JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Article

pubs.acs.org/JACS

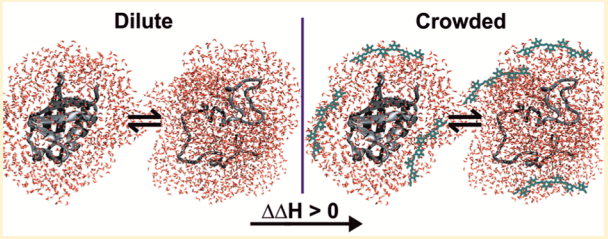
Protein Stabilization by Macromolecular Crowding through Enthalpy Rather Than Entropy

Michael Senske, Lisa Törk, Benjamin Born,[†] Martina Havenith, Christian Herrmann,[‡] and Simon Ebbinghaus*

Department of Physical Chemistry II, Ruhr-Universität Bochum, 44780 Bochum, Germany

Supporting Information

ABSTRACT: The interior of the cell is a densely crowded environment in which protein stability is affected differently than in dilute solution. Macromolecular crowding is commonly understood in terms of an entropic volume exclusion effect based on hardcore repulsions among the macromolecules. We studied the thermal unfolding of ubiquitin in the presence of different cosolutes (glucose, dextran, poly(ethylene glycol), KCl, urea). Our results show that for a correct dissection of the cosolute-induced changes of the free energy into its enthalpic and entropic contributions, the temperature dependence of the heat capacity change needs to be explicitly taken into account. In contrast to the prediction by the excluded volume theory, we observed an enthalpic stabilization and an entropic destabilization for glucose, dextran, and poly(ethylene glycol). The enthalpic stabilization mechanism induced by the macromolecular crowder dextran was similar to the enthalpic stabilization mechanism of its monomeric building block glucose. In the case of poly(ethylene glycol), entropy is dominating over enthalpy leading to an overall destabilization. We propose a new model to classify cosolute effects in terms of their enthalpic contributions to protein stability.



INTRODUCTION

The inner of the cell is a densely crowded (up to a volume fraction of 40%[1]) heterogeneous solvent consisting of a versatile set of organic and inorganic compounds including macromolecules, osmolytes and ions.[2] These cosolutes modify the Gibbs free energy of unfolding ΔG_u (eq 1) either by changing the enthalpy or entropy of unfolding ΔH_u and ΔS_u, respectively. An increase in protein stability by cosolutes can be either caused by an increase of ΔH_u or a decrease of ΔS_u.

$$\Delta G_u(T) = \Delta H_u - T\Delta S_u \quad (1)$$

Cosolute effects that alter protein stability are commonly classified as (i) macromolecular crowders, (ii) bioprotective osmolytes, and (iii) chemical denaturants.

Macromolecular crowders like proteins, DNA, dextran, poly(ethylene glycol) (PEG), or Ficoll are believed to cause a volume exclusion and thereby are thought to restrict the conformational accessible states of a protein due to hard-core repulsions between the crowders and the protein. The excluded volume theory predicts a loss of configurational entropy of a protein in the presence of inert macromolecules as the thermodynamic driving force.[3,4] The entropic loss is usually larger for the protein's extended and more flexible denatured state compared to the compact folded state resulting in an overall stabilization of the protein.

Bioprotective osmolytes like methylamines, polyols, or sugars stabilize the native conformation of globular proteins relative to the unfolded state under external stresses such as dehydration, temperature and pH variations, high salinity, and high concentrations of denaturants.[5−7] This stabilization is explained by an unfavorable interaction of the protein and the cosolute leading to a preferential exclusion of the osmolyte from the protein surface and a preferential hydration of the protein.[8] As the solvent accessible surface area (SASA) is usually increased for the unfolded state, the folding equilibrium is shifted toward the native state. Recent studies suggest that the stabilizing osmolytic effect is an indirect enthalpic stabilization mechanism which is mediated by the interstitial hydration water between the protein and the excluded osmolyte.[9−15]

Chemical denaturants like guanidine hydrochloride and urea destabilize proteins mainly via direct interactions.[16,17] Since the larger SASA of the unfolded protein allows more attractive interactions of the protein with the denaturant, the unfolded state is thermodynamically favored compared to the native state. The attractive interactions between the protein and chemical denaturants are characterized by a reduction of ΔH_u.[9,16,18]

These classes of cosolutes show distinct physical and chemical properties, explaining why they are thought to act differently on protein folding stability. The diversity of the underlying thermodynamic mechanisms can be analyzed by quantifying the enthalpic and entropic contributions to the Gibbs free energy.[19,20] This approach has recently lead to new insights, in particular on how macromolecular crowders act on protein folding stability. A decrease in enthalpy ΔH_u was shown to counteract the expected decrease in entropy ΔS_u, associated with

Received: March 31, 2014
Published: June 3, 2014

   dx.doi.org/10.1021/ja503205y | J. Am. Chem. Soc. 2014, 136, 9036−9041

the excluded volume effect.[21−23] Moreover, stabilizing enthalpic effects that even dominate the entropic contributions have been identified.[20,24]

Using a combination of circular dichroism (CD) spectroscopy and differential scanning calorimetry (DSC), we systematically study the underlying thermodynamic mechanism of different cosolutes (dextran, glucose, PEG, KCl, and urea) on the ubiquitin folding equilibrium. We discuss their effects in terms of the excess enthalpy $\Delta\Delta H_u$ and entropy $\Delta\Delta S_u$ with respect to dilute solution and introduce an enthalpy-based classification scheme. For the accurate dissection of $\Delta\Delta G_u$, we show that it is crucial to take the temperature dependence of the heat capacity change ΔC_p explicitly into account. In particular, we focus on dextran which is commonly used to mimic the macromolecular crowding phenomenon *in vitro*.[25] We specifically address the role of its macromolecular nature by comparing it directly to its monomeric equivalent glucose since the excluded volume effect depends on the crowder radius.[26−28] Dextran stabilizes ubiquitin by an osmolytes-like preferential hydration mechanism. We find that PEG, used as a macromolecular crowder[29] and a precipitating[30] or crystallizing[31] agent, acts via a combination of preferential hydration and preferential binding.

