BIOCHEMISTRY
including biophysical chemistry & molecular biology

Rapid Report

pubs.acs.org/biochemistry

Unexpected Effects of Macromolecular Crowding on Protein Stability

Laura A. Benton,[†] Austin E. Smith,[†] Gregory B. Young,[‡] and Gary J. Pielak*[,†,‡,§]

[†]Department of Chemistry, [‡]Department of Biochemistry and Biophysics, and [§]Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599, United States

Supporting Information

ABSTRACT: Most theories about macromolecular crowding focus on two ideas: the macromolecular nature of the crowder and entropy. For proteins, the volume excluded by the crowder favors compact native states over expanded denatured states, enhancing protein stability by decreasing the entropy of unfolding. We tested these ideas with the widely used crowding agent Ficoll-70 and its monomer, sucrose. Contrary to expectations, Ficoll and sucrose have approximately the same stabilizing effect on chymotrypsin inhibitor 2. Furthermore, the stabilization is driven by enthalpy, not entropy. These results point to the need for carefully controlled studies and more sophisticated theories for understanding crowding effects.

The influence of concentrated solutions of macromolecules on protein stability is thought to arise from two phenomena: hard-core repulsion and chemical interactions. The repulsive interaction, which is always stabilizing, has been emphasized in crowding theories for more than 30 years.[1,2] Although a role for chemical interactions has been acknowledged in theories of crowding,[3] there are few examples of experiments aimed at assessing these interactions.[4−7] Because hard-core repulsions involve only the arrangement of molecules, they affect the entropic component of protein stability. Chemical interactions are expected to act enthalpically.

Unfortunately, information about the nature of macromolecular crowding effects is incomplete. First, there is limited evidence showing that the source of crowding effects resides in the macromolecular nature of the crowding molecule, because controls comparing the monomer to its macromolecular counterpart are rarely performed. Second, although several recent modeling and simulation studies consider chemical interactions,[8−10] experimentally derived information is limited. We addressed both limitations by using the cross-linked polymer Ficoll-70 and its monomer, sucrose, as cosolutes and nuclear magnetic resonance (NMR)-detected amide−proton exchange experiments[11,12] to quantify the stability of chymotrypsin inhibitor 2 (CI2) as a function of temperature.

Opening free energies ($\Delta G^{\circ\prime}_{op}$) were measured in dilute solution, at Ficoll concentrations of 100, 200, and 300 g/L, and in 200 g/L sucrose at 20, 25, 30, 37, and 45 °C. We used ^{1}H saturation transfer NMR experiments[13] to show that the cosolutes have an insignificant effect on the intrinsic rate of exchange and activation energy of exchange. The materials and methods used and the complete data sets are provided as Supporting Information.

As expected, Ficoll increased $\Delta G^{\circ\prime}_{op}$ at all temperatures compared to that in the dilute solution. The results are superimposed on the structure[14] in Figure 1. The Fersht

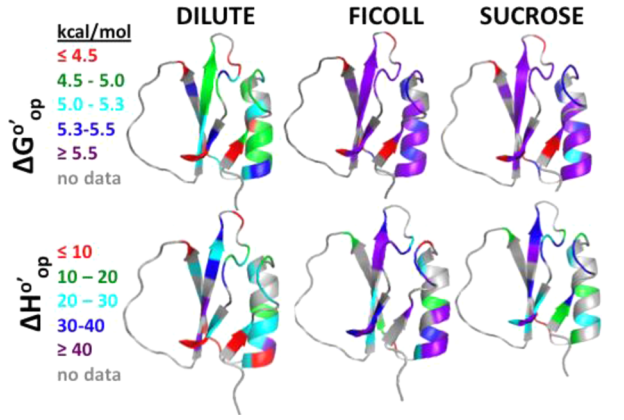

Figure 1. Equilibrium thermodynamic data superimposed on the structure of CI2 [50 mM sodium acetate (pH 5.4)]. $\Delta G^{\circ\prime}_{op}$ (37 °C) and $\Delta H^{\circ\prime}_{op}$ in dilute solution, 300 g/L Ficoll, and 200 g/L sucrose. It was not possible to obtain data for the extended loop, shown on the left-hand side of the structures, because the residues are always exposed to solvent and exchange too quickly.

laboratory identified residues whose amide protons exchange only on global unfolding.[15] For these residues $\Delta G^{\circ\prime}_{op}$ is equivalent to the denaturation free energy, $\Delta G^{\circ\prime}_{D}$. The averages and standard deviations of the $\Delta G^{\circ\prime}_{op}$ values for the observed globally unfolding residues at 37 °C are listed in Table 1.

