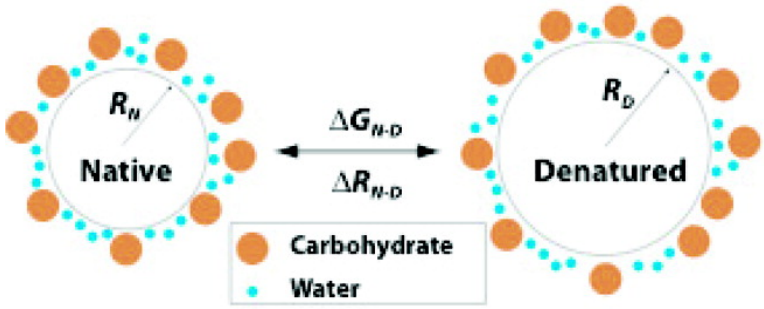
Communication

Simultaneous Determination of Structural and Thermodynamic Effects of Carbohydrate Solutes on the Thermal Stability of Ribonuclease A

Thomas F. O'Connor, Pablo G. Debenedetti, and Jeffrey D. Carbeck

J. Am. Chem. Soc., **2004**, 126 (38), 11794-11795• DOI: 10.1021/ja0481777 • Publication Date (Web): 04 September 2004



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 1 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
COMMUNICATIONS

Published on Web 09/04/2004

Simultaneous Determination of Structural and Thermodynamic Effects of Carbohydrate Solutes on the Thermal Stability of Ribonuclease A

Thomas F. O'Connor, Pablo G. Debenedetti, and Jeffrey D. Carbeck*

Department of Chemical Engineering, Princeton University, Princeton, New Jersey 08544

Received March 30, 2004; E-mail: jcarbeck@princeton.edu

The production, processing, and utilization of proteins, in nature and in biotechnology, occur under nonideal solution conditions. Nature uses high concentrations of organic molecules to protect organisms from dehydration.[1] In vivo, biomolecular interactions occur in crowded intracellular environments.[2,3] In vitro, the addition of high concentrations of sugars increases the stability of the native state of proteins over denatured states.[4] This enhancement of stability typically increases with concentration and molecular weight of the sugar. The mechanisms by which sugars increase stability are not completely understood. This communication describes a study of the effects of fructose and sucrose on the thermal stability of ribonuclease A (RNase) using capillary electrophoresis (CE) and protein charge ladders, collections of proteins that differ incrementally in number of chemically modified charged groups.[5] Because this approach provides information on both the thermodynamics (i.e., the free energy, ΔG_{N-D}, of denaturation) and structural changes (i.e., the effective hydrodynamic radius, R_H, of proteins in both the native and denatured states) associated with stability, it allows a simple microscopic interpretation of the effects of sugars on the stability of RNase.

We used CE to measure ΔG_{N-D} of RNase at 25 °C and pH 8.4 in the presence of different concentrations of sucrose and fructose.[6−8] Specifically, the fraction of protein in the native and denatured states was determined as a function of temperature by measuring shifts in electrophoretic mobility. We used a two-state model of denaturation, a reasonable approximation for RNase,[9] to determine ΔG_{N-D}. Values of ΔG_{N-D} and melting temperature, T_m, are shown in Table 1.

Values of T_m increase modestly in the presence of the sugars: 4.5 °C for the addition of 0.8 M sucrose and 5.4 °C for the addition of 1.52 M fructose. Effects of sugar on values of ΔG_{N-D} are significant. At the largest concentrations of sucrose (0.8 M) and fructose (1.52 M), ΔG_{N-D} is 3 kcal/mol greater than in the absence of sugar, an increase of ∼36%.

To further quantify the effects of sugars on stability, we calculated values of $\Delta\Delta G$ by subtracting values of ΔG_{N-D} measured in buffer from values obtained in the presence of sugar; values of $\Delta\Delta G$ are plotted as a function of molar concentration in Figure 1. The results are qualitatively consistent with previous studies:[4] (i) values of $\Delta\Delta G$ increase approximately linearly with concentration of sugar; (ii) the larger sugar, sucrose, has a greater stabilizing effect on RNase than fructose at a given molar concentration. This second effect can be quantified by the gradient of ΔG_{N-D} with concentration of sugar, which yields values of 3.6 kcal/mol per mole of sucrose and 2.0 kcal/mol per mole of fructose.

We used the combination of CE and charge ladders to measure values of R_H of RNase in the native and denatured states.[7] Values of electrophoretic mobility of the rungs of the charge ladder were fit to Henry's model of electrophoresis. The results are presented in Table 1. The value of R_H for the native state in the absence of sugars was confirmed independently using pulse-field gradient NMR.[10] Thermal denaturation results in a change in R_H of ∼6 Å, independent of the concentration of sugars.

To rationalize the effects of sugars on the stability of RNase we used the simplest possible model of the free energy of solvation of proteins: contributions of sugars to the enthalpy of solvation are ignored; only effects of entropy are considered. This situation is described by scaled particle theory (SPT),[11] where the solution is modeled as a mixture of hard spheres of different size.

