Protein Stability

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Protein Stability in Reverse Micelles

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Abstract: *The N-terminal SH3 domain of the Drosophila signal transduction protein drk was encapsulated in reverse micelles. Both the temperature of maximum stability and the melting temperature decreased on encapsulation. Dissecting the temperature-dependent stability into enthalpic and entropic contributions reveals a stabilizing enthalpic and a destabilizing entropic contribution. These results do not match the expectations of hard-core excluded volume theory, nor can they be wholly explained by interactions between the head groups in the reverse micelle and the test protein. We suggest that geometric constraints imposed by the reverse micelles need to be considered.*

Understanding the effect of confinement is essential for explaining protein behavior during biosynthesis (ribosome exit tunnel), misfolding (chaperones), turnover (proteasome), and transport across membranes (translocon). Confinement is also important for industrial applications such as enzyme stabilization and drug delivery.[1] Even though confined and crowded environments are intrinsic to biology, they are absent from most studies of proteins, which are conducted in simple buffer solutions.[2]

Efforts to explain biomolecular interactions in crowded environments have attracted the attention of researchers for decades, but the landscape of crowding research has shifted dramatically in the past few years. The long-accepted explanation for differences in protein chemistry between buffer and the cytoplasm focused on hard-core repulsions, a purely entropic effect.[3] It is now known that repulsions are often diminished or even overwhelmed by nonspecific attractive interactions between the test protein and the macromolecules (and perhaps metabolites) in the cytoplasm and in cosolute solutions in vitro.[4]

Confinement is easy to envision. The test protein is trapped in a cavity just large enough to contain it and some hydrating water. A widely accepted model is that spherical confinement stabilizes globular proteins because there is no room to unfold.[5] This is a purely entropic effect because it deals only with the arrangement of molecules. We test this idea by using the tools of equilibrium thermodynamics to assess the effects of encapsulation in reverse micelles on protein stability.

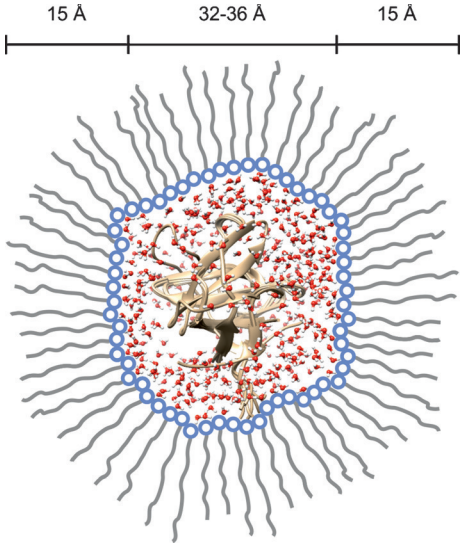

Figure 1. Representation of a CTA Br reverse micelle containing SH3 (2A36.pdb).[12b] Dimensions are from the data in Table 1. One reverse micelle contains one protein molecule and about 300 H_2O molecules.

Reverse micelles comprise an aqueous core surrounded by a layer of surfactant embedded in a bulk organic solvent (Figure 1). The spontaneously formed micelles, the inner core diameter of which increases with increasing water loading ratio, $W_0 = \frac{[H_2O]}{[surfactant]}$,[6] have attracted researchers from many fields.

For instance, they are used as low viscosity protein hosts for protein NMR spectroscopy,[7] to study the properties of confined peptides[8] and proteins,[9] and as model systems for confined water.[10]

Protein stability is defined as the modified standard state Gibbs free-energy of unfolding, $\Delta G_u^{0'}$, which equals $-RT\ln(K_u)$, where R is the gas constant, T is the absolute temperature, and K_u the equilibrium constant ($K_u = [\text{Unfolded}]/[\text{Folded}]$). In Equation (1), $\Delta H_u^{0'}$ and $\Delta S_u^{0'}$ are the temperature-dependent modified standard state enthalpy and entropy of unfolding, respectively. Positive $\Delta G_u^{0'}$ values indicate an excess of folded over unfolded protein.

$$\Delta G_u^{0'}(T) = \Delta H_u^{0'}(T) - T\Delta S_u^{0'}(T) \quad (1)$$

The temperature dependence of $\Delta G_u^{0'}$ for a globular protein is bell-shaped with a maximum at T_s. The curve crosses the abscissa at the temperature of cold denaturation, T_c and the melting temperature, T_m. Expressions for the temperature dependence are obtained by using Kirchhoff's equations [Eqs. (2) and (3)], where T_{ref} is a reference temper-

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$$\Delta H_u^{0'}(T) = \Delta H_u^{0'}(T_{ref}) + \int_{T_{ref}}^{T} \Delta C_p^{0'}\, dT \quad (2)$$

$$\Delta S_u^{0'}(T) = \Delta S_u^{0'}(T_{ref}) + \int_{T_{ref}}^{T} \frac{\Delta C_p^{0'}}{T}\, dT \quad (3)$$

ature and $\Delta C_p^{0'}$ is the modified standard-state heat capacity of unfolding, which we assume is temperature-independent over the range studied.

