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Reverse Micelle Encapsulation as a Model for Intracellular Crowding

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Abstract: Reverse micelles are discrete nanoscale particles composed of a water core surrounded by surfactant. The amount of water within the core of reverse micelles can be easily manipulated to directly affect the size of the reverse micelle particle. The water loading capacity of reverse micelles varies with temperature, and water can be shed if reverse micelles are exposed to low temperatures. The use of water shedding from the reverse micelle provides precise and comprehensive control over the amount of water available to solvate host molecules. Proteins encapsulated within reverse micelles can be studied to determine the effects of confinement and excluded volume. The data presented here provide an important bridge between commonly employed dilute in vitro studies and studies of the effects of a crowded environment, as found in vivo. Ubiquitin was encapsulated within bis(2-ethylhexyl) sodium sulfosuccinate AOT reverse micelles under various degrees of confinement and was compared with an analogously reconstituted sample of ubiquitin in the commonly used molecular crowding agent bovine serum albumin. The effects of encapsulation were monitored using chemical shift perturbation analysis of the amide ^{1}H and ^{15}N resonances. The results also reconcile alternative interpretations of protein cold denaturation within reverse micelles.

Introduction

Reverse micelles (RMs) are composed of surfactants that assemble around a nanosized aqueous core in an apolar solvent (Figure 1). Aqueous buffers and protein solutions can be transferred to the water interior of the reverse micelle particle and hosted in a nativelike environment.[1,2] Reverse micelles have been used to enhance the sensitivity and resolution of NMR spectra of encapsulated macromolecules by exploiting the relatively low viscosity of the bulk solvent to enhance the overall tumbling rate.[1,3] The enhancement arises due to the increased rate of tumbling, which scales the rate of transverse relaxation and produces a significant increase in the resolution and sensitivity. More recently, the use of reverse micelles has been extended to investigate the biophysical properties of encapsulated proteins, including low-temperature unfolding and the effects of confinement on protein and water dynamics. These applications represent promising new avenues for development of the technology and elucidation of challenging biophysical questions.[4−7]

The cellular environment is highly crowded compared to relatively dilute in vitro conditions. For example, *Escherichia coli* cytoplasm has approximately 300−400 mg/mL of protein and nucleic acids, which is significantly greater than concentrations traditionally used for solution NMR samples, which are typically about 15 mg/mL of protein.[8,9] Even the extracellular environment can be relatively crowded, as is illustrated by the fact that blood plasma generally has about 80 mg/mL of protein.[8] Excluded volume is defined as the space that is excluded from occupation by a specific probe macromolecule because that space is occupied by other macromolecules. The concept was developed by Flory to describe the end-to-end distance distribution of flexible polymers.[10] Confinement is a closely related concept, which is in some ways a more intuitive description of effects due to entrapment within a limited space (cavity, chamber, cell, etc.). The effects of macromolecular crowding and confinement are anticipated to be similar, since both reduce the available volume. Regardless of the nomenclature, the excluded volume of crowded solutions influences several important physical properties of proteins, including protein folding and stability, the effective volume of the folded protein, protein aggregation, and local protein dynamics.[6,8,9,11−14] Moreover, under crowded conditions, such as those found within