The extent of Ficoll-induced stabilization increases with Ficoll concentration. The average $\Delta G^{\circ\prime}_{op}$ increases by 0.5 ± 0.1 and 0.9 ± 0.2 kcal/mol in 100 and 300 g/L Ficoll, respectively (Table 1). This stabilization is consistent with prior

Table 1. Average $\Delta G^{\circ\prime}_{op}$ Values at 37 °C and $\Delta H^{\circ\prime}_{op}$ Values in Kilocalories per Mole for Globally Exchanging Residues[a]

	dilute solution	100 g/L Ficoll	300 g/L Ficoll	200 g/L sucrose
$\Delta G^{\circ\prime}_{op}$	4.9 ± 0.1	5.4 ± 0.1	5.8 ± 0.2	5.4 ± 0.1
$\Delta H^{\circ\prime}_{op}$	26 ± 1	35 ± 3	42 ± 3	35 ± 5

[a]Uncertainties are standard deviations of the mean.

Received: July 7, 2012
Revised: November 19, 2012
Published: November 20, 2012

observations. For instance, Ficoll increases the $\Delta G^{\circ\prime}_{D}$ of creatine kinase[16] and FKBP[17] and the melting temperature (T_m) of apoflavodoxin,[18] cytochrome *c*,[19] apocalmodulin,[20] ubiquitin,[21] and phosphoglycerate kinase.[22] Dextran, a glucose-based polymer, increases the $\Delta G^{\circ\prime}_{D}$ of ubiquitin,[23] lysozyme,[4] FKBP,[17] glutaredoxin 2,[24] and the molten globule of cytochrome *c*[4] and the T_m of cytochrome *c*.[19] Likewise, $\Delta G^{\circ\prime}_{op}$ values for CI2 are increased by polyvinylpyrrolidone (PVP).[25,26]

Sucrose also stabilizes CI2 (Figure 1). The data in Table 1 indicate that the stabilizing effect of 200 g/L sucrose is approximately equivalent to that of 100 g/L Ficoll. Stabilization by sugars is well-known. For instance, sugars and other polyols increase the $\Delta G^{\circ\prime}_{D}$ of cytochrome *c*[27,28] and its molten globule,[29] ubiquitin,[23] lysozyme,[30] and α-chymotrypsin.[31] Although it is known that the monomer of PVP destabilizes CI2,[25] that both glucose and dextran stabilize ubiquitin,[23] and that sucrose has no effect on phosphoglycerate kinase,[22] this "monomer test" is not often performed.

The observation that sucrose and the sucrose-based polymer have similar effects on stability led us to question the idea that the Ficoll-induced stabilization is entirely due its macromolecular nature. To obtain more information, we measured the van't Hoff enthalpies of opening ($\Delta H^{\circ\prime}_{op}$) by plotting $\Delta G^{\circ\prime}_{op}/T$, where T is absolute temperature, for each residue against $1/T$ and assessing the slope. The resulting $\Delta H^{\circ\prime}_{op}$ data are superimposed onto the structure in Figure 1, and the averages for the globally unfolding residues are listed in Table 1.

Ficoll increases $\Delta H^{\circ\prime}_{op}$ in general (Figure 1) and for the globally unfolding residues (Table 1), and the increases are larger at higher Ficoll concentrations. A Ficoll-induced increase in the enthalpy of denaturation ($\Delta H^{\circ\prime}_{D}$) has also been reported for apocalmodulin,[20] but a decrease is observed for ubiquitin.[6] Because the increase in $\Delta H^{\circ\prime}_{op}$ reported in Table 1 is greater than the increase in $\Delta G^{\circ\prime}_{op}$, the Ficoll-induced stabilization of CI2 is entirely enthalpic, counter to theoretical expectations.

We then measured $\Delta H^{\circ\prime}_{op}$ in sucrose (Figure 1). The same effect is observed (Table 1); the average increase in $\Delta H^{\circ\prime}_{op}$ is larger than the increase in $\Delta G^{\circ\prime}_{op}$. These results are consistent with the observations that sucrose stabilizes α-chymotrypsin,[31] a β-hairpin peptide,[7] and lysozyme[30] by increasing $\Delta H^{\circ\prime}_{D}$.

