used a new technique, pressure perturbation calorimetry, to measure the temperature dependence of a solute's partial compressibility. Using fundamental thermodynamic relations, we converted these data to the pressure dependence of the partial heat capacity to examine the impact of protein stabilizing and denaturing solutes on water structure by applying the classic two-state mixture model for water. Contrary to widely held expectations, we found no correlation between a solute's impact on water structure and its effect on protein stability. Our results indicate that efforts to explain solute effects should focus on other hypotheses, including those based on preferential interaction and excluded volume.

Introduction

Globular proteins, nature's most functionally diverse macromolecules, are only marginally stable. Even under physiological conditions, a delicate balance of forces combine to give proteins a maximum stability of only a few kilocalories per mole,[1] a value near the dissociation energy of a few hydrogen bonds.[2] Solutes can have large effects on proteins.[3] For instance, urea,[4,5] urea derivatives,[4] and guanidinium chloride[6] denature proteins, while sugars[7,8] and glycine derivatives[7,9] can double a protein's stability. It is important to understand the mechanism by which solutes exert these large effects. One fundamental and recurring hypothesis is that solutes act by altering water structure.[2,10−17] We test this hypothesis by applying thermodynamic analysis to calorimetric data on aqueous solutions of protein stabilizers and denaturants and by using a two-state mixture model for water.

Water can be conceptualized as a mixture of two rapidly interconverting species; a less dense, more structured species, and a more dense, less structured species.[18−24] Although this two-state mixture approximation has been criticized for its simplicity,[25,26] it has proven useful in understanding the volumetric properties of solutions.[23] According to this model and Le Chatelier's principle, increasing either the temperature or the pressure increases the fraction of the more dense, less structured species at the expense of the other species.[20,21,27] Because it takes heat to break the structure, a decrease in structure will decrease the heat capacity, C_p, of pure water, making C_p inversely proportional to P at constant T $[(\partial C_p/\partial P)_T < 0]$.

An analogous idea applies to a solute's effect on water structure. One model of solute hydration states that the differences between bulk water and hydration water arise from a

[†] Department of Chemistry.
[‡] Department of Biochemistry and Biophysics.
[§] Lineberger Cancer Research Center.

(1) Pace, C. N. *Trends Biochem. Sci.* **1990**, *15*, 14−17.
(2) Creighton, T. E. *Proteins, Structures and Molecular Properties*, 2nd ed.; W. H. Freeman and Company: New York, 1996.
(3) Hofmeister, F. *Arch. Exp. Pathol. Pharmakol.* **1888**, *24*, 247−260.
(4) Poklar, N.; Lah, N.; Oblak, M.; Vesnaver, G. *Acta Chim. Slov.* **1999**, *46*, 315−322.
(5) Tanford, C. *J. Am. Chem. Soc.* **1964**, *86*, 2050−2059.
(6) Tanford, C. *Adv. Protein Chem.* **1970**, *24*, 1−95.
(7) Liu, Y.; Bolen, D. W. *Biochemistry* **1995**, *34*, 12884−12891.
(8) Davis-Searles, P. R.; Morar, A. S.; Saunders, A. J.; Erie, D. A.; Pielak, G. J. *Biochemistry* **1998**, *37*, 17048−17053.
(9) Santoro, M. M.; Liu, Y.; Khan, S. M. A.; Hou, L.-X.; Bolen, D. W. *Biochemistry* **1992**, *31*, 5278−5283.
(10) Vanzi, F.; Madan, B.; Sharp, K. *J. Am. Chem. Soc.* **1998**, *120*, 10748−10753.
(11) Zou, Q.; Bennion, B. J.; Daggett, V.; Murphy, P. K. *J. Am. Chem. Soc.* **2002**, *124*, 1192−1202.
(12) Noto, R.; Martorana, V.; Emanuele, A.; Fornili, S. L. *J. Chem. Soc., Faraday Trans.* **1995**, *91*, 3803−3808.
(13) Plumridge, T. H.; Waigh, R. D. *J. Pharm. Pharmacol.* **2003**, *54*, 1155−1179.
(14) Schiffer, C. A.; Dötsch, V. *Curr. Opin. Biotechnol.* **1996**, *7*, 428−432.
(15) Washabaugh, M.; Collins, K. D. *J. Biol. Chem.* **1986**, *27*, 12477−12485.
(16) von Hippel, P. H.; Wong, K.-Y. *J. Biol. Chem.* **1965**, *240*, 3909−3923.
(17) Schleich, T.; von Hippel, P. H. The Effects of Neutral Salts on Macromolecules. In *Structure and Stability of Biological Macromolecules*; Timasheff, S. N., Fasman, G. D., Eds.; Marcel Dekker: New York, 1969; Vol. 2, pp 417−574.
(18) Röntgen, W. K. *Ann. Phys. Chem.* **1892**, *45*, 91−97.
(19) Frank, H. S.; Wen, W.-Y. *Discuss. Faraday Soc.* **1957**, *24*, 133−140.
(20) Vedmuthu, M.; Singh, S.; Robinson, G. W. *J. Phys. Chem.* **1994**, *98*, 2222−2230.
(21) Hepler, L. G. *Can. J. Chem.* **1969**, *47*, 4613−4617.
(22) Lin, L. N.; Brandts, J. F.; Brandts, J. M.; Plotnikov, V. *Anal. Biochem.* **2002**, *302*, 144−160.
(23) Chalikian, T. V. *J. Phys. Chem. B* **2001**, *105*, 12566−12578.
(24) Hura, G.; Head-Gordon, T. *Chem. Rev.* **2002**, *102*, 2651−2670.
(25) Kauzmann, W. *L'Eau Syst. Biol., Colloq. Int. C. N. R. S.* **1975**, *246*, 63−71.
(26) Endo, H. *J. Chem. Phys.* **1980**, *72*, 4324−4326.
(27) Urquidi, J.; Cho, C. H.; Singh, S.; Robinson, G. W. *J. Mol. Struct.* **1999**, *485−486*, 363−371.

