Power-law dependence of the melting temperature of ubiquitin on the volume fraction of macromolecular crowders

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The dependence of the melting temperature increase (ΔT_m) of the protein ubiquitin on the volume fraction (φ) of several commonly used macromolecular crowding agents (dextran 6, 40, and 70 and ficoll 70) was quantitatively examined and compared to a recently developed theoretical crowding model, i.e., $\Delta T_m \sim (R_g/R_c)^\alpha \varphi^{\alpha/3}$. We found that in the current case this model correctly predicts the power-law dependence of ΔT_m on φ but significantly overestimates the role of the size (i.e., R_c) of the crowding agent. In addition, we found that for ubiquitin the exponent α is in the range of 4.1–6.5, suggesting that the relation of $\alpha = 3/(3\nu - 1)$ is a better choice for estimating α based on the Flory coefficient (ν) of the polypeptide chain. Taken together these findings highlight the importance of improving our knowledge and theoretical treatment of the microcompartmentalization of the commonly used model crowding agents. © 2011 American Institute of Physics. [doi:10.1063/1.3556671]

I. INTRODUCTION

Cellular compartments contain high concentrations of macromolecules (80–400 g/L), which are expected to affect the thermodynamics and kinetics of many biological processes that take place in such crowded environments.^{1–3} Thus, many *in vitro* experiments^{4–21} and computer simulations^{10,14,15,21–29} have been carried out to determine, for example, the influence of macromolecular crowding on the energy landscape of protein folding and protein–protein interactions. Additionally, several models have been developed, aiming to provide a theoretical basis for understanding the effect of macromolecular crowding on various biological events.^{1,23,30–35} In the case of protein folding, while the existing experimental results are consistent with the theoretical prediction that an inert macromolecular crowder will destabilize the unfolded state of proteins via a repulsive depletion force arising from the volume occupied by the macromolecular cosolutes,^{1,23,30–33,35} quantitative comparisons of the experimentally observed and theoretically predicted crowding effects are hardly made.³⁶ A rare exception is the recent work of Zhou and coworkers,^{14,15} which showed that in some cases their theoretical model is able to provide a quantitative fit to the experimental results.¹⁴ Thus, there is a need for further experimental validation of the existing crowding models.^{23,30–35}

The scaling laws of polymer physics^{37,38} predict that the thermal melting temperature (T_m) of a protein that is subject to confinement exhibits a simple power-law dependence on the size of the confining space, i.e., $\Delta T_m \sim (R_g/R)^\alpha$; where α is a constant, R_g is the radius of gyration of the unfolded protein in the absence of confinement, and R is a characteristic length of the confining cavity. Indeed, recent computer simulations have shown that such a power-law dependence exists for proteins confined in spherical^{39,40} or cylindrical cavities.^{40–42} Based on this model, Cheung and Thirumalai²³

further proposed that the effect of macromolecular crowding on a protein's T_m could be quantified by evaluating the underlying confinement effect, which was shown to be a function of the radius (R_c) and volume fraction (φ) of the crowder. For $0 < \varphi < 0.1$, they proposed that the confinement effect is equivalent to that arising from a spherical pore of radius $R = (4\pi/3\varphi)^{1/3} R_c$, namely:

$$\Delta T_m \sim \left(\frac{R_g}{R_c} \right)^\alpha \varphi^{\alpha/3}. \quad (1)$$

While it has been shown in previous studies that T_m increases nonlinearly with φ ,^{6,15} there is no rigorous experimental test of Eq. (1). In addition, there is some ambiguity about how to relate the exponent α in Eq. (1) to the Flory coefficient (ν) of a volume-excluded random coil polymer chain ($\nu \approx 0.6$).^{37,38} For example, in the case of confining a polymer to a spherical cavity, theory predicts $\alpha = 3/(3\nu - 1)$; whereas in the case of confinement to slits or capillaries, it is found $\alpha = 1/\nu$.³⁸ Though the latter expression was adopted in a theoretical analysis of simulations of proteins in crowded environments,^{23,26} it is not clear whether such choice is also applicable to other crowding systems. Furthermore, we note that Eq. (1) was tested via simulations only for a special case where $R_c = R_g$; its application to more complex scenarios could be complicated by other factors such as protein–protein association^{23,43} and/or entropically driven phase separation.^{23,44}