MATERIALS AND METHODS

Sample Preparation. Ubiquitin wildtype (98% purity from bovine erythrocytes, Sigma-Aldrich, Germany), Dextran 40 (AppliChem, Germany), glucose (AppliChem, Germany), PEG 20 (Merck, Germany), KCl (Avantor Performance Materials, The Netherlands), and urea (Avantor Performance Materials, The Netherlands) were used without further purification. Stock solutions of lyophilized protein (10 mg/mL) were prepared in aqueous solutions of different pH, ranging from pH 2−3.5. We conducted the measurements at low pH at which the unfolding transition is highly reversible while its native structure is preserved.[32] Folding reversibility is a prerequisite to analyze equilibrium thermodynamics. Thus, ubiquitin is commonly studied as a model system for protein folding in the low pH regime.[28,33,34] Protein concentration was determined by the weight of the lyophilized protein and its given purity (98%). Ubiquitin stock solution was then added to solutions of varying concentrations of cosolutes. The pH was adjusted by HCl and was controlled before each measurement. To calculate the volume fraction of the cosolutes in the respective solutions, we determined the partial specific volumes of the cosolutes (Table ST1) by density measurements using a DMA 58 density meter (Anton Paar, Austria).[35] All data were analyzed with Mathematica 9 (Wolfram Mathematica).

Circular Dichroism Spectroscopy. CD measurements were carried out at ubiquitin concentrations of 0.3−0.4 mg/mL using a J-815 spectrophotometer (Jasco, Germany). Cells of 1 mm path length were used for the protein melts and were sealed with Teflon stoppers to slow down the rate of evaporation. Ubiquitin unfolding was measured at 204 nm at a heating rate of 60 K/h. The signal of the blank solution was subtracted, and a two-state transition model[36] (Supporting Information, eq 1) was fitted to the sigmoidal CD unfolding curves to extract ubiquitin's transition temperature T_m and its van't Hoff enthalpy of unfolding ΔH_u (Tables ST2 and ST3).

Differential Scanning Calorimetry. DSC thermograms (Figure SF1) were recorded at the same heating rate as the CD measurements (60 K/h) by a VP-DSC instrument (MicroCal). For DSC scans, a protein concentration of 0.5 mg/mL was used. After blank subtraction, the data were normalized to the protein amount and a two-state transition model was fitted to the data (Supporting Information, eq 5) to extract T_m, ΔH_u, and the heat capacity change upon unfolding ΔC_p of ubiquitin.

Thermodynamic Analysis. Thermal unfolding curves of ubiquitin were recorded by CD spectroscopy for the various cosolvents at different concentrations and pH values to determine ubiquitin's melting temperature T_m and the corresponding enthalpic change upon unfolding ΔH_u. Kirchhoff's laws describe the temperature dependence of the standard enthalpy change ΔH^0 and the standard entropy change ΔS^0. Both parameters depend on the standard heat capacity change ΔC_p^0, which is also temperature dependent. Since the CD and DSC measurements were not conducted at standard conditions, we apply Kirchhoff's laws assuming that $\Delta H^0 \approx \Delta H_u$, $\Delta S^0 \approx \Delta S_u$, and $\Delta C_p^0 \approx \Delta C_p$ (eqs 2 and 3). At $T = T_m$, $\Delta G_u = 0$ holds resulting in $\Delta S_u(T_m) = \Delta H_u(T_m)/T_m$. Inserting eqs 2 and 3 into eq 1, eq 4 is obtained for the temperature dependence of ΔG_u.

$$\Delta H_u(T) = \Delta H_u(T_m) + \int_{T_m}^{T} \Delta C_p \, dT \tag{2}$$

$$\Delta S_u(T) = \Delta S_u(T_m) + \int_{T_m}^{T} \frac{\Delta C_p}{T} \, dT \tag{3}$$

$$\Delta G_u(T) = \Delta H_u(T_m)\left(1 - \frac{T}{T_m}\right) + \int_{T_m}^{T} \Delta C_p \, dT - T\int_{T_m}^{T} \frac{\Delta C_p}{T} \, dT \tag{4}$$

Here, we used Kirchhoff's equations to determine a temperature dependent expression for the heat capacity change $\Delta C_p(T)$ of ubiquitin in dilute solution and in the presence of cosolutes by measuring $\Delta H_u(T)$. Therefore, we recorded ΔH_u at different T_m by varying the pH[33,37−39] and fitted a second-order polynomial (Table ST4) to the data to obtain a linear function for $\Delta C_p(T)$.[33,34] We validated the determination of ΔC_p from the pH-dependent $\Delta H_u - T_m$ curves by DSC. Therefore, we measured ΔC_p pH-dependently in dilute solution by DSC (Figure SF2b). ΔC_p was shown to be pH-independent in the analyzed pH range.[33]