10.1021/ja039335h CCC: $27.50

varying ratio between the more dense and the less dense water species.[23] A "structure-making" solute increases the fraction of the less dense species at the expense of the more dense species in the solute's hydration water. A "structure-breaking" solute has the opposite effect. The sign of $(\partial\bar{C}_p/\partial P)_T$, where $\bar{C}_p$ represents the partial molar heat capacity, indicates whether a solute makes $[(\partial\bar{C}_p/\partial P)_T < 0]$ or breaks $[(\partial\bar{C}_p/\partial P)_T > 0]$ water structure.[21] For example, increasing the pressure shifts the equilibrium of a structure-making solute's hydration water toward the denser, less structured species. This shift decreases the structure-making solute's $\bar{C}_p$, making $(\partial\bar{C}_p/\partial P)_T$ negative. Conversely, increasing the pressure leaves a structure-breaking solute less bulk water structure to break, making $\bar{C}_p$ negative and $(\partial\bar{C}_p/\partial P)_T$ positive. As shown by Loren Hepler[21] and as reiterated in the Supporting Information, relating $(\partial\bar{C}_p/\partial P)_T$ to the partial specific volume, $\bar{V}$, and temperature, T, gives the thermodynamic relation[21]

$$\left(\frac{\partial\bar{C}_p}{\partial P}\right)_T = -T\left(\frac{\partial^2\bar{V}}{\partial T^2}\right)_P \qquad (1)$$

Hepler used eq 1 to assess a solute's effect on water structure in 1969,[21] and Neal and Goring performed a similar analysis the next year,[28] but little progress has been made because the measurements to obtain $(\partial\bar{V}/\partial T)_P$ and its derivative are tedious. Pressure perturbation calorimetry (PPC),[22] a new technique, is superior to previously employed methods because it allows the collection of large amounts of highly accurate data in a short time. In PPC, a N_2 pressurization device is fitted to a differential scanning calorimeter, and pressure pulses of ~350 kPa are alternatively applied to the sample and reference cells. The differential heat provides information about the difference in compressibility between the sample and the reference and, hence, about $\bar{\alpha}$ of the sample. Data are collected as a function of temperature to provide the coefficient of thermal expansion, $\bar{\alpha} = 1/\bar{V}(\partial\bar{V}/\partial T)_p$, as a function of temperature.[22] Defining equations for PPC have been presented,[22,29] and are available in the Supporting Information. Measuring $\bar{\alpha}$ simplifies the determination of $(\partial\bar{C}_p/\partial P)_T$. Substituting the definition of $\bar{\alpha}$ into eq 1 gives

$$\left(\frac{\partial\bar{C}_p}{\partial P}\right)_T = -T\left(\frac{\partial(\bar{V}\bar{\alpha})}{\partial T}\right)_P \qquad (2)$$

Several hypotheses have been offered to explain a solute's effect on protein stability, here defined in terms of the equilibrium constant for the reaction $N \leftrightarrows D$, where N represents the native, biologically active state, and D represents the denatured, inactive state.[30,31] One of these hypotheses states that stabilizers and destabilizers of globular proteins act indirectly by altering water structure.[2,10−17] According to this hypothesis, structure makers decrease protein stability and structure breakers increase stability. Despite the popularity of the water structure hypothesis,[2,10−17] there is a lack of data that quantifies a solute's effect on water structure. To test the hypothesis, we used PPC and various thermodynamic relations to determine the sign of $(\partial\bar{C}_p/\partial P)_T$ and its temperature dependence for a number of protein stabilizers and denaturants.