Arg 46 and Arg 48 are exceptions in that the $\Delta H^{\circ\prime}_{op}$ values for these (non-globally unfolding) residues are large (<40 kcal/mol) and decrease steeply with increasing Ficoll concentration (Supporting Information). Both residues are involved in interactions with the extended loop: the amide proton of Arg 46 forms a salt bridge with Glu 41, and the amide proton of Arg 48 forms a hydrogen bond with Thr 39.[14] A likely explanation for the decrease in $\Delta H^{\circ\prime}_{op}$ is that crowding deforms the flexible loop, weakening these hydrogen bonds.

Importantly, for all other residues, $\Delta H^{\circ\prime}_{op}$ either increases or remains approximately unchanged with increasing Ficoll concentration. The average $\Delta H^{\circ\prime}_{op}$ for the global unfolding residues increases by 9 ± 4 and 16 ± 4 kcal/mol in 100 and 300 g/L Ficoll, respectively (Table 1). Sucrose has a similar effect.

Except for the increase in stability, our observations run counter to what is expected from simple ideas about macromolecular crowding. First, the monomer and its macromolecular counterpart have nearly the same stabilizing effect. Second, theory predicts that stabilization arises from a decrease in the entropy of unfolding, but the observed increase is entirely enthalpic; in fact, the entropic effect is destabilizing.

This enthalpy-driven stabilization could be rationalized if Ficoll or sucrose interacted strongly with the native state. The situation would be akin to ligand binding where complex formation pulls the equilibrium away from the denatured state. Three lines of evidence, however, indicate that the cosolutes do not form such complexes. First, significant (>0.02 ppm[26]) Ficoll-induced changes in the backbone chemical shift vector[32] are observed for only four positions; two (Glu 25 and Thr 39) are in the flexible loop, and one (Gln 22) has a side chain exposed to solvent (Supporting Information). Second, relaxation-based NMR experiments designed to expose weak interactions[33] indicate that Ficoll interacts weakly, if at all, with CI2.[34] Third, analysis of second virial coefficients indicates that sucrose interacts weakly with proteins.[35] The possibility that the crowding-induced stabilization arises because of denatured state compaction[36] cannot be assessed from our data.

An alternative explanation is that Ficoll and sucrose stabilize CI2 via preferential hydration.[37] As discussed by Bolen and coworkers,[38,39] the $\Delta G^{\circ\prime}$ for transferring the peptide bonds of a protein from water to a sugar solution is unfavorable. The stabilization induced by preferential hydration arises because the denatured state exposes more peptide bonds than the native state. Consistent with our data, the stability enhancement does not require the cosolute to be a macromolecule and occurs without strong interactions between the cosolute and the protein. In addition, the hallmark of preferential hydration is an increase in $\Delta H^{\circ\prime}_{D}$ that is partially offset by an increase in the entropy of unfolding,[5] which is what we observe. Nevertheless, this explanation is not entirely satisfying because preferential hydration can be rationalized in terms of the simple theory for macromolecular crowding.[28]

There is no doubt that hard-core repulsions play a role, because at 300 g/L, Ficoll[36,40] and sucrose[41] occupy approximately 20% of the volume. Our data indicate that other phenomena are also important, and that these phenomena can overwhelm the effects of hard-core repulsions. Although not all macromolecule cosolutes may act in a manner described here, our data show the need for refinements in explanations for the effects of macromolecular crowding. One source of refinements will probably involve enthalpy−entropy compensation.[42]

■ ASSOCIATED CONTENT

Supporting Information

Tables of thermodynamic parameters and intrinsic exchange rates and a figure with chemical shift changes. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Department of Chemistry, University of North Carolina, Chapel Hill, NC 27599. Phone: (919) 966-3671. E-mail: gary_pielak@unc.edu.

Funding

Supported by National Science Foundation Grant MCB-1051819.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Andrew Miklos for help in the early phase of the project and Elizabeth Pielak for comments on the manuscript.