Herein, we aim to test both the validity of Eq. (1) at physiologically relevant volume fractions of various crowding agents and the appropriate choice of the exponent α . This is accomplished by examining how the thermal melting temperature of ubiquitin⁴⁵ varies with φ using model crowding agents of different chemical structures and molecular weights, i.e., dextran 6 (6 kDa), dextran 40 (40 kDa), dextran 70 (68 kDa), and ficoll 70 (68 kDa). Ubiquitin is a 76-residue, single domain protein consisting of both α and β secondary structures, whose folding/unfolding thermodynamics in

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dilute solution have been well characterized.^{46–51} We find that the ΔT_m of ubiquitin exhibits a power-law dependence on the volume fraction of these crowders as predicted by Eq. (1) and that setting $\alpha = 3/(3\nu - 1)$ results in a reasonable prediction of how ΔT_m changes with ϕ . Interestingly, we find that the increase in the thermal melting temperature of ubiquitin is independent of the molecular weight of the dextrans used in the current study, which is surprising as the hydrodynamic radii of these dextran molecules are significantly different. Thus, taken together these results suggest that the microcompartmentalization of such polymer solutions is different from that created by hard spheres with a radius equivalent to the hydrodynamic radius of the crowder molecule.

II. MATERIALS AND METHODS

A. Materials

All materials were used as received. D₂O (D, 99.96%) and deuterium chloride (D, 99.5%) were purchased from Cambridge Isotope Laboratories (Andover, MA), 6-kDa and 40-kDa dextrans and bovine ubiquitin were obtained from Sigma-Aldrich (St. Louis, MO), 68-kDa dextran was obtained from Fisher Scientific (Pittsburg, PA). Ficoll was purchased from GE Healthcare Biosciences (Piscataway, NJ).

B. Sample preparation

The T_m of ubiquitin at $pH > 4$ is higher than 90 °C.⁴⁷ Therefore, all experiments were performed at $pH^* 1$. This pH condition was chosen because it is known to effectively decrease the thermal stability of ubiquitin without inducing any structural changes to the folded state.⁴⁹ An appropriate amount of crowding agent was weighed out on an analytical balance (Denver Instrument, M-220, Bohemia, NY) and dissolved in an appropriate amount of DCl solution (~125 mM in D₂O), dispensed via a pipette (EP2100–100R, Eppendorf, Hauppauge, NY). The amount of DCl solution to be added was determined as follows: In separate experiments, 1.0, 1.5, and 2.0 g of crowding agent was added to a 5 mL volumetric flask (precision ± 0.02 mL) and the volume of Millipore water needed to get a total volume of 5 mL was determined via an Eppendorf pipette. The volume of added water was then subtracted from the total volume of 5 mL to calculate the volume occupancy of the polymer. The volume fraction of polymer (V_{polymer} in %) was then plotted against the concentration of the polymer ($\text{conc}_{\text{polymer}}$ in g/L), and the data was fit by a linear equation, $V_{\text{polymer}} = \text{conc}_{\text{polymer}}m$, which was used to calculate the amounts of DCl solution needed to prepare the protein samples. The slopes m were found to be 0.0651, 0.0633, 0.0632, and 0.0659 for dextran 6, dextran 40, dextran 70, and ficoll 70, respectively. The polymer solutions were vortexed and sonicated for about 10 min, then added to dry, deuterium exchanged ubiquitin powder. The resultant protein solution was then vortexed and sonicated for another 5 min before use.

C. Fourier transform infrared (FTIR) measurements

FTIR spectra were collected on a Nicolet Magna-IR 860 spectrometer with 2 cm^{-1} resolution. The details of

the sample cell (56 μm optical pathlength), temperature control, and data acquisition protocol have been described elsewhere.⁵² The corresponding temperature-dependent FTIR spectra (in the range of 1550–1800 cm^{-1}) obtained under a specific solution condition were analyzed using the singular value decomposition (SVD)⁵³ function within MATLAB. Based on the absorbance at 1624 cm^{-1} , the reversibility of the thermally induced unfolding transitions of ubiquitin investigated herein was estimated to be >90%.