The heat capacity change of ubiquitin in the presence of cosolvents $\Delta C_{p,cos}(T)$ is very similar to its value in dilute solution $\Delta C_{p,dilute}(T)$ (only dextran caused a slightly stronger temperature dependence of $\Delta C_p(T)$). Therefore, we used the assumption $\Delta C_{p,cos}(T) \approx \Delta C_{p,dilute}(T)$ to calculate ΔG_u, ΔH_u, and ΔS_u by Kirchhoff's laws, because $\Delta C_{p,cos}(T)$ does not span the entire measured temperature range. Data for cosolute concentrations for which $\Delta C_{p,cos}(T)$ could be used for analysis are in agreement with the extrapolation using $\Delta C_{p,dilute}(T)$ (Tables ST5 and ST6). We extrapolated the enthalpic and entropic values for each cosolute concentration to the melting temperature in dilute solution. We subtracted ΔG_u, ΔH_u, and ΔS_u for ubiquitin in the dilute solution from the results for all cosolvents yielding the respective excess parameter $\Delta\Delta X_u = \Delta X_{u,cos} - \Delta X_{u,dilute}$ with $X = H, S, G$. Errors of the primary parameters ΔH_u and T_m are representative of the 68% confidence interval of the mean. This error of the mean was derived from at least three repeated measurements using a Student's *t*-distribution, which considers the limited repetition of measurements leading to slightly larger errors in comparison to a Gaussian distribution. The errors of the primary parameters were then propagated via the Gaussian error propagation law into the respective excess parameter errors.

RESULTS

Cosolute Induced Changes of the Melting Temperature and van'T Hoff Enthalpy of Ubiquitin. The thermal unfolding curves of ubiquitin in different cosolutes are compared to dilute solution at the respective pH (Figure 1a). We varied the cosolute concentration and determined the corresponding van't Hoff enthalpies ΔH_u as well as the melting temperatures T_m (Figure 1b−d). Glucose and dextran at constant pH caused an increase of T_m of ubiquitin (Figure 1a). The shift in T_m was accompanied by an increase of ΔH_u (Figure 1b) which was observed over the entire pH range (only pH 2 and 2.75 are depicted in Figure 1b for clarity).

While glucose and dextran stabilized the native state of ubiquitin relative to the denatured state, PEG caused a destabilization, which was detected as a decrease of T_m of

dx.doi.org/10.1021/ja503205y | J. Am. Chem. Soc. 2014, 136, 9036−9041

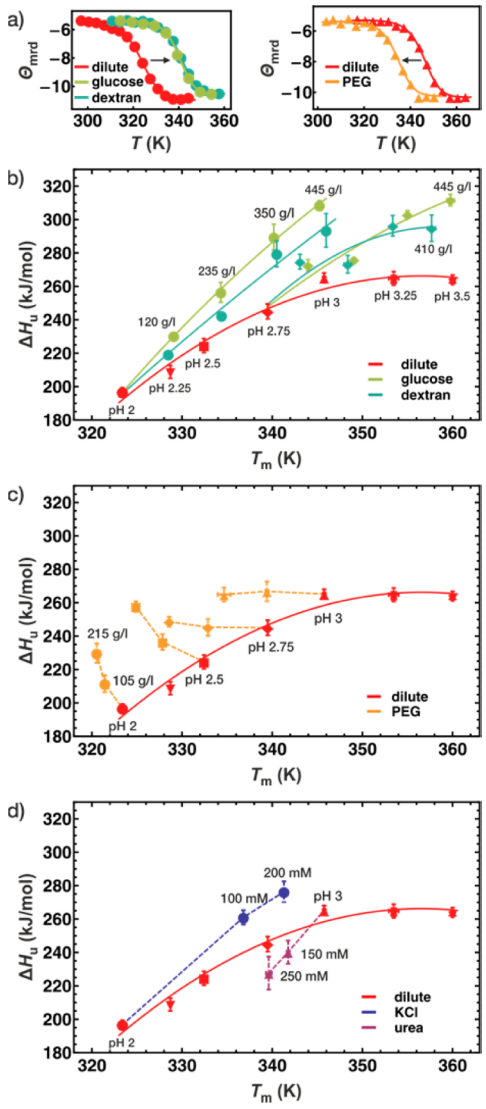


Figure 1. (a) CD-unfolding curves of ubiquitin in dilute and crowded solutions (glucose and dextran, 350 g/L, pH 2; PEG, 215 g/L, pH 3). (b–d) The van't Hoff enthalpy of unfolding ΔH_u is plotted versus the melting temperature T_m of ubiquitin in solutions of glucose, dextran, PEG, KCl, and urea in comparison to the dilute aqueous solution (red). Solid lines represent second-order polynomial fits to the measured data points, and dashed lines connect data points to guide the eye. Symbols represent measurements at different pH, and colors different cosolutes.

ubiquitin upon addition of PEG (Figure 1a). In analogy to glucose and dextran, we found an increase of the van't Hoff enthalpy in the presence of PEG in comparison to the dilute solution (Figure 1c). At pH 2 and 2.5, ΔH_u increases with decreasing T_m. In contrast to glucose and dextran, the cosolute effect of PEG on ubiquitin is found to be pH-dependent. With decreasing pH, we found a decrease of ΔT_m and an increase of ΔH_u. Similar to glucose and dextran, the stabilization of ubiquitin by KCl was accompanied by an increase of ΔH_u (Figure 1d). In contrast, urea destabilized ubiquitin and caused a reduction of ΔH_u (Figure 1d).

To evaluate the cosolute induced changes on ΔG_u, ΔH_u, and ΔS_u, we calculated the respective excess parameters $\Delta\Delta X_u$ using the assumption $\Delta C_{p,cos}(T) \approx \Delta C_{p,dilute}(T)$ (details described in the Materials and Methods).