REFERENCES

(1) Minton, A. P. (1981) Excluded volume as a determinant of macromolecular structure and reactivity. *Biopolymers 20*, 2093−2120.
(2) Minton, A. P., and Wilf, J. (1981) Effect of macromolecular crowding upon the structure and function of an enzyme: Glyceraldehyde-3-phosphate dehydrogenase. *Biochemistry 20*, 4821−4826.
(3) Zhou, H. X., Rivas, G., and Minton, A. P. (2008) Macromolecular crowding and confinement: Biochemical, biophysical, and potential physiological consequences. *Annu. Rev. Biophys. 37*, 375−397.
(4) Sasahara, K., McPhie, P., and Minton, A. P. (2003) Effect of dextran on protein stability and conformation attributed to macromolecular crowding. *J. Mol. Biol. 326*, 1227−1237.
(5) Politi, R., and Harries, D. (2010) Enthalpically driven peptide stabilization by protective osmolytes. *Chem. Commun. 46*, 6449−6451.
(6) Wang, Y., Sarkar, M., Smith, A. E., Krois, A. S., and Pielak, G. J. (2012) Macromolecular crowding and protein stability. *J. Am. Chem. Soc. 134*, 16614−16618.
(7) Sukenik, S., Sapir, L., Politi, R., and Harries, D. (2013) Diversity in the mechanisms of cosolute action on biomolecular processes. *Faraday Discuss.*,.
(8) Cheung, M. S., and Thirumalai, D. (2006) Nanopore-protein interactions dramatically alter stability and yield of the native state in restricted spaces. *J. Mol. Biol. 357*, 632−643.
(9) McGuffee, S. R., and Elcock, A. H. (2010) Diffusion, crowding & protein stability in a dynamic molecular model of the macterial cytoplasm. *PLoS Comput. Biol. 6*, e1000694.
(10) Feig, M., and Sugita, Y. (2012) Variable interactions between protein crowders and biomolecular solutes are important in understanding cellular crowding. *J. Phys. Chem. 116*, 599−605.
(11) Englander, S. W., and Kallenbach, N. R. (1983) Hydrogen exchange and structural dynamics of proteins and nucleic acids. *Q. Rev. Biophys. 16*, 521−655.
(12) Miklos, A. C., Li, C., and Pielak, G. J. (2009) Using NMR-detected backbone amide ^{1}H exchange to assess macromolecular crowding effects on globular-protein stability. *Methods Enzymol. 466*, 1−18.
(13) Wang, A., Robertson, A. D., and Bolen, D. W. (1995) Effects of a naturally occurring compatible osmolyte on the internal dynamics of ribonuclease A. *Biochemistry 34*, 15096−15104.
(14) McPhalen, C. A., and James, M. N. G. (1987) Crystal and molecular structure of the serine proteinase inhibitor CI-2 from barley seeds. *Biochemistry 26*, 261−269.
(15) Itzhaki, L. S., Neiva, J., and Fersht, A. (1997) Amide proton exchange in CI2 probed by denaturants and temperature. *J. Mol. Biol. 270*, 89−98.
(16) Wang, Y., He, H., and Li, S. (2010) Effect of Ficoll 70 on thermal stability and structure of creatine kinase. *Biochemistry (Moscow, Russ. Fed.) 75*, 648−654.
(17) Batra, J., Xu, K., and Zhou, H.-X. (2009) Nonadditive effects of mixed crowding on protein stability. *Proteins: Struct., Funct., Bioinf. 77*, 133−138.
(18) Stagg, L., Zhang, S.-Q., Cheung, M. S., and Wittung-Stafshede, P. (2007) Molecular crowding enhances native structure and stability of α/β protein flavodoxin. *Proc. Natl. Acad. Sci. U.S.A. 104*, 18976−18981.
(19) Christiansen, A., Wang, Q., Samiotakis, A., Cheung, M. S., and Wittung-Stafshede, P. (2010) Factors defining effects of macromolecular crowding on protein stability: An in vitro/in silico case study using cytochrome *c*. *Biochemistry 49*, 6519−6530.
(20) Wang, Q., Liang, K.-C., Czader, A., Waxham, M. N., and Cheung, M. S. (2011) The effect of macromolecular crowding, ionic strength and calcium binding on calmodulin dynamics. *PLoS Comput. Biol. 7*, e1002114.
(21) Waegele, M. M., and Gai, F. (2011) Power-law dependence of the melting temperature of ubiquitin on the volume fraction of macromolecular crowders. *J. Chem. Phys. 134*, 095104.