Table 1. Solute Effects on Protein Stability and $(\partial\bar{C}_p/\partial P)_T$ at 25 °C

compound	effect on stability [a]	$(\partial\bar{C}_p/\partial P)_T \times 10^{-10}$ at 25 °C, cal × atm^{-1} × K^{-1} [b]
$(NH_4)_2SO_4$	++[c]	11.1
NH_4Cl	+[c]	8.89
guanidinium Cl	− −[c,d]	6.35
guanidinium SCN	− − −[c]	6.39
N-methylglycine (sarcosine)	++[e,f]	4.74
urea	−[g,h]	4.88
glucose	+[i]	2.62
N-trimethylglycine (betaine)	++[f]	2.34
trehalose	+[i,j]	1.62
sucrose	+[e,i]	1.72
glycerol	+,−[i]	1.68
stachyose	+[i]	1.30
melezitose	+[i]	0.44
1,3-dimethylurea	−[g]	−0.32
trimethylamine *N*-oxide dihydrate	+++[f]	−2.08
1,3-diethylurea	−[g]	−1.44
2-propanol	*k*	−6.50

[a] (+) indicates stabilizing, (−) indicates destabilizing. The number of symbols is related to the magnitude of the effect. [b] Uncertainty is ±0.2 × 10^{-10} cal × atm^{-1} × K^{-1}. [c] Reference 16. [d] Reference 6. [e] Reference 7. [f] Reference 9. [g] Reference 4. [h] Reference 5. [i] Reference 8. [j] Reference 35. [k] Not applicable because 2-propanol is not commonly used to affect protein stability.

Experimental Section

Solutes were purchased from Fisher, Sigma, and Mallinckrodt and dissolved in distilled deionized water. Density measurements were made on a vibrating tube densitometer (DMA 5000, Anton Paar). PPC was performed on a MicroCal VP-DSC, equipped with a pressurizing system.[22] PPC data were reproduced at least once. The ΔP used in PPC experiments was 350 kPa. Origin 5.0 software (Microcal) was used for data integration to give the Δq (see Supporting Information for more detail). SigmaPlot 2000 (SPSS) was used for analysis of $\bar{V}$, $\bar{\alpha}$, and $-T(\partial(\bar{V}\bar{\alpha})/\partial T)_P$. $\bar{V}$ values were obtained from literature values[32,33] and from the concentration dependence of the density.[32] These values, as well as values of $\bar{\alpha}$ at 25 °C are compiled in the Supporting Information. PPC data were collected for all the solutes at a concentration of 20.0 g/L. We also made measurements for 2.00 M solutions of $(NH_4)_2SO_4$, guanidinium SCN, sucrose, and trimethylamine *N*-oxide (Supporting Information, Figure 1s). The temperature dependence of $\bar{V}$ was small (increase of ≤15% from 20 to 70 °C), and therefore it was not included in data analysis. $\bar{V}$ was assumed to equal the partial specific volume at infinite dilution because apparent partial volumes at concentrations below a few percent are close to the partial volume at infinite dilution.[22]

Results

The 17 solutes chosen for study were $(NH_4)_2SO_4$, NH_4Cl, guanidinium Cl, guanidinium SCN, sarcosine, urea, glucose, trehalose, sucrose, betaine, glycerol, stachyose, melezitose, 1,3-dimethylurea, trimethylamine *N*-oxide dihydrate, 1,3-diethylurea, and 2-propanol. Most solutes were chosen for their known stabilizing or destabilizing effects. Literature citations concerning the effects of the solutes on protein stability are given in Table 1. 2-Propanol was chosen because it is a clear example of a