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(1) Flynn, P. F. *Prog. Nucl. Magn. Reson. Spectrosc.* **2004**, *45*, 31–51.
(2) Wand, A. J.; Babu, C. R.; Flynn, P. F.; Milton, M. J. In *Biological Magnetic Resonance*; Krishna, N. R., Berliner, L. J., Eds.; Springer: New York, 2003; Vol. 20, p 121−160.
(3) Flynn, P. F.; Milton, M. J.; Babu, C. R.; Wand, A. J. *J. Biomol. NMR* **2002**, *23*, 311–316.
(4) Van Horn, W. D.; Simorellis, A. K.; Flynn, P. F. *J. Am. Chem. Soc.* **2005**, *127*, 13554–13560.
(5) Simorellis, A. K.; Flynn, P. F. *J. Am. Chem. Soc.* **2006**, *128*, 9580–9581.
(6) Simorellis, A. K.; Van Horn, W. D.; Flynn, P. F. *J. Am. Chem. Soc.* **2006**, *128*, 5082–5090.
(7) Babu, C. R.; Hilser, V. J.; Wand, A. J. *Nat. Struct. Mol. Biol.* **2004**, *11*, 352–357.
(8) Ellis, R. J. *Curr. Opin. Struct. Biol.* **2001**, *11*, 114–119.
(9) Zimmerman, S. B.; Trach, S. O. *J. Mol. Biol.* **1991**, *222*, 599–620.
(10) Flory, P. J. *Principles of Polymer Chemistry*; Cornell University Press: Ithaca, NY, 1953.
(11) Snoussi, K.; Halle, B. *Biophys. J.* **2005**, *88*, 2855–2866.
(12) Tokuriki, N.; Kinjo, M.; Negi, S.; Hoshino, M.; Goto, Y.; Urabe, I.; Yomo, T. *Protein Sci.* **2004**, *13*, 125–133.
(13) van den Berg, B.; Ellis, R. J.; Dobson, C. M. *EMBO J.* **1999**, *18*, 6927–6933.

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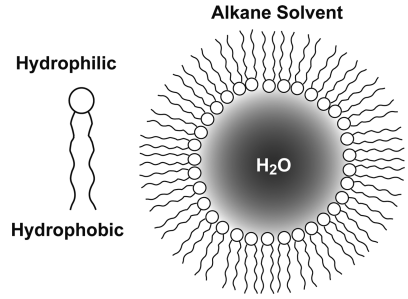

Figure 1. Schematic model of a reverse micelle forming surfactant and the resulting reverse micelle particle. Reverse micelle protein encapsulation and the resulting nanoarchitecture represents an extremely confining environment. The effects of the excluded volume in this distinct platform are compared to macromolecular crowding with solution NMR.

cells, translational diffusional rates (D_t) are slowed by approximately 1 order of magnitude.[8,15]

The critical parameter for formation of reverse micelles is the water loading (w_0), which is defined as the molar ratio of water to surfactant. Water loading directly influences the physical attributes of the reverse micelle particle, including the size of the particle, the degree of solvation of a hosted molecule, and the encapsulation efficiency.[4,6,16,17] When reverse micelles are exposed to low temperature (e.g., −30 °C), water is expelled from the interior of the particle and collects as ice in the bottom of the sample container in a process called water shedding.[4,6] As water is shed from the reverse micelle, the water pool, and therefore the entire particle size, becomes smaller. When the sample is returned to room temperature, water passively returns to the reverse micelle water core at a rate of about 1 unit of w_0 per day; however, that rate can be greatly accelerated by simple agitation of the sample. The ability to precisely control the w_0 through shedding and reequilibration of water provides the flexibility to probe the effects of confinement in a systematic way. The data presented here establish the utility of reverse micelles applied to investigation of crowding and confinement studies. Chemical shift perturbation analysis of spectra recorded for human ubiquitin that is confined within AOT reverse micelles is compared to spectra recorded for ubiquitin crowded with bovine serum albumin (BSA), and the results reveal very similar trends, indicating that RMs are a valid, and thus highly attractive, mimetic for crowding studies. RM-based confinement studies thus should be an excellent general vehicle for examining the influence of in vivo like conditions on the structure and dynamics of functional biomacromolecules.

The analysis of time-dependent changes in the chemical shifts of resonances and their intensities lends important insights into previous low-temperature reverse micelle studies of cold denaturation.[4,7,18] We describe a new phenomenon that we term "protein shedding". Protein shedding occurs exclusively when low ionic strength reverse micelle samples are exposed to low temperatures. This phenomenon has important implications related to the use of reverse micelle encapsulation as a vehicle to investigate low-temperature unfolding, e.g. cold denaturation.