(22) Dhar, A., Samiotakis, A., Ebbinghaus, S., Nienhaus, L., Homouz, D., Gruebele, M., and Cheung, M. S. (2010) Structure, function, and folding of phosphoglycerate kinase are strongly perturbed by macromolecular crowding. *Proc. Natl. Acad. Sci. U.S.A. 107*, 17586−17591.
(23) Roberts, A., and Jackson, S. E. (2007) Destabilised mutants of ubiquitin gain equal stability in crowded solutions. *Biophys. Chem. 128*, 140−149.
(24) Kuhnert, D. C., Gildenhuys, S., and Dirr, H. W. (2008) Effect of macromolecular crowding on the stability of monomeric glutaredoxin 2 and dimeric glutathione transferase A1-1. *S. Afr. J. Sci. 104*, 76−80.
(25) Charlton, L. M., Barnes, C. O., Li, C., Orans, J., Young, G. B., and Pielak, G. J. (2008) Residue-level interrogation of macromolecular crowding effects on protein stability. *J. Am. Chem. Soc. 130*, 6826−6830.
(26) Miklos, A. C., Li, C., Sharaf, N. G., and Pielak, G. J. (2010) Volume exclusion and soft interaction effects on protein stability under crowded conditions. *Biochemistry 49*, 6984−6991.
(27) Saunders, A. J., Davis-Searles, P. R., Allen, D. L., Pielak, G. J., and Erie, D. A. (2000) Osmolyte-induced changes in protein conformational equilibria. *Biopolymers 53*, 293−307.
(28) Davis-Searles, P. R., Saunders, A. J., Erie, D. A., Winzor, D. J., and Pielak, G. J. (2001) Interpreting the effects of small uncharged solutes on protein-folding equilibria. *Annu. Rev. Biophys. Biomol. Struct. 30*, 271−306.
(29) Davis-Searles, P. R., Morar, A. S., Saunders, A. J., Erie, D. A., and Pielak, G. J. (1998) Sugar-induced molten-globule model. *Biochemistry 37*, 17048−17053.
(30) Gekko, K. (1982) Calorimetric study on thermal denaturation of lysozyme in polyol-water mixtures. *J. Biochem. 91*, 1197−1204.
(31) Attri, P., Venkatesu, P., and Lee, M.-J. (2010) Influence of osmolytes and denaturants on the structure and enzyme activity of α-chymotrypsin. *J. Phys. Chem. B 114*, 1471−1478.
(32) Davison, T. S., Nie, X., Ma, W., Lin, Y., Kay, C., Benchimol, S., and Arrowsmith, C. H. (2001) Structure and functionality of a designed p53 dimer. *J. Mol. Biol. 307*, 605−617.
(33) Li, C., and Pielak, G. J. (2009) Using NMR to distinguish viscosity effects from nonspecific protein binding under crowded conditions. *J. Am. Chem. Soc. 131*, 1368−1369.
(34) Wang, Y., Li, C., and Pielak, G. J. (2010) Effects of proteins on protein diffusion. *J. Am. Chem. Soc. 132*, 9392−9397.
(35) Weatherly, G. T., and Pielak, G. J. (2001) Second virial coefficients as a measure of protein−osmolyte interactions. *Protein Sci. 10*, 12−16.
(36) Hong, J., and Gierasch, L. M. (2010) Macromolecular crowding remodels the energy landscape of a protein by favoring a more compact unfolded state. *J. Am. Chem. Soc. 132*, 10445−10452.
(37) Timasheff, S. N. (1993) The control of protein stability and association by weak interactions with water: How do solvents affect these processes? *Annu. Rev. Biophys. Biomol. Struct. 22*, 67−97.
(38) Street, T. O., Bolen, D. W., and Rose, G. D. (2006) A molecular mechanism for osmolyte-induced protein stability. *Proc. Natl. Acad. Sci. U.S.A. 103*, 13997−14002.
(39) Auton, M., Rösgen, J., Sinev, M., Holthauzen, L. M. F., and Bolen, D. W. (2011) Osmolyte effects on protein stability and solubility: A balancing act between backbone and side-chains. *Biophys. Chem. 159*, 90−99.
(40) Lavrenko, P. N., Mikriukova, O. I., and Okatova, O. V. (1987) On the separation ability of various Ficoll gradient solutions in zonal centrifugation. *Anal. Biochem. 166*, 287−297.
(41) Lee, J. C., and Timasheff, S. N. (1981) The stabilization of proteins by sucrose. *J. Biol. Chem. 256*, 7193−7201.
(42) Lumry, R., and Rajender, S. (1970) Enthalpy-entropy compensation phenomena in water solutions of proteins and small molecules: A ubiquitous property of water. *Biopolymers 9*, 1557−1559.