(28) Neal, J. L.; Goring, D. A. I. *J. Phys. Chem.* **1970**, *74*, 658−664.
(29) Kujawa, P.; Winnik, F. M. *Macromolecules* **2001**, *34*, 4130−4135.
(30) Lumry, R.; Biltonen, R.; Brandts, J. F. *Biopolymers* **1966**, *4*, 917−944.
(31) Cohen, D. S.; Pielak, G. J. *Protein Sci.* **1994**, *3*, 1253−1260.
(32) Durchschlag, H. Specific Volumes of Biological Macromolecules and Other Molecules of Biological Interest. In *Thermodynamic Data for Biochemistry and Biotechnology*; Hinz, H.-J., Ed.; Springer-Verlag: Berlin, 1986; pp 45−182.
(33) Weast, R. C. *Handbook of Chemistry and Physics*, 54th ed.; CRC Press: Cleveland, OH, 1973.

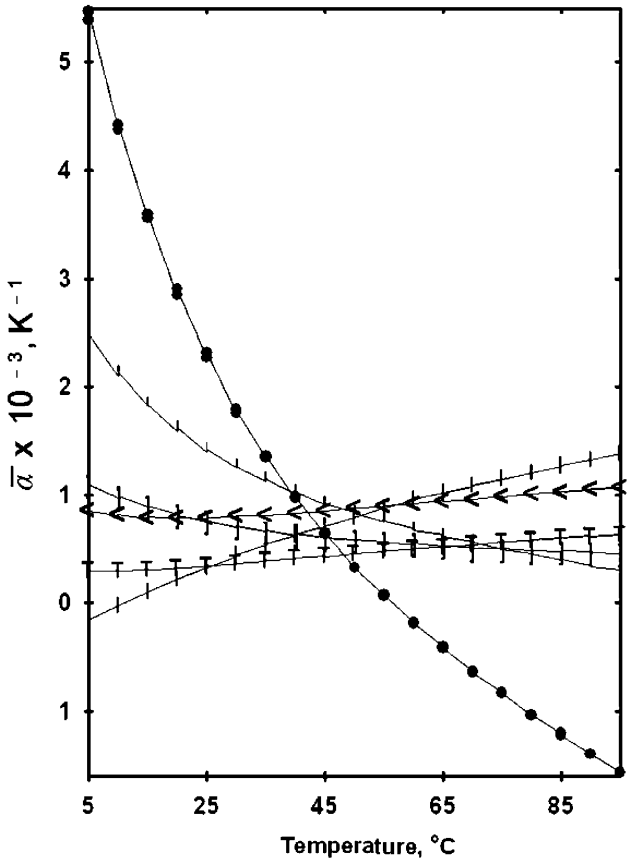

Figure 1. Plots of $\bar{\alpha}$ versus temperature for 20 g/L aqueous solutions of $(NH_4)_2SO_4$ (●), guanidinium Cl (′), sucrose (]), 1,3-dimethylurea (<), trimethylamine *N*-oxide dihydrate (T), and 2-propanol (|).