Cosolute Induced Changes of the Free Energy of Ubiquitin Unfolding. Ubiquitin is stabilized in the presence of glucose, dextran, and KCl ($\Delta\Delta G_u > 0$) and destabilized in solutions of PEG and urea ($\Delta\Delta G_u < 0$) (Figure 2). We observed

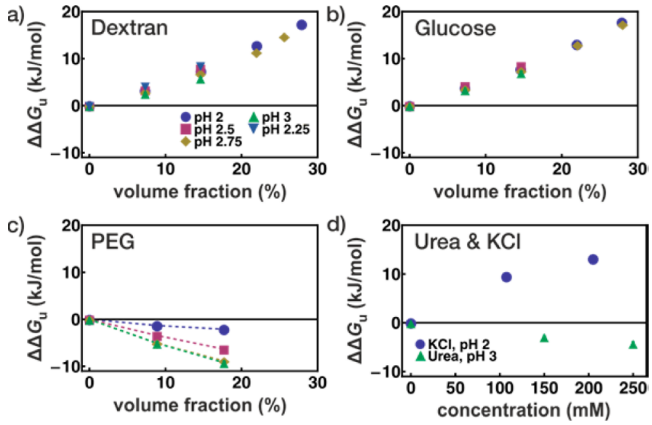


Figure 2. Excess free energy of ubiquitin unfolding $\Delta\Delta G_u$ ($\Delta\Delta G_u = \Delta G_{u,cos} - \Delta G_{u,dilute}$) as a function of the volume fraction or concentration for all tested cosolutes. Data points in (c) are connected by dashed lines to indicate the systematic pH trend, which was absent for glucose and dextran. Symbols in (b) and (c) have the same meaning as in (a). The temperatures (in K) to which $\Delta G_{u,cos}$ of ubiquitin was extrapolated are 323.38 ± 0.13 (pH 2), 328.70 ± 0.08 (pH 2.25), 332.42 ± 0.12 (pH 2.5), 339.50 ± 0.18 (pH 2.75), and 345.75 ± 0.54 (pH 3).

a linear volume fraction dependence of $\Delta\Delta G_u$ with deviations from linearity for glucose and dextran beyond a volume fraction of 20%. As mentioned above, the cosolute effect of PEG is pH-dependent. The higher the pH, the lower $\Delta\Delta G_u$ of ubiquitin in solutions of PEG is.

Cosolute Induced Changes of the Enthalpy of Ubiquitin Unfolding. We found an enthalpic stabilization of ubiquitin in the presence of glucose, dextran, PEG, and KCl ($\Delta\Delta H_u > 0$) and an enthalpic destabilization in the presence of urea ($\Delta\Delta H_u < 0$) (Figure 3). Similar to the concentration

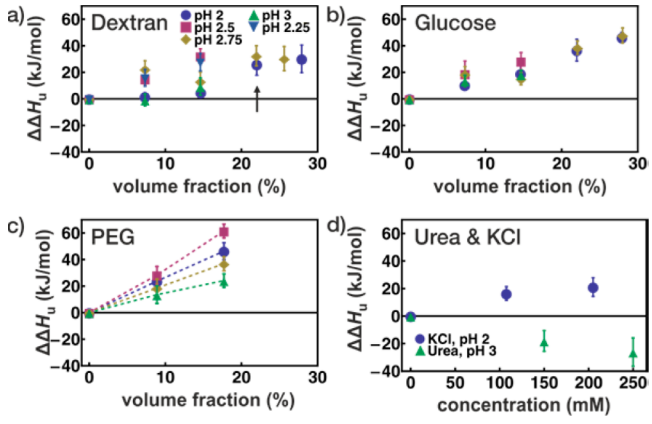


Figure 3. Excess enthalpy of ubiquitin unfolding $\Delta\Delta H_u$ ($\Delta\Delta H_u = \Delta H_{u,cos} - \Delta H_{u,dilute}$) as a function of the volume fraction or concentration for all tested cosolutes. At volume fractions of dextran of ~22%, marked by an arrow in (a), the excess enthalpy reaches a constant value. Data points in (c) are connected by dashed lines to indicate the systematic pH trend, which was not evident for glucose and dextran. Symbols in (b) and (c) have the same meaning as in (a). The temperatures to which $\Delta H_{u,cos}$ of ubiquitin was extrapolated correspond to the values listed in the caption of Figure 2.

dx.doi.org/10.1021/ja503205y | J. Am. Chem. Soc. 2014, 136, 9036–9041

dependence of the free energy, glucose, PEG, and urea cause a linear increase or decrease of $\Delta\Delta H_u$, while in case of dextran, the excess enthalpy reached a maximum around a volume fraction of 20%.

In addition, we observed the same systematic pH-dependence of $\Delta\Delta H_u$ in solutions of PEG as observed for $\Delta\Delta G_u$. The excess enthalpy of ubiquitin in PEG solutions at high pH (pH 2.75 and 3) is smaller than at low pH (pH 2 and 2.5). A systematic pH dependence could not be observed for glucose and dextran.

Cosolute Induced Changes of the Entropy of Ubiquitin Unfolding. Ubiquitin is entropically destabilized in solutions of glucose, dextran, PEG, and KCl ($\Delta\Delta S_u > 0$), and entropically stabilized by urea ($\Delta\Delta S_u < 0$) (Figure 4). While in solutions of glucose, $\Delta\Delta S_u$ increases over the entire concentration range, a maximum of $\Delta\Delta S_u$ can be observed in solutions of dextran. This maximum coincides with the onset of nonlinearity of $\Delta\Delta G_u$ (Figure 2) and the beginning of the plateau for $\Delta\Delta H_u$ (Figure 3).