Values of $\Delta\Delta G$ were calculated using SPT (solid lines in Figure 1). SPT predicts the free energy of solvation of the protein in terms of the work of forming a cavity in the solution large enough to

Table 1. Effects of Carbohydrates on the Stability and Hydrodynamic Radius of RNase[a]

	T_m (°C)	ΔG_{N-D} (kcal/mol)	$R_{H,N}$ (Å)	$R_{H,D}$ (Å)
buffer	62.6 (0.1)	8.2 (0.1)	21.2 (0.3)	27.5 (0.3)
0.15 M sucrose	63.6 (0.1)	9.1 (0.2)		
0.31 M sucrose	64.7 (0.2)	9.5 (0.2)	21.9 (0.3)	28.2 (0.3)
0.47 M sucrose	65.9 (0.1)	10.2 (0.2)		
0.63 M sucrose	66.5 (0.2)	10.6 (0.2)	22.2 (0.3)	28.0 (0.3)
0.80 M sucrose	67.1 (0.2)	11.1 (0.2)		
0.29 M fructose	63.9 (0.1)	9.1 (0.2)		
0.57 M fructose	64.5 (0.2)	9.5 (0.2)	21.7 (0.3)	28.0 (0.3)
0.89M fructose	66.0 (0.2)	10.1 (0.2)		
1.21 M fructose	66.5 (0.1)	10.5 (0.2)	22.0 (0.3)	28.0 (0.3)
1.52 M fructose	68.0 (0.2)	11.2 (0.2)		

[a] T_m is the melting temperature, ΔG_{N-D} is the free energy of denaturation of RNase at 25 °C, $R_{H,N}$ is the hydrodynamic radius of the protein in the native state and $R_{H,D}$ in the denatured state. Values in parentheses are estimates of standard error of the data.

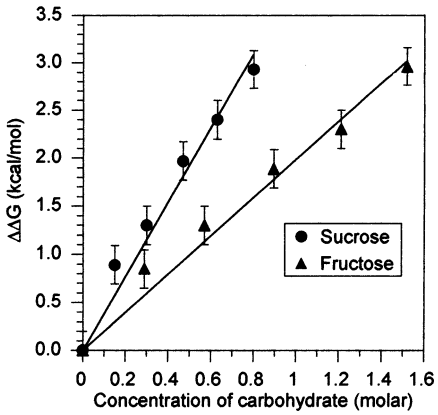


Figure 1. Values of $\Delta\Delta G$, the difference in ΔG_{N-D} measured in buffer and in the presence of sugars, for RNase at 25 °C as a function of molar concentration of sugars. Lines are predictions using scaled particle theory with radii of 1.38 Å for water, 3.25 Å for fructose, and 4.30 Å for sucrose. R_H values of 21 Å for native RNase and 28 Å for denatured RNase were used for the hard sphere radii of the protein. All experiments were conducted at pH 8.4 in 25 mM Tris, 192 mM Gly, and 30 mM NaCl.

* To whom correspondence should be addressed.

10.1021/ja0481777 CCC: $27.50

accommodate the protein. The reversible work of cavity formation, w (kcal/mol), is expressed as a third-order polynomial in the radius of the cavity, R (eq 1), where the coefficients A, B, and C depend on the size and concentration of sugars;[12] P is pressure.

$$w = A + BR + CR^2 + P4/3\pi R^3 \quad (1)$$

We used eq 1 to calculate the excess work of cavity formation, w_{exs}: the difference in w for RNase in water and the sugar solution. Because P is constant, the term proportional to R^3 cancels. We calculated $\Delta\Delta G$ as the difference of w_{exs} for the protein in the native and denatured states. In this way, SPT predicts the change in stability of RNase due to the contributions the sugars make to the entropy of solvation of the protein in the native and denatured states.

We used a version of SPT that treats water explicitly.[13] This approach requires values of hard sphere radii for water, sugars, and RNase in both the native and denatured states. In previous studies, values of radii for proteins were estimated from solvent-accessible surface areas (SASAs).[4,14] Radii of native proteins were calculated from SASAs estimated from crystal structures; radii of denatured proteins were calculated by assuming SASAs twice those of the native proteins. In contrast, we used measured values of R_H of RNase in the native and denatured states as estimates of the hard sphere radii. We used 1.38 Å for water's hard sphere radius.[14] Sugar radii were adjusted to fit the experimental data. Doing so yielded values of 4.3 Å for sucrose and 3.25 Å for fructose, in good agreement with values reported previously[15] of 3.9−4.5 Å for sucrose and 3.2−3.9 Å for glucose, a monosaccharide similar in size to fructose.