Inserting Equations (2) and (3) into (1) at T_m, where $\Delta G_u^{0'} = 0$ and $\Delta S_u^0(T_m) = \Delta H_u^0(T_m)/T_m$, or at T_s, where $\Delta S_u^{0'}(T_s) = 0$ and integrating, gives Equations (4) and (5), respectively.[11]

$$\Delta G_u^{0'}(T) = \Delta H_u^{0'}(T_m)\left(1 - \frac{T}{T_m}\right) + \Delta C_p^{0'}\left[(T - T_m) - T\ln\left(\frac{T}{T_m}\right)\right] \quad (4)$$

$$\Delta G_u^{0'}(T) = \Delta H_u^{0'}(T_s) + \Delta C_p^{0'}\left[(T - T_s) - T\ln\left(\frac{T}{T_s}\right)\right] \quad (5)$$

Here, we quantify the effects of confinement by using the 7 kDa *N-term*inal SH3 domain of the *Drosophila* signal transduction protein drk, which exists in a dynamic equilibrium between the folded state and the unfolded ensemble.[12] The protein has one tryptophan residue. The spectrum of the 5-fluorotryptophan-labeled[13] protein has only two ^{19}F NMR resonances: one from the folded state, the other from the unfolded ensemble.[12a] The area under each resonance is proportional to its concentration.

To probe the effect of confinement on stability, we incorporated the protein into reverse micelles systems with different internal properties. Those made from cetyltrimethylammonium bromide (CTA Br) provided a cationic interior, and those made from decyl-1-*rac*-glycerol (10MAG) plus lauryldimethylamine-*N*-oxide (LDAO) provided a zwitterionic interior. The CTA^+ head group is a trimethyl ammonium ion (with a Br^- counterion). The head group on LDAO is a trimethylamine oxide (TMAO)-like zwitterion, and 10MAG is uncharged. Thus, the CTA Br reverse micelles have a higher charge density.

To understand the effects of encapsulation we first examined the size of the reverse micelles through the effects of encapsulation on the rotational motion of the protein (Table 1). ^{19}F NMR data and the Einstein–Stokes relation [Eq. (6)] were used to estimate the size of protein-containing

$$\tau_m = \frac{4\pi\eta r_x^3}{3k_B T} \quad (6)$$

reverse micelles, r_{rm}, from the rotational correlation time, τ_m, of SH3 using the viscosity of the bulk solvent (isooctane),[14] η, Boltzmann's constant, k_B, and the absolute temperature, T. τ_m was determined from the longitudinal and transverse relaxation times, T_1 and T_2, respectively[16] as described in the Supporting Information. Assuming partial specific volumes of $0.7\ L\,g^{-1}$ for protein and $1\ L\,g^{-1}$ for water, each micelle contains one protein molecule and about 300 H_2O molecules. These data were used to construct the dimensions shown in Figure 1. The interior is approximately the same size as the hydrated protein (r_H) in buffer (Table 1). There is no dependence on W_0 for either system, consistent with data,[7e] showing that the size of the protein-containing reverse micelles cannot be tuned by changing W_0 because it is the protein that determines the amount of H_2O in the micelle.

Table 1: Rotational correlation times, τ_m, of folded SH3 in buffer (pH 7.0) and in reverse micelles at three water loadings (W_0).

	τ_m, ns (at T, K)	r_{rm}, [Å]	r_{aq}, [Å]
buffer	3.3 ± 0.5 (298)	–	r_H: 15.4 ± 0.1
CTA Br			
W_0 15	15.5 ± 4.3 (283)	29.4 ± 0.3	14.0 ± 0.3
W_0 20	13.7 ± 3.1 (283)	28.2 ± 0.2	12.8 ± 0.2
W_0 25	21.8 ± 5.4 (277)	31.9 ± 0.3	16.5 ± 0.3
10MAG/LDAO			
W_0 15	6.6 ± 1.5 (303)	24.5 ± 0.2	12.5 ± 0.2
W_0 20	6.1 ± 1.1 (298)	23.3 ± 0.1	11.3 ± 0.1
W_0 25	9.0 ± 1.6 (283)	24.5 ± 0.1	12.5 ± 0.1

[a] τ_m could not be measured at a common temperature, because the different micelles have differential effects on protein stability. Measurement temperatures are given in parentheses. Radii of the micelles, r_{rm}, and the aqueous phases, r_{aq}, were calculated using Equation (6), the viscosity of isooctane[14] and the contour length of CTA Br[15] (15.4 Å) and 10MAG/LDAO (ca. 12 Å). Equation (6) was also used to obtain the radii of the hydrated protein, r_H.