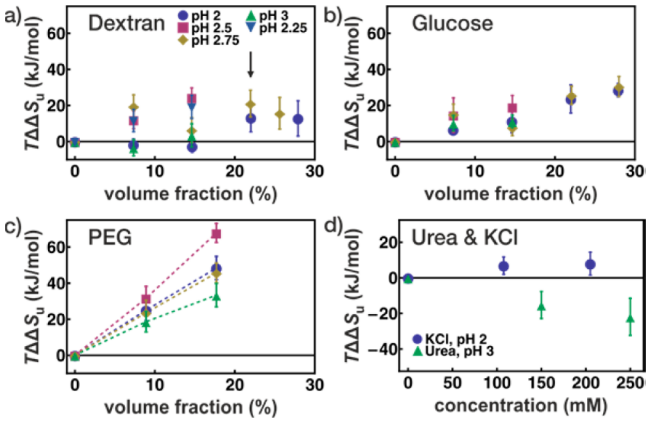


Figure 4. Excess entropy of ubiquitin unfolding $\Delta\Delta S_u$ ($\Delta\Delta S_u = \Delta S_{u,cos} - \Delta S_{u,dilute}$) as a function of the volume fraction or concentration for all tested cosolutes. The arrow in (a) marks the starting point of a decreasing excess entropy. Data points in (c) are connected by dashed lines to indicate the systematic pH trend, which was not observed for glucose and dextran. Symbols in (b) and (c) have the same meaning as in (a). The temperatures to which $\Delta S_{u,cos}$ of ubiquitin was extrapolated correspond to the values listed in the caption of Figure 2.

The pH-dependence of $\Delta\Delta G_u$ and $\Delta\Delta H_u$ in solutions of PEG is also reflected in the volume fraction dependence of $\Delta\Delta S_u$. However, the pH-dependent decrease of $\Delta\Delta S_u$ at pH 2.75 and pH 3 is less pronounced than the decrease of $\Delta\Delta H_u$.

DISCUSSION

Glucose and Dextran Stabilize Ubiquitin by an Enthalpic Solvent-Mediated Mechanism. The interpretation of enthalpic and entropic cosolute effects crucially depends on the knowledge of the temperature dependence of ΔC_p. In the following we compare the results of extrapolating enthalpy and entropy of ubiquitin in solutions of glucose and dextran by Kirchoff's laws (eq 2 and 3). Thereby, we use a temperature independent value for ΔC_p and a temperature dependent value for ΔC_p for the same extrapolation. The results for ubiquitin at pH 2.75 are compared in Figure 5.

When inserting the temperature independent ΔC_p (1.5 kcal/mol·K)[21,23] into Kirchhoff's laws, an entropic stabilization along with an enthalpic destabilization results which was previously interpreted as an excluded volume effect by Wang et al. and Zhou.[21,23] Both used the same constant ΔC_p value as input which was determined at temperatures below 50 °C (323 K).

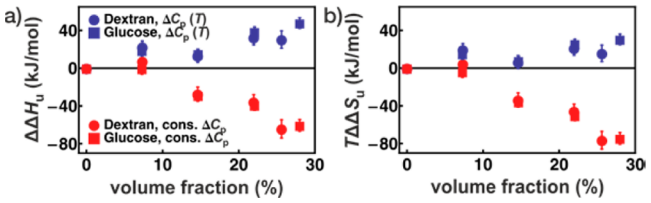


Figure 5. Comparison of $\Delta\Delta H_u$ and $\Delta\Delta S_u$ of ubiquitin in solutions of glucose and dextran at pH 2.75 calculated using the temperature dependent expression $\Delta C_{p,dilute}(T)$ and a temperature independent value for ΔC_p (1.5 kcal/mol·K).[21,23] Data at other pH values show the same trend and are omitted for clarity.

In contrast, our results show an enthalpic stabilization of ubiquitin in the presence of the branched polymer dextran and its corresponding monomeric osmolyte glucose by taking into account the temperature dependence of ΔC_p. The fact that the excess entropy $\Delta\Delta S_u$ (Figure 4) is positive over the entire concentration range for both crowders is contradictory to the expectation of a macromolecular crowding effect.[23] This rules out the excluded volume effect as the main stabilization mechanism. However, for high concentrations, a decrease in $\Delta\Delta S_u$, in line with the excluded volume theory, is observed for dextran which balances a further increase of $\Delta\Delta H_u$ (Figure 3). In summary, the stabilization of ubiquitin in the presence of dextran is enthalpic up to volume fractions of 28% (445 g/L) with an additional entropic contribution for high volume fractions (>20%). Nevertheless, $\Delta\Delta S_u$ remains positive, i.e., destabilizing in the entire concentration range because of the enthalpy–entropy compensation[40,41] as a consequence of an enthalpy-mediated stabilization.