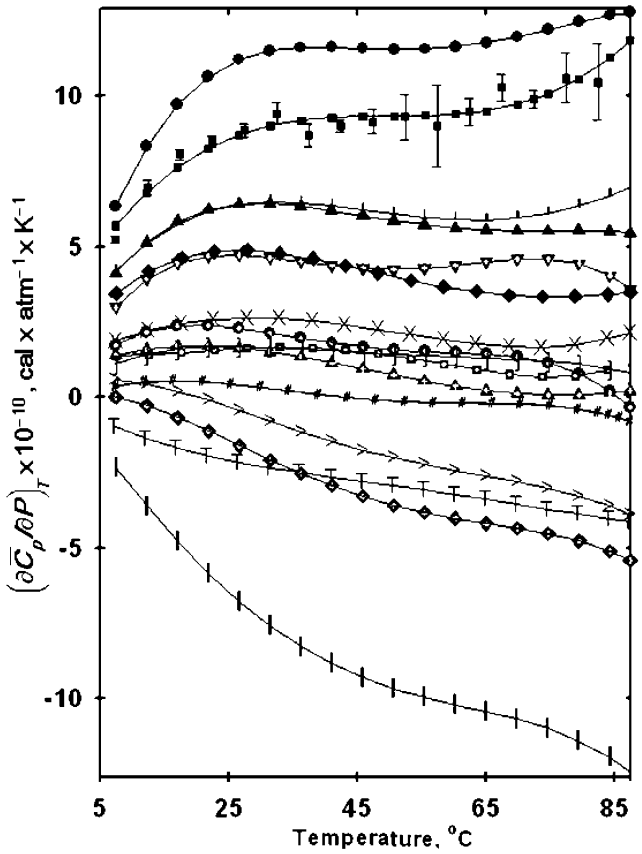

Figure 2. $(\partial \bar{C}_p/\partial P)_T$ and its temperature dependence for 20 g/L aqueous solutions of $(NH_4)_2SO_4$ (●), NH_4Cl (■), guanidinium Cl (′), guanidinium SCN (▲), sarcosine (▽), urea (◆), glucose (×), betaine (○), sucrose (]), trehalose (□), glycerol (△), melezitose (#), 1,3-dimethylurea (>), trimethylamine *N*-oxide dihydrate (T), 1,3-diethylurea (◇), and 2-propanol (|). A smoothed curve of no theoretical significance has been drawn through the data. The symbols only serve as a guide to the eye. Datapoints and the fit are shown in Supporting Information. The error bars shown for NH_4Cl are representative of the uncertainty in all samples and were determined from repetition of the experiment. Data begin at 7.5 °C.

structure maker;[28] it is not commonly used to affect protein stability. The urea derivatives were chosen to compare a known stability trend[4] with effects on water structure. Most of the other solutes have been extensively studied; there is a limited amount of work on the effects of urea derivatives[4] and guanidinium SCN[16] on protein stability.

The temperature dependence of $\Delta q_{(\text{sample}/H_2O)}$ (Supporting Information, eq 18s) was measured for the solutes by using PPC. These data were converted to $\bar{\alpha}$ versus temperature and then to $(\partial \bar{C}_p/\partial P)_T$ versus temperature by using equations given in the Supporting Information. Representative plots of $\bar{\alpha}$ versus temperature are presented in Figure 1. Plots of $(\partial \bar{C}_p/\partial P)_T$ versus temperature for 16 solutes are presented in Figure 2. Stachyose is not shown in Figure 2 because its $(\partial \bar{C}_p/\partial P)_T$ values are within the error of other sugars. Representative plots of $(\partial \bar{C}_p/\partial P)_T$ versus temperature for 2.00 M solutes and the raw data used in preparing $\bar{\alpha}$ versus temperature plots are provided in the Supporting Information. Table 1 shows the $(\partial \bar{C}_p/\partial P)_T$ values for all 17 solutes at 25 °C. Our results are in accord with those of Hepler[21] and Neal and Goring[28] for the solutes common between the studies (glucose, sucrose, urea, and 2-propanol).

As explained in the Introduction, a positive $(\partial \bar{C}_p/\partial P)_T$ indicates solute-induced structure breaking, and negative values indicate structure making. By these criteria, at 25 °C trimethylamine *N*-oxide, 1,3-dimethylurea, 1,3-diethylurea, and 2-propanol are structure makers, and the 13 other solutes are structure breakers. Experiments conducted at a higher solute concentration (2.00 M) do not change our conclusions (Supporting Information). Inspection of Table 1 shows no correlation between stability effects and the sign of $(\partial \bar{C}_p/\partial P)_T$. Comparing the $(\partial \bar{C}_p/\partial P)_T$ data in Table 1, specific for 25 °C, with the full range of temperatures (Figure 2) shows that this lack of correlation is not an artifact of the temperature selected to compile Table 1.

The water structure hypothesis[2,10−17] predicts that structure-breaking solutes stabilize proteins and that structure-making solutes destabilize proteins. Our data show that this prediction is not borne out whether we consider related solutes or look at the solutes as a whole. As previously reported,[4] urea is a stronger denaturant than 1,3-dimethylurea, which is a stronger denaturant than 1,3-diethylurea, but all three are weaker than guanidinium salts. If the water structure hypothesis were true, urea and its two derivatives would be structure makers, and urea would be a stronger structure maker than either of its derivatives. Our data show that urea breaks water structure and that both 1,3-dimethylurea and 1,3-diethylurea make structure in water. If the water structure hypothesis were correct, the zwitterionic solutes would have different effects. Trimethylamine *N*-oxide, because it is a structure maker, would destabilize proteins, while the two glycine derivatives, because they are structure breakers, would stabilize proteins. Yet all three stabilize proteins. Again, if the hypothesis were true, guanidinum chloride and guanidinum thiocyante would have the same (stabilizing) effect. However,

both compounds are known to be destabilizing, and guanidinum thiocyanate is known to be more destabilizing than guanidinum chloride. Also, both the protein-stabilizing chaotrope, $(NH_4)_2SO_4$,[34] and the protein-destabilizing kosmotrope, guanidinium SCN,[34] are structure breakers. This is significant because the words "chaotrope" and "kosmotrope" were introduced to signify a structure-breaking salt or a structure-making salt, respectively.[34] Finally, trehalose, despite claims of its "exceptional" structuring of water when compared with other carbohydrates,[35] has about the same effect as sucrose. In summary, our data show there is no direct correlation between a solute's effect on water structure, as defined by the simple two-state model for water, and its effect on protein stability.