The temperature dependence of the SH3 stability was then measured in buffer alone,[17] buffered micelles, and cosolute–buffer solutions.[17] Unfolding is reversible (see Figure S1 in the Supporting Information). Equations (4) and (5) were fit to the $\Delta G_u^{0'}$ values to obtain T_s, $\Delta H_u^{0'}(T_s)$ [or $\Delta H_u^{0'}(T_m)$], $\Delta C_p^{0'}$, and T_m (Table S1). Water loading has a small effect on the stability in CTA Br and an insignificant effect in 10MAG/LDAO reverse micelles, consistent with the data on its effect on reverse micelle size. In addition, only minor changes in stability curves were observed for reverse micelles prepared at different 10MAG/LDAO ratios (Figure S2).

Both reverse micelle systems lower the stability of SH3 compared to its stability in buffer alone above 265 K and 290 K, respectively (Figure 2). This observation is in sharp contrast to the stabilizing effect expected from simple hard-core excluded volume effects. Thus, an effect besides hard-core repulsions between the protein and the inside of the micelles is controlling the stability of the encapsulated proteins.

To assess the effect of the reverse micelle environment on SH3 structure we acquired ^{19}F and ^{15}N-^1H HSQC spectra. The ^{19}F spectra were compared at the stability extremes shown in Figure 2. The only resonances that change with temperature are from the folded and the unfolded forms. These and other data[7d] confirm the lack of protein–alkane solvent interactions. The ^{15}N-^1H HSQC data on SH3 in buffer and in both reverse micelle systems (Figure S3) indicate that encapsulation maintains the structural integrity of SH3, an observation consistent with studies of other proteins.[7b]

To understand the effects of encapsulation on the thermodynamics of unfolding we evaluated the influence of encapsulation on T_s and T_m (compared to buffer) by using the formalism of H.-X. Zhou.[4g] Both reverse micelle systems

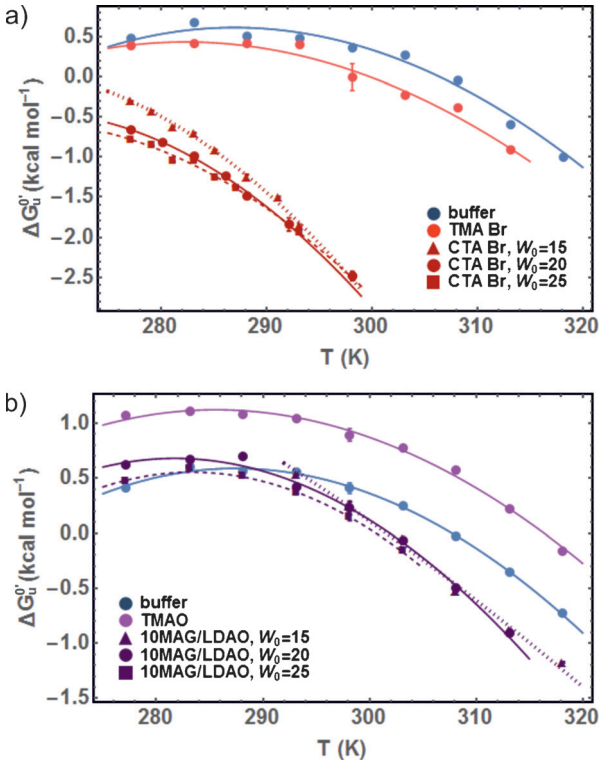

Figure 2. a) Temperature dependence of SH3 stability in CTA Br reverse micelles, tetramethylammonium bromide (TMA Br) (3.7 mol kg^{-1}, $W_0 = 15$), and buffer at pH 7.6, b) 10MAG/LDAO reverse micelles (pH 7.0), TMAO (50 g L^{-1}, pH 7.2) and buffer (pH 7.0). Measurements in 10MAG/LDAO reverse micelle at water loadings of 15 and 25 could not be obtained at low and high temperatures, respectively, because of precipitation. See the Supporting Information for details about uncertainties.