To further elucidate the role of the macromolecular crowding effect, we analyzed the excess free energy $\Delta\Delta G_u$ as a function of the volume fraction of the crowders. The excluded volume theory predicts a power-law dependence of $\Delta\Delta G_u$ and T_m on the volume fraction and the crowder radius.[26−28] However, glucose and dextran, even though they have very different crowder radii, showed a quantitatively similar and linear volume fraction dependence of $\Delta\Delta G_u$ (0.58 vs 0.55 kJ/mol per % volume fraction, Figure 2) with small deviations from linearity beyond 20% volume fraction. This linear increase of $\Delta\Delta G_u$ is characteristic for protein stabilizers like osmolytes[9,42] but not expected for macromolecular crowders. The nonlinearity of $\Delta\Delta G_u$ obtained beyond 20% volume fraction of dextran indicates an excluded volume contribution. This also coincides with the reduction of $\Delta\Delta S_u$ and the maximum of $\Delta\Delta H_u$ at the same concentrations. The striking similarities between glucose and dextran on ubiquitin folding are in line with a previous study of Roberts and Jackson,[43] who measured similar Gibbs energy changes of ubiquitin in solutions of glucose and dextran at pH 7.4, as well as with a study of Waegele and Gai, who observed no dependence of ΔT_m on the polymer size of dextran at pH 1.[28]

The nearly identical enthalpic stabilization of ubiquitin in solutions of either glucose or dextran indicates that the chemical properties of dextran are mainly responsible for the induced stabilization rather than its polymeric composition or polymer size and thus its excluded volume effect. We therefore suggest that the enthalpic stabilization, a hallmark for osmolytes,[9,10] for glucose and dextran results from an osmolyte-like mechanism which is mediated by the solvent: Protein stabilization by osmolytes is believed to occur via an indirect mechanism as

dx.doi.org/10.1021/ja503205y | J. Am. Chem. Soc. 2014, 136, 9036−9041

osmolytes are preferentially excluded from the protein surface resulting in a preferentially hydrated protein.[8] Recent experimental and computational studies have supported the important role of solvation for an osmolyte-mediated stabilization of proteins.[11−15] On a molecular level, the solvent-mediated stabilization can be understood as an optimization of hydrogen bonds as a consequence of cosolute-induced distortions in the hydrogen bond network of the hydration water of the protein.[12]

PEG Is an Intermediate between a Stabilizing Osmolyte and a Chemical Denaturant. Similar to glucose and dextran, PEG also induced positive $\Delta\Delta H_u$ and $\Delta\Delta S_u$ values. However, compared to glucose and dextran the entropic contribution (Figure 4c) dominates the enthalpic contribution (Figure 3c) leading to an overall destabilization of ubiquitin (Figure 2c). A destabilizing effect of PEG has been previously observed for a class of proteins such as lysozyme, chymotrypsinogen, and β-lactoglobulin and has been attributed to direct protein-PEG interactions.[44,45] However, direct ubiquitin−PEG contacts alone cannot explain the observed positive enthalpy change $\Delta\Delta H_u$, since direct cosolute contacts should cause a decrease of ΔH_u rather than the experimentally observed increase.[21,23] We demonstrated this by determining ΔH_u of ubiquitin in the presence of urea, which is known to destabilize proteins by direct binding to the protein.[16,17] Indeed, we observed a destabilization of ubiquitin which was accompanied by a negative enthalpy change $\Delta\Delta H_u$ (Figure 3), in line with previous studies.[9,18]

We suggest that the positive enthalpy change $\Delta\Delta H_u$ of ubiquitin in solutions of PEG can be attributed to the preferential hydration model, similar to glucose and dextran, with a contribution of an enthalpic stabilization which is mediated by the solvent. However, compared to carbohydrates, PEG is more hydrophobic with less hydrogen-bonding capacities which increases its potential for unspecific attractive hydrophobic interactions with the protein. The fact that the cosolute effect of PEG is pH-dependent indicates that PEG has both a stabilizing enthalpic effect, likely mediated by the solvation water, as well as a destabilizing enthalpic effect due to attractive interactions with the protein. The lower the pH, the more positive charges are present at the protein surface decreasing PEG's capability to form hydrophobic contacts to the protein. At pH 2, ubiquitin is characterized by a high density of positive charges resulting in a minor destabilization by PEG (see $\Delta\Delta G_u$ in Figure 2) but a large increase in $\Delta\Delta H_u$ (Figure 3). When increasing the pH from pH 2 to pH 3, a drastic increase of ΔT_m was observed in the presence of PEG. The reduction of $\Delta\Delta H_u$ exceeds the decrease in $T\Delta\Delta S_u$, indicating a contribution of enthalpic destabilization which can be assigned to attractive protein-PEG interactions. We attribute the drastic increase in ΔT_m when increasing the pH from 2 to 3 to the breaking of ubiquitin's two salt bridges.[46,47] Due to this breaking, two additional positive charges are being exposed to the solvent.

For urea, it is was shown that it fits nearly perfectly into the water network.[48] This excludes distortions in the water hydrogen-bonding network as the origin of a solvent mediated enthalpic stabilization.[12] Furthermore, attractive urea-protein contacts might be of higher affinity compared to PEG-protein contacts leading to larger destabilizing contribution to ΔH_u and resulting in an overall negative excess enthalpy.