Discussion

The data in Table 1 and Figure 2 indicate that a solute's effect on water structure is not the determining factor in its effect on protein stability. There are, however, other hypotheses for explaining a solute's effect on protein stability. One hypothesis considers the volume occupied by the solute. Every solute molecule takes up space in the solution, leaving less space for the protein. Referred to as the excluded volume effect, this decrease in the space available to the protein shifts the N ⇆ D equilibrium toward the state with the least surface area.[36,37] Because N has less surface area than D, the excluded volume effect is always stabilizing. This effect is colligative; a protein destabilizer must overcome it.

Another hypothesis considers the solute's affinity for a protein's surface. If this affinity is energetically favored over solute hydration, the N ⇆ D equilibrium will be shifted to the protein state with the most surface area. Because D has more surface area, a solute interacting more favorably with a protein's surface than with water will destabilize a protein.[38−41] Conversely, a solute whose hydration is energetically favored over its affinity for a protein's surface will indirectly stabilize a protein through the excluded volume effect.[7,40] A third hypothesis concerns a solute's ability to attenuate or accentuate the hydrophobic effect by increasing or decreasing the solubility of a protein's hydrophobic core. Urea decreases a protein's stability by increasing the solubility of a protein's hydrophobic core.[42−44] The final effect of a solute on protein stability is probably a combination of all the effects described above.

In conclusion, we have shown that there is no direct correlation between a solute's effect on water structure and its effect on protein stability. Future efforts to explain solute effects should focus on other hypotheses, including those based on preferential interaction and excluded volume.

Acknowledgment. This work was supported by the National Science Foundation (MCB 0212939) and the Smallwood Foundation. We thank Dorothy Erie for insights into excluded volume and Matthew Redinbo, Lee Pederson, Elizabeth Pielak, and the Pielak group for comments on the manuscript. The UNC Macromolecular Interactions Facility is supported by grants from the North Carolina Biotechnology Center and the National Institutes of Health.

Supporting Information Available: Derivations of eq 1 and $\bar{\alpha}$ from $(\partial Q_{rev}/\partial P)_T$, and a table of $\bar{V}$ and $\bar{\alpha}$ at 25 °C. Representative plots of $(\partial \bar{C}_p/\partial P)_T$ versus temperature for 2.00 M aqueous solutions, $\Delta q_{(sample/H_2O)}$ versus temperature for 20.0 g/L aqueous solutions, and $(\partial \bar{C}_p/\partial P)_T$ versus temperature, and the fourth order linear regressions used for producing Figure 2 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA039335H

(34) Collins, K. D.; Washabaugh, M. W. *Q. Rev. Biophys.* **1985**, *18*, 323−422.
(35) Kaushik, J. K.; Bhat, R. *J. Biol. Chem.* **2003**, *278*, 26458−26465.
(36) Minton, A. P. *Biopolymers* **1981**, *20*, 2093−2120.
(37) Davis-Searles, P. R.; Saunders, A. J.; Erie, D. A.; Winzor, D. J.; Pielak, G. J. *Annu. Rev. Biophys. Biomol. Struct.* **2001**, *30*, 271−306.
(38) Myers, J. K.; Pace, C. N.; Scholtz, M. *Protein Sci.* **1995**, *4*, 2138−2148.
(39) Robinson, D. R.; Jencks, W. P. *J. Am. Chem. Soc.* **1965**, *87*, 2462−2470.
(40) Schellman, J. A. *Biophys. J.* **2003**, *85*, 108−125.
(41) Courtenay, E. S.; Capp, M. W.; Record, T. M. *Protein Sci.* **2001**, *10*, 2485−2597.
(42) Alonso, D. O. V.; Dill, K. A. *Biochemistry* **1991**, *30*, 5974−5985.
(43) Zou, Q.; Habermann-Rottinghaus, S. M.; Murphy, K. *Proteins: Struct., Funct., Genet.* **1998**, *31*, 107−115.
(44) Muller, N. *J. Phys. Chem.* **1990**, *94*, 3856−3859.