Materials and Methods

Protein Preparation and Purification. The plasmid cw-ubq encoding human ubiquitin was transformed into chemically competent BL21 (DE3) expression cells (Invitrogen Corp., Carlsbad, CA). Transformants were selected by ampicillin resistance and cultured at 37 °C on M9 minimal media, with the sole nitrogen source being $^{15}NH_4Cl$. The growth was monitored at 600 nm, and at an optical density (OD_{600}) of ∼0.6, the cells were induced to overexpress ^{15}N-labeled human ubiquitin by adding an aliquot of IPTG to generate a final concentration of 0.5 mM. The culture was grown for an additional 4 h postinduction (terminal $OD_{600} \sim 1.3$). Cells were harvested by centrifugation at 5000 rpm for 15 min at 4 °C and then stored at −80 °C. The cells were thawed and incubated for 30 min in Ni-binding buffer (500 mM NaCl, 20 mM sodium phosphate, pH 7.4) containing lysozyme and 1 mM phenylmethylsulfonyl fluoride. The cell extract was sonicated and then cleared by centrifugation at 15 000 rpm for 1 h at 4 °C. The cell extract supernatant was cold-filtered through a 0.45 μm filter (Pall Corp., East Hills, NY). The extract containing C-terminal 6× His-tagged ubiquitin was affinity-purified by a Ni-IDA column (Amersham Biosciences, Piscataway, NJ). The ubiquitin-containing elutant was concentrated and the buffer was exchanged by a stirred cell (Millipore, Billerica, MA) into 150 mM NaCl, 50 mM sodium phosphate, pH 7.2, and then further purified by gel filtration chromatography (HiPrep 16/60 Sephacryl S-100 HR; Amersham Biosciences, Piscataway, NJ). The resulting ubiquitin solution was then concentrated and exchanged into ddH_2O (>18 MΩ using the Biocel by Millipore, Billerica, MA) as above. After exchange into ddH_2O, the ubiquitin solution was aliquoted into 5 mg quantities and lyophilized. The yield of the protein following purification was approximately 20−30 mg of pure protein per liter of cell culture.

Reverse Micelle Sample Preparation. AOT was purchased from Sigma-Aldrich (St. Louis, MO)and used without further purification. Approximately 0.029 g of AOT and 650 μL of *n*-pentane-d_{12} (Cambridge Isotope Laboratories Inc., Andover, MA) were added to a screw-cap NMR tube (Wilmad LabGlass, Buena, NJ) to make a 100 mM AOT solution. ^{15}N isotopically enriched ubiquitin (5 mg) was hydrated in either ddH_2O or the appropriate buffer. The protein solution was added to the 100 mM AOT solution and shaken vigorously for several minutes. Two types of reverse micelle samples were prepared, which were identical except for the ionic strength of the reconstitution buffer used to prepare the samples. One buffered, salt containing sample was generated using 50 mM NaOAc, pH 5.0, and 1.0 M NaCl to probe the effects of varied levels of confinement and determine the stability of the ternary system at low temperature. The pH of the NaOAc buffer was adjusted with HCl. Other unbuffered samples were made with ddH_2O, which were used to investigate the process of protein shedding. Note that as described in the Results, under elevated ionic strength conditions, protein shedding was abolished.

BSA-Crowded Sample Preparation. Purified (≥99%) bovine serum albumin (BSA) was purchased from Sigma-Aldrich and used without further purification. Molecularly crowded samples consisted of 5 mg of ^{15}N labeled ubiquitin and 36.9 or 74.8 mg of BSA in 650 μL total volume of ddH_2O and 12% D_2O, generating ∼1:1 and ∼1:2 molar ratios of ubiquitin:BSA, respectively. Importantly, since BSA has a molecular weight approximately 7 times larger than ubiquitin, it yields biologically relevant crowding conditions.