Contribution of an Enthalpic Solvent-Mediated Mechanism to the Stabilization of Ubiquitin by KCl. An enthalpic stabilization at low concentrations of KCl similar to glucose and dextran was observed at pH 2 (Figure 1). The T_m-shift is weakened at the isoelectric point of ubiquitin (pH 6.8)[49] (Figure SF3). Our experimental results are in line with a previous calorimetric study of ubiquitin in solutions of different salts at pH 2.[34] The authors assign the stabilizing enthalpic effect to a direct binding of the anions to the positively charged and exposed residues of ubiquitin leading to an increase of ΔH_u. The pH-dependency of the T_m-shift illustrates the role of electrostatic interactions in the KCl-induced stabilization of ubiquitin. The concentration dependent slope of the change in enthalpy ΔH_u with the melting temperature T_m of KCl resembles the slopes of glucose and dextran at pH 2 (Figure 1). This suggests a water mediated enthalpic contribution as an additional component for the stabilization of ubiquitin in KCl. Such a combination of both mechanisms was recently also used to analyze folding of a small peptide in the presence of various salts.[19]

CONCLUSION

In conclusion, we classify the observed cosolute effects according to the differences in the excess enthalpy $\Delta\Delta H_u$ with respect to changes of T_m (Figure 6). The addition of glucose, dextran and KCl increased both ΔH_u and T_m. For PEG solutions, we find a positive $\Delta\Delta H_u$ which is, however, accompanied by a decrease of T_m. Urea shows the opposite trend compared to glucose, dextran, and KCl as it decreases both, ΔH_u and T_m. Thus, PEG can be seen as an intermediate between a stabilizing osmolyte and a denaturant.

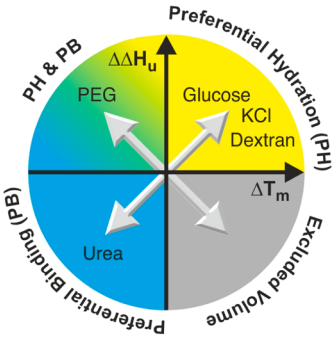


Figure 6. An enthalpic view to classify effects of cosolutes on the folding equilibrium of ubiquitin. Cosolute effects are classified into four categories (preferential hydration, excluded volume, preferential binding, preferential hydration and preferential binding) according to their effects on ΔH_u and T_m.

On the basis of our results, we introduce a new classification model for cosolute effects using four main categories: (i) Preferential hydration of the protein is a solvent-mediated enthalpic stabilization by an exclusion of the cosolute from the direct contact with the protein. (ii) Ideal excluded volume effects only lead to a decrease of ΔS_u, while ΔH_u remains unchanged. Real volume crowders can have a certain possibility for unspecific attractive interactions with the protein leading to a negative $\Delta\Delta H_u$. (iii) Preferential binding of a cosolute to a protein surface is characterized by a decrease of both, ΔH_u and T_m. (iv) A combination of preferential hydration of the protein and preferential binding of the cosolute to the protein results in a negative ΔT_m and a positive $\Delta\Delta H_u$. Cosolutes which belong to this class preferentially bind to the protein surface resulting in a decrease of ΔH_u and T_m. In addition, those solutes also induce a solvent-mediated enthalpic stabilization with a positive contribution to ΔH_u resulting in an overall positive excess enthalpy $\Delta\Delta H_u$.

dx.doi.org/10.1021/ja503205y | J. Am. Chem. Soc. 2014, 136, 9036−9041

Previous experimental studies of proteins in the presence of macromolecular crowders[24,25,44,50,51] and stabilizing osmolytes[9,10] can be interpreted within this framework. We could show that all cosolute effects can be rationalized by their enthalpic changes questioning the entropic-centered view in the field of macromolecular crowding. Future studies need to address how the complex mixture of cosolutes in the cell affects biochemical reactions.

■ ASSOCIATED CONTENT

Supporting Information

Description of data analysis for CD and DSC measurements. Numerical values of thermodynamic excess parameters calculated via a cosolute dependent and cosolute independent heat capacity change are given. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

Simon.Ebbinghaus@rub.de

Present Addresses

[†]Department of Biological Regulation, Weizmann Institute of Science, Rehovot 76100, Israel.

[‡]Department of Physical Chemistry I, Ruhr-Universität Bochum, 44780 Bochum, Germany.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Roland Winter for helpful discussions and Magdalena Groß for help with the CD experiments. We acknowledge funding from the Ministry of Innovation, Science and Research of the State of North Rhine-Westphalia (Rückkehrerprogramm) and the Cluster of Excellence RESOLV (EXC 1069) funded by the German Research Foundation (DFG). M.S. is supported by the Verband der Chemischen Industrie. B.B. acknowledges financial support from the Human Frontier Science Program (LT000336/2011).