III. RESULTS AND DISCUSSION

A. Temperature dependence of the amide I' band of ubiquitin

The thermal unfolding transitions of ubiquitin under dilute and crowded conditions were quantitatively assessed using infrared spectroscopy. In particular, the amide I' band of the protein under each condition, which has been shown to be a sensitive reporter of both α -helical^{54,55} and β -sheet structures,⁵² was collected over a wide temperature range. As shown (Fig. 1), the amide I' band of ubiquitin in dilute aqueous solution loses/gains intensity in the lower/higher frequency region of the spectrum with increasing temperature, indicative of its usefulness in monitoring protein unfolding. To extract quantitative thermodynamic information from these spectra, we analyzed them via SVD (Ref. 53) following Tokmakoff and coworkers, who have shown that the second

FIG. 1. Temperature dependent FTIR spectra (a) and difference FTIR spectra (b) of ubiquitin at $pH^* 1$ in D₂O. The lowest and highest temperatures in this case were 1.6 and 82.6 °C, respectively.

FIG. 2. Thermal melting curves constructed from the normalized second-component coefficients obtained by singular value decomposition analysis of the temperature dependent FTIR spectra of ubiquitin under different solution conditions, as indicated. The data points below 30 °C are not shown for clarity. Lines are fits of these data to Eq. (2) and the resultant fitting parameters are listed in Table I.

singular value, $C_2(T)$, is a good reporter of the unfolding transition of ubiquitin.^{50,51} As shown (Fig. 2), the thermal melting curve thus obtained, indeed, shows a sigmoid shape, consistent with a cooperative unfolding transition.^{50,51} Thus, these data were further fit to the following two-state model,

$$\frac{C_2(T)}{C_2(T_{\text{ref}})} = A_2 + \frac{(A_1 - A_2)}{(1 + \exp[(T - T_m)/W])}, \quad (2)$$

where T is temperature, T_m and W represent the midpoint and width of the sigmoidal transition, respectively. In addition, $A_1 = mT + b_1$ and $A_2 = mT + b_2$ represent the folded and unfolded baselines, respectively. As shown (Fig. 2), the fit is satisfactory and the resultant T_m , 66.2 ± 1.1 °C, is in good agreement with that obtained in previous studies.^{49,51} We note that fitting Eq. (2) to the thermal melting curve determined using the absorbance at 1624 cm^{-1} , which contains predominantly contributions from both α -helical and β -sheet structures, yields essentially identical results, thus further corroborating the notion that the thermal unfolding transition of ubiquitin occurs between two conformational ensembles.

B. Dependence of T_m on crowder concentration

Following the method discussed above, the thermal melting curves of ubiquitin under different crowding conditions were also determined and fitted to Eq. (2). As shown (Fig. 2 and Table I), the T_m of ubiquitin increases

with increasing crowder concentration, a trend that has been observed in other experimental studies^{6,10,21} and also computer simulations.^{21,23,28} While such an increase is consistent with the excluded volume effect of the crowding agents, which preferentially destabilizes the unfolded state of proteins,^{1,23,30–33} a quantitative assessment of the underlying relation between T_m and the degree of crowding (or volume fraction of the crowder, ϕ) was rarely performed. As shown (Fig. 3), the T_m of ubiquitin increases nonlinearly with increasing ϕ (within the experimentally accessible range of crowder concentrations). In fact, the data can be well described by Eq. (1) (Fig. 3 and Table II), suggesting that the theoretical model of Cheung and Thirumalai,²³ which is based on considerations of the entropy loss associated with confining an unstructured polymer to a spherical pore,^{37,38} possibly captures the essential physics underlying the crowding effect. What is more interesting, however, is that the exponents (i.e., α) extracted from the fits of the data obtained with different crowding agents fall within the range of 4.1–6.5 (Table II). As discussed above, polymer theory provides two possible expressions for α , one is based on confinement to a spherical cavity, which provides repulsive boundaries in all three spatial dimensions, and the other is based on confinement to a capillary/slit, which restricts the polymer in two-dimensions/one-dimension.³⁸ Using the Flory coefficient for an unstructured excluded-volume polymer in a good solvent, which yields $\nu \approx 0.6$, the theoretically predicted values of

TABLE I. Midpoints (T_m) and widths (W) of the thermal denaturation curves of ubiquitin obtained under different crowding conditions by fitting the experimental IR data to Eq. (2). The standard deviations for all reported melting temperatures and widths are estimated to be ± 1.1 and ± 0.3 °C, respectively. These estimates are based on five independent measurements of the denaturation of ubiquitin in dilute aqueous solution.