NMR Spectroscopy. The bulk of the experiments were recorded on a Varian INOVA 500 MHz ^{1}H NMR spectrometer using either a Varian broad band indirect single-axis (*z*-coil) PFG probe or a Varian broad band triple-resonance (HCN), triple-axis (*z*-coil) PFG probe. The single-axis PFG probe was designed for use over a broad temperature range, from −150 to 100 °C, and was employed for all low-temperature experiments, including those documenting low-temperature phenomena. The HCN PFG probe was used because of the higher signal-to-

(14) Munishkina, L. A.; Cooper, E. M.; Uversky, V. N.; Fink, A. L. *J. Mol. Recognit.* **2004**, *17*, 456–464.
(15) Konopka, M. C.; Shkel, I. A.; Cayley, S.; Record, M. T.; Weisshaar, J. C. *J. Bacteriol.* **2006**, *188*, 6115–6123.
(16) Babu, C. R.; Flynn, P. F.; Wand, A. J. *J. Biomol. NMR* **2003**, *25*, 313–323.
(17) Leser, M. E.; Luisi, P. L. *Chimia* **1990**, *44*, 270–282.
(18) Pometun, M. S.; Peterson, R. W.; Babu, C. R.; Wand, A. J. *J. Am. Chem. Soc.* **2006**, *128*, 10652–10653.

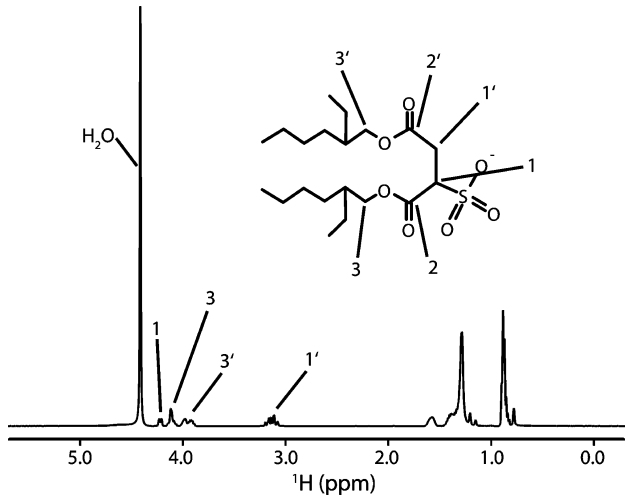

Figure 2. One-dimensional ^{1}H NMR spectrum of AOT, annotated with the resonance assignments. The sample was composed of 100 mM AOT, 5 mg ubiquitin, $w_0 = 9.9$ (measured), and 1 M NaCl in *n*-pentane-d_{12} at 20 °C.

noise ratio than available on the single-axis PFG probe, employed in the studies relating to confinement at 20 and 30 °C. To aid in peak assignment of the molecular crowded sample with a molar ratio of 1:2 ubiquitin to BSA, a Varian INOVA 600 MHz ^{1}H NMR spectrometer and Varian triple-resonance cold probe were used. Chemical shift data for the encapulated ubiqutin sample was obtained using a sensitivity-enhanced version of the heternonuclear single-quantum correlation experiment (HSQC).[19] Chemical shift data for the BSA-crowded ubiquitin sample was recorded using a transverse-relaxation enhanced (TROSY)[20] version of the HSQC experiment, which rendered narrower resonance line widths, thus providing more precise chemical shift measurements. The sample temperature was calibrated using the chemical shift difference (Δ ppm) between the two resonances of a neat methanol standard sample to within $\pm$0.1 °C of the desired temperature.[21] One dimensional ^{1}H spectra were used to directly establish the w_0 (see Figure 2). All reverse micelle confinement experiments were conducted at 20 °C, while the BSA crowding experiments were recorded at 30 °C. BSA crowding experiments were conducted at the higher temperatures to improve spectral resolution resulting from the increase in solution viscosity arising from the added BSA. Water and protein shedding experiments were conducted at −30 °C.