■ REFERENCES

(1) Zimmerman, S. B.; Trach, S. O. *J. Mol. Biol.* **1991**, *222*, 599−620.
(2) Ellis, R. J. *Trends Biochem. Sci.* **2001**, *26*, 597−604.
(3) Minton, A. P. *Biopolymers* **1981**, *20*, 2093−2120.
(4) Zhou, H. X.; Rivas, G.; Minton, A. P. *Annu. Rev. Biophys.* **2008**, *37*, 375−397.
(5) Yancey, P. H.; Clark, M. E.; Hand, S. C.; Bowlus, R. D.; Somero, G. N. *Science* **1982**, *217*, 1214−1222.
(6) Somero, G. N. *Physiology* **1986**, *1*, 9−12.
(7) Lippert, K.; Galinski, E. A. *Appl. Microbiol. Biotechnol.* **1992**, *37*, 61−65.
(8) Timasheff, S. N. *Annu. Rev. Biophys. Biomol. Struct.* **1993**, *22*, 67−97.
(9) Attri, P.; Venkatesu, P.; Lee, M. J. *J. Phys. Chem. B* **2010**, *114*, 1471−1478.
(10) Politi, R.; Harries, D. *Chem. Commun. (Cambridge, U.K.)* **2010**, *46*, 6449−6451.
(11) Heyden, M.; Brundermann, E.; Heugen, U.; Niehues, G.; Leitner, D. M.; Havenith, M. *J. Am. Chem. Soc.* **2008**, *130*, 5773−5779.
(12) Gilman-Politi, R.; Harries, D. *J. Chem. Theory Comput.* **2011**, *7*, 3816−3828.
(13) Hunger, J.; Tielrooij, K. J.; Buchner, R.; Bonn, M.; Bakker, H. J. *J. Phys. Chem. B* **2012**, *116*, 4783−4795.
(14) Bruzdziak, P.; Panuszko, A.; Stangret, J. *J. Phys. Chem. B* **2013**, *117*, 11502−11508.
(15) Harada, R.; Tochio, N.; Kigawa, T.; Sugita, Y.; Feig, M. *J. Am. Chem. Soc.* **2013**, *135*, 3696−3701.
(16) Canchi, D. R.; Garcia, A. E. *Annu. Rev. Phys. Chem.* **2013**, *64*, 273−293.
(17) Moeser, B.; Horinek, D. *J. Phys. Chem. B* **2014**, *118*, 107−114.
(18) Makhatadze, G. I.; Privalov, P. L. *J. Mol. Biol.* **1992**, *226*, 491−505.
(19) Sukenik, S.; Sapir, L.; Gilman-Politi, R.; Harries, D. *Faraday Discuss.* **2013**, *160*, 225−237 discussion 311−227.
(20) Sukenik, S.; Sapir, L.; Harries, D. *Curr. Opin. Colloid Interface Sci.* **2013**, *18*, 495−501.
(21) Wang, Y.; Sarkar, M.; Smith, A. E.; Krois, A. S.; Pielak, G. J. *J. Am. Chem. Soc.* **2012**, *134*, 16614−16618.
(22) Minton, A. P. *Biopolymers* **2013**, *99*, 239−244.
(23) Zhou, H. X. *FEBS Lett.* **2013**, *587*, 394−397.
(24) Benton, L. A.; Smith, A. E.; Young, G. B.; Pielak, G. J. *Biochemistry* **2012**, *51*, 9773−9775.
(25) Sasahara, K.; McPhie, P.; Minton, A. P. *J. Mol. Biol.* **2003**, *326*, 1227−1237.
(26) Cheung, M. S.; Klimov, D.; Thirumalai, D. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 4753−4758.
(27) Sakaue, T.; Raphael, E. *Macromolecules* **2006**, *39*, 2621−2628.
(28) Waegele, M. M.; Gai, F. *J. Chem. Phys.* **2011**, *134*, 095104.
(29) Sasaki, Y.; Miyoshi, D.; Sugimoto, N. *Nucleic Acids Res.* **2007**, *35*, 4086−4093.
(30) Juckes, I. R. *Biochim. Biophys. Acta* **1971**, *229*, 535−546.
(31) McPherson, A., Jr. *J. Biol. Chem.* **1976**, *251*, 6300−6303.
(32) Colley, C. S.; Griffiths-Jones, S. R.; George, M. W.; Searle, M. S.; Clark, I. P. *Chem. Commun. (Cambridge, U.K.)* **2000**, 1493−1494.
(33) Wintrode, P. L.; Makhatadze, G. I.; Privalov, P. L. *Proteins* **1994**, *18*, 246−253.
(34) Makhatadze, G. I.; Lopez, M. M.; Richardson, J. M., 3rd; Thomas, S. T. *Protein Sci.* **1998**, *7*, 689−697.
(35) Wandrey, C.; Bartkowiak, A.; Hunkeler, D. *Langmuir* **1999**, *15*, 4062−4068.
(36) Greenfield, N. J. *Nat. Protoc.* **2006**, *1*, 2527−2535.
(37) Privalov, P. L. *Adv. Protein Chem.* **1979**, *33*, 167−241.
(38) Shortle, D.; Meeker, A. K.; Freire, E. *Biochemistry* **1988**, *27*, 4761−4768.
(39) Becktel, W. J.; Schellman, J. A. *Biopolymers* **1987**, *26*, 1859−1877.
(40) Liu, L.; Yang, C.; Guo, Q. X. *Biophys. Chem.* **2000**, *84*, 239−251.
(41) Chodera, J. D.; Mobley, D. L. *Annu. Rev. Biophys.* **2013**, *42*, 121−142.
(42) O'Brien, E. P.; Ziv, G.; Haran, G.; Brooks, B. R.; Thirumalai, D. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 13403−13408.
(43) Roberts, A.; Jackson, S. E. *Biophys. Chem.* **2007**, *128*, 140−149.
(44) Lee, L. L.; Lee, J. C. *Biochemistry* **1987**, *26*, 7813−7819.
(45) Crowley, P. B.; Brett, K.; Muldoon, J. *ChemBioChem* **2008**, *9*, 685−688.
(46) Vijay-Kumar, S.; Bugg, C. E.; Cook, W. J. *J. Mol. Biol.* **1987**, *194*, 531−544.
(47) Ibarra-Molero, B.; Loladze, V. V.; Makhatadze, G. I.; Sanchez-Ruiz, J. M. *Biochemistry* **1999**, *38*, 8138−8149.
(48) Funkner, S.; Havenith, M.; Schwaab, G. *J. Phys. Chem. B* **2012**, *116*, 13374−13380.
(49) Tofaris, G. K.; Razzaq, A.; Ghetti, B.; Lilley, K. S.; Spillantini, M. G. *J. Biol. Chem.* **2003**, *278*, 44405−44411.
(50) Mittal, S.; Singh, L. R. *PLoS One* **2013**, *8*, No. e78936.
(51) Erlkamp, M.; Grobelny, S.; Winter, R. *Phys. Chem. Chem. Phys.* **2014**, *16*, 5965−5976.