Crowder	Concentration (g/L)	T_m (°C)	W (°C)
None	0.0	66.2	4.0
Dextran 6	200	67.9	3.5
Dextran 6	250	71.5	3.8
Dextran 6	300	70.7	3.6
Dextran 6	350	73.3	3.7
Dextran 6	400	73.2	3.9
Dextran 40	200	68.0	3.8
Dextran 40	300	70.9	4.4
Dextran 40	400	74.7	4.3
Dextran 70	200	67.8	3.8
Dextran 70	300	70.3	3.6
Dextran 70	400	73.5	3.5
Ficoll 70	200	67.2	4.0
Ficoll 70	300	67.7	3.3
Ficoll 70	400	69.3	3.6

α are 1.67 and 3.75 for capillary/slit and spherical confining geometries, respectively. Thus, the experimentally determined value of α suggests that the crowding effect of these macromolecular crowders is more similar to the confinement exerted by a spherical cavity than the one of a capillary/slit. This finding is remarkable as intuitively we might think that the confinement effect exerted by a flexible and dynamic crowding agent would bear more similarity to those of a capillary/slit confining geometry rather than the ones of a spherical cavity. The question arises, however, whether the power-law dependence observed herein applies universally to different proteins, as observed in simulation studies of the confinement effect by Takagi *et al.*,⁴¹ or if it is unique to a specific protein as shown in simulations by Rathore *et al.*³⁹ While more experiments are needed to answer this question, the few existing experimental data seem to suggest that setting $\alpha = 1/\nu$ provides too small a value for α and ΔT_m . For example, Sasahara and Minton⁶ found a quadratic dependence of the thermal melting temperature of hen egg-white lysozyme (129 residues) on crowder concentration, which is in good agreement with our results as their data essentially suggest an α value of 6. Similarly, we find that the experimental data of Stagg *et al.*¹⁰ on flavodoxin (148 residues) can also be fit by Eq. (1), yielding a value of about 3.7 for α .

While it is noted that residual structures in the unfolded state could also lead to a larger α due to $\nu < 0.6$, this seems to be unlikely for ubiquitin. Simulation studies have shown that if partially folded structures are populated under a crowded environment the width of the unfolding transition would show a broadening with increasing crowder concentration,^{23,28} due to a decrease in the cooperativity of the folding–unfolding transition. As shown (Table I), our results indicate that the width of the thermal unfolding transition of ubiquitin does not show any measurable dependence on the concentration of the crowding agents used, which further corroborates the

two-state model used to analyze the temperature melting curves and suggests that the large α value observed is unlikely to be caused by residual structures in the unfolded state of ubiquitin.

C. Dependence of T_m on crowder size and structure

According to excluded volume models,^{1,23,30–33} destabilization of the unfolded state of proteins by crowding is a consequence of reduction of the space available to the polypeptide chain, as confinement effectively eliminates conformations whose radii of gyration are larger than the size of the confining space. The radius of the most probable spherical volume that is void of crowders in a solution at a volume fraction ϕ of crowders is given by $(4\pi/3\phi)^{1/3}R_c$.²³ Thus, there should be a functional dependence of the destabilization of the unfolded state on the size of the crowder as predicted by Eq. (1). For example, using the experimentally determined hydrodynamic radii⁵⁷ of dextran 70 ($R_{c,D70} = 6.49$ nm) and dextran 40 ($R_{c,D40} = 4.78$ nm), and an exponent $\alpha = 3.75$, the ratio of $\Delta T_{m,D40}/\Delta T_{m,D70} \sim (R_{c,D70}/R_{c,D40})^\alpha$ is estimated to be about 3.1. Similarly, using the hydrodynamic radius of dextran 10 kDa ($R_{c,D10} = 1.86$ nm),⁵⁷ one predicts that $\Delta T_{m,D10}/\Delta T_{m,D70}$ is about 108 for $\alpha = 3.75$ and about 8 for $\alpha = 1.67$. Though the constant of proportionality in Eq. (1), which is equal to the inverse of the entropy of unfolding will lower the calculated ratios somewhat, the data clearly do not support such a strong dependence for ubiquitin. As a matter of fact, the ratio is found to be close to unity (Table I). Such a stark difference between theoretical prediction and experiment cannot be attributed to experimental uncertainty, but must arise from other reasons. Similarly, other experimental studies have also found either no¹¹ or a weak^{14,21} dependence of the thermal stability of proteins on the crowder size. The origin of the observed discrepancy may be because when mapping a given crowded solution onto a spherical cavity via $R = (4\pi/3\phi)^{1/3}R_c$, void formation due to density fluctuations in the crowded solution is not taken into account. In other words, protein conformations with $R_g > R$ are strictly not allowed according to such mapping relationship, but they could exist in a real crowded solution which is subject to density fluctuations. Interestingly, the probability of void formation has been shown to be a function of the number density (ρ_c) of the crowder, i.e., $(1 - \phi)/\rho_c$.¹⁸ Alternatively, these findings may not indicate a failure of the theory, but rather suggest that picturing the crowder molecule as a hard