lower these temperatures (Figure 2, Table S1). A lower T_s with no overall change in free energy (i.e., a horizontal shift) corresponds to an enthalpic stabilization counteracted by an entropic destabilization. In addition, the curve for CTA Br reverse micelles is slightly vertically downshifted, indicating an enthalpic destabilization (Table S2). However, this shift does not fully compensate the enthalpic stabilization that accompanies the lower T_s. The enthalpic stabilization of SH3 in CTA Br reverse micelles is even stronger than in 10MAG/LDAO reverse micelles for which the stability is not vertically downshifted (Tables S1 and S2). This enthalpic stabilization with compensating entropic destabilization resembles data from studies where proteins were found to be stabilized enthalpically in the presence of polyols, sugars, and even polymers.[18]

The enthalpic stabilization and entropic destabilization in reverse micelles further contradicts expectations from theories based on hard-core effects, which predict no change of enthalpy and a stabilizing change in entropy.[3b,19] Therefore, models built on volume-based entropic stabilization cannot explain our observations in reverse micelles even when direct interactions between the protein and the interior are considered, because such considerations predict a shift of T_s to higher temperatures.[4g]

Next, we compared the effects of reverse micelles to those of solutions containing models of the reverse micelle head groups. Comparing the cationic CTA Br reverse micelles to tetramethylammonium bromide (TMA Br) solutions suggests that a cosolute effect between the head group and the protein plays a role because both the reverse micelles and the model compounds decrease $\Delta G^{0'}_u(T)$ compared to buffer (Figure 2a). However, this cosolute effect cannot completely explain the effect of confinement, because TMA Br cannot account for the strong T_s decrease in CTAB reverse micelles compared to buffer. The lack of a coherent explanation is not surprising because the fixed arrangement and high density of head groups directed towards the protein in micelles is not reproduced in bulk solutions containing models of the head groups.

We also explored head group effects by comparing the effects of CTA Br reverse micelles to those of urea, because tetraalkylammonium halides are protein denaturants.[20] Urea has favorable enthalpic interactions with protein surfaces and destabilizes proteins because the unfolded state exposes more surface than the native state (Table S1). The CTA Br reverse micelles and TMA Br in solution both result in a vertical downshift compared to buffer [similar to the effect of urea (Table S1)], indicating a contribution from attractive chemical interactions. This effect is independent of the net charge of SH3, because decreasing the pH from near neutrality, where the protein bears a net charge of −6, to pH 4.2, where the protein bears a net charge of +2, does not alter the effects of encapsulation (Figure S4).

The zwitterionic head group of LDAO is analogous to the osmolyte TMAO. Encapsulation in 10MAG/LDAO reverse micelles results in a vertical downshift compared to TMAO, which is inconsistent with the stabilizing effect of TMAO (Figure 2b). TMAO stabilizes proteins because it is excluded from the protein surface.[21] Thus, encapsulation increases the attractive interactions between the protein and the inside surface of the reverse micelle. Increasing the number of TMAO-like head groups in the micelle does not increase SH3 stability indicating a micelle effect rather than a cosolute effect as the major driving force (Figure S2).

Our results for SH3 in the cationic and zwitterionic reverse micelles (Figure 2) contrast the calorimetric data of proteins in anionic sodium bis(2-ethylhexyl)sulfosuccinate (AOT) reverse micelles.[9] Most proteins studied in AOT reverse micelles are characterized by an enthalpic destabilization and an increase in T_s (entropic stabilization), regardless of protein pI[9b] An example is shown in (Figure S5). Unfortunately, AOT does not maintain the structural integrity of most encapsulated proteins.[7a]

In summary, our observations and those of others[9b] are in sharp contrast with predictions of the hard-core excluded volume theory, which predicts only entropic stabilization. Chemical interactions between the group on the interior of the micelle and the test protein also do not wholly explain the effects on encapsulation. As pointed out by Van Horn et al.,[22] the geometry of the chemical interactions may play a role. Whereas the cosolute models of the surfactant head groups can approach the protein from any angle, in reverse micelles, the protein is faced with a concave surface with

a radius almost matching its convex surface. Furthermore, the confined water molecules may play a major role because this water is strongly affected by the micelle–protein interface.[10] Opposite observations, shifts to higher T_s and $\Delta G_u^{0'}(T)$ values, have been observed in high concentration of synthetic polymers and protein crowders.[18d] Although a unified explanation is not yet available, all these data point to the inadequacy of explanations based solely on hard-core excluded volume.

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