Clearly, neither water nor the sugars are realistically spheres with no enthalpic interactions with the protein. Nevertheless, as shown in Figure 1, this simple model is sufficient to describe quantitatively the effects of these sugars on the stability of RNase. There is no need, in this case, to refer to quasichemical concepts such as the exchange of solutes with water at specific sites on the protein.[16]

Physical insight can be gained by examining the relative contributions of the different terms in eq 1. This analysis shows that the R^2 term contributes 90−95% of the total value of w_{exs}. This result is consistent with previous observations that values of w_{exs} are approximately proportional to the area of the protein−solvent interface.[17,18] Sucrose has a greater impact on ΔG_{N-D} than fructose at the same molar concentration. According to SPT, this difference reflects the fact that sucrose is larger than fructose and thereby makes a greater contribution to the (unfavorable) entropy of interface formation at a given molar concentration.

The effect of the disaccharide sucrose on the stability of RNase that we measured ($\Delta\Delta G$ of 3.6 kcal/mol per mole of sugar) is almost twice as large as the value reported previously for the disaccharide trehalose ($\Delta\Delta G$ of 1.9 kcal/mol per mole of sugar).[17] We used SPT to rationalize these differences. The two sugars are the same size; according to SPT, they should have the same impact on the stability of RNase. The difference can be accounted for entirely by differences in the radius of RNase under different solution conditions. The trehalose measurements were done under acidic conditions, where the radius of RNase has been measured as 19.5 Å in the native state and 23.5 Å in the denatured state.[9] Using these values of radii and 4.3 Å for the radius of trehalose, SPT predicts the measured value of $\Delta\Delta G$ of 1.9 kcal/mol per mol of trehalose.

From these results we conclude that the effects of sugars on the stability of RNase are primarily the result of an increase in the free energy of creating the protein−solvent interface; in SPT, this effect is described entirely by an increase in the entropy of interface formation in the presence of sugars, relative to pure water. The impact of sugars on protein stability is strongly dependent on the size and concentration of the sugar and on the size (conformation) of proteins in both the native and denatured states; the conformation of proteins can change significantly with the conditions of the solution (pH, temperature, and ionic strength). Although these sugars have a significant impact on the stability of RNase, they have little effect on the hydrodynamic size, and presumably the conformation, of RNase in either native or denatured states. CE, when combined with protein charge ladders, yields in a single set of experiments both the thermodynamic and structural information that is essential for this microscopic interpretation of the effects of sugars on protein stability. Understanding the role of solutes on protein stability will have an impact on the selection of sugars and other additives for the formulation of protein pharmaceuticals and biocatalysts for improved stability and shelf life; it will also contribute to our understanding of protein stability in vivo.

Acknowledgment. The authors thank the National Science Foundation (Contract CTS-0134429) and the Camille and Henry Dreyfus Foundation for financial support of this work.

Supporting Information Available: Thermodynamic analysis of electrophoretic mobility data and details of the scaled particle theory. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Crowe, J. H.; Crowe, L. M. *Nat. Biotechnol.* **2000**, *18*, 145−146.
(2) Zimmerman, S. B.; Trach, S. O. *J. Mol Biol.* **1991**, *222*, 599−620.
(3) Ellis, R. J.; Minton, A. P. *Nature* **2003**, *425*, 27−28.
(4) 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.
(5) Carbeck, J. D.; Colton, I. J.; Gao, J. M.; Whitesides, G. M. *Acc. Chem. Res.* **1998**, *31*, 343−350.
(6) Hilser, V. J.; Freire, E. *Anal. Biochem.* **1995**, *224*, 465−485.
(7) Negin, R. S.; Carbeck, J. D. *J. Am. Chem. Soc.* **2002**, *124*, 2911−2916.
(8) Separations were performed on a 47 cm capillary (40 cm from inlet to detector; i.d. 50 μm) coated with polydiallydimethylammonium chloride. Detection was by direct UV absorbance at 214 nm. The concentration of RNase A (Sigma) in all experiments was 30 μM.
(9) Corbett, R. J.; Roche, R. S. *Biochemistry* **1984**, *23*, 1888−1894.
(10) Wilkins, D. K.; Grimshaw, S. B.; Receveur, V.; Dobson, C. M.; Jones, J. A.; Smith, L. J. *Biochem.* **1999**, *38*, 16424−16431.
(11) Reiss, H.; Frisch, H. L.; Helfland, E.; Lebowitz, J. L. *J Am. Chem. Soc.* **1960**, *32*, 119−124.
(12) These coefficients are defined in Supporting Information.
(13) Berg, O. C. *Biopolymers* **1990**, *30*, 1027−1037.
(14) Saunders, A. J.; Davis-Searles, P. R.; Allen, D. L.; Pielak, G. J.; Erie, D. A. *Bioploymers* **2000**, *53*, 293−307.
(15) Tang, K. E. S.; Bloomfield, V. A. *Biophys. J.* **2000**, *79*, 2222−2234.
(16) Schellman, J. A., *Biophys. Chem.* **1990**, 37, 121−140.
(17) Lee, J. C.; Timasheff, S. N. *J. Biol. Chem.* **1981**, *256*, 7193−7201.
(18) Baynes, B. M.; Trout, B. L. *J Phys. Chem. B.* **2003**, *107*, 14058−14067.

JA0481777