TABLE II. Parameters obtained from fitting the data in Fig. 3 to Eq. (1), i.e., $T_m(\phi) = T_m^d + C\phi^\alpha$, where T_m^d is the thermal melting temperature of ubiquitin in D₂O. The standard deviations of the fits were estimated using the jackknife method (Ref. 56) and R is the Pearson product moment correlation coefficient.

Crowder	C	α	R^2
Dextran 6	48.5 ± 31.5	4.1 ± 1.2	0.89
Dextran 40	164.0 ± 43.5	6.5 ± 0.4	1.00
Dextran 70	133.7 ± 48.2	6.3 ± 0.6	1.00
Ficoll 70	41.6 ± 36.7	5.9 ± 2.3	0.98

FIG. 3. Thermal melting temperature (T_m) of ubiquitin as a function of the volume fraction (ϕ) of different crowding agents, as indicated. Lines are fits of these data to Eq. (1), i.e., $T_m(\phi) = T_m^d + C\phi^{\alpha/3}$, and the resultant fitting parameters are listed in Table II. The standard deviations of the data points were assumed to be the same as those for the melting temperature in dilute solution (see Table I).

sphere of a given radius and the polymer solution as a suspension of hard spheres is an oversimplification. In particular, it seems that the effective “crowding radii” of dextrans of different molecular weights are not as different as one might expect based on their hydrodynamic radii, at least for proteins that have a similar size to ubiquitin. For example, it is well known that dextran and ficoll polymers undergo compression as concentration is increased.^{58–60} At high concentrations, the degree of compression was found to be dependent on molecular weight,⁵⁸ providing a possible explanation for the observed insensitivity of ΔT_m on dextran molecular weight. Taken together, our results highlight the need for experimental and theoretical efforts to gain a more detailed picture of the microscopic structure and dynamics of these polymer solutions.

Moreover, numerous experimental studies have compared the efficacy of various polymers as a crowding agent.^{4,14,15,17,21} For example, previous studies have found that dextran 70 is more effective than ficoll 70 in stabilizing the folded state of proteins.^{14,15,21} As shown (Table I and Fig. 3), our results are in agreement with this finding. Though the most probable geometric shapes of either dextran 70 or ficoll 70 are not definitely determined experimentally, it is typically postulated that dextran 70 assumes a rodlike shape whereas ficoll 70 has a spherelike geometry.²¹ Thus, under identical solution conditions dextran 70 should exert a larger confinement effect to the unfolded polypeptide chain than

ficoll 70, because according to the excluded-volume theory the size of the most probable interstitial space found between rod-shaped objects is smaller than that created by spherical particles of similar molecular weight.³⁰

IV. CONCLUSIONS

In summary, we demonstrated the power-law dependence of the thermal melting temperature of ubiquitin on the crowder volume fraction, a theoretical prediction that has not been experimentally verified. The exponent α of the power-law was found to be in the range of 4.1–6.5 for both dextran and ficoll polymers. This result suggests, as previously proposed, that the effect of macromolecular crowding on protein folding stability can be modeled by confinement of the protein of interest to a spherical cavity.²³ However, in contrast to theoretical prediction, but in agreement with other experimental studies,^{11,14,21} we found that the stabilization arising from crowding shows a weak, if any, dependence on the size of the crowding agents used in the current study. Taken together, these findings suggest that the microscopic compartmentalization of these polymer solutions is different from what is expected based on the hydrodynamic radii of the crowders and underscore the need for further improvement of existing crowding theories.

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