All ^{15}N HSQC experiments were recorded with direct and indirect dimension acquisition times of 64 and 51 ms, respectively. The ^{15}N HSQC experiment is relatively sensitive and has become a standard assay of the folded state of polypeptides, because it monitors resonances of amide groups throughout the protein.[22−24] Structural perturbations in the protein manifest themselves in spectra as a range of effects, from relatively minor chemical shift changes in the resonances, to larger chemical shift and intensity perturbation, to complete disappearance of the resonance.

Results

Water Loading and Water Shedding. Water loading, w_0, is the molar ratio of water to surfactant. This ratio dictates the size of the reverse micelle particle as well as protein hydration and other physical properties of the RM that in turn affect protein structure and dynamics.[25,26] It should also be noted that w_0 capacity depends on the ionic strength of the aqueous core.[4,16,17] Moreover, reverse micelle encapsulation uses relatively small volumes of water (tens of microliters) and hygroscopic surfactants, which makes water loading values based exclusively on formulation unreliable. Therefore, experimental measurement of the w_0 is crucial.

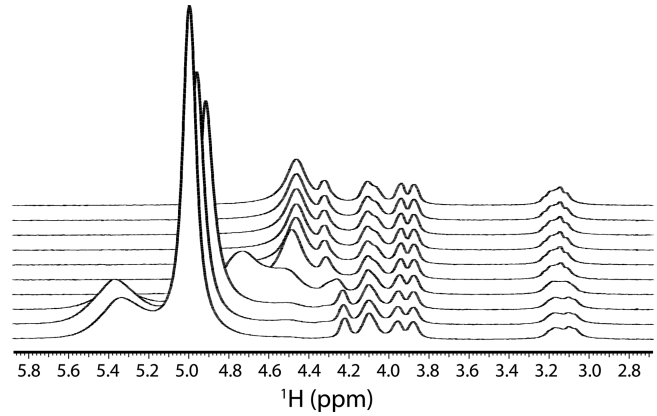

Figure 3. ^{1}H NMR data recorded at 500 MHz (^{1}H) showing that water is shed from the protein-containing reverse micelle particle. The sample is composed of 100 mM AOT, 5 mg ubiquitin, w_0 initial = 21.75, and w_0 final = 3.64. Spectra were recorded at 1 min intervals for a total time of 10 min, until equilibrium was reached.

The w_0 is readily and accurately determined based on the ratio of the integral of the resonance intensities of water and the AOT C1′ methylene protons derived from a ^{1}H 1D NMR spectrum (see Figure 2).

Exposure of reverse micelles to low temperatures has been shown to decrease the w_0 capacity of a given sample.[4] The temperature-driven change in water loading is termed water shedding and has been extensively characterized in simple reverse micelle samples, e.g. in the absence of encapsulated protein.[6] In addition, we find that this phenomenon also persists in the presence of coencapsulated proteins. The behavior of reverse micelles, with respect to water shedding, is essentially the same with or without protein. The process of water shedding in the presence of ubiquitin under low ionic strength conditions, as monitored using a time-series of one-dimensional ^{1}H spectra, is shown in Figure 3. Initially, two resonances at ∼5.4 and ∼5.0 ppm are observed, which are assigned to the ^{1}H resonance of water transiently dissolved in *n*-pentane and encapsulated within the reverse micelle water, respectively. Exposure to low temperature causes the water to withdraw from the reverse micelle interior and collect at the bottom of the NMR sample tube in the form of ice (Figure 3). Under low ionic strength the water shedding reaches equilibrium within minutes, e.g., less than 6 min, whereas, under elevated ionic strength conditions, the kinetics of water shedding proceeds more slowly and requires tens of minutes to reach equilibrium.[6]

Procedure for Systematically Exploring Protein Confinement within Reverse Micelles. As stated previously, water shedding can be used to systematically vary the amount of water

(19) Lewis, K. E.; Keifer, P.; Saarinen, T. *J. Am. Chem. Soc.* **1992**, *114*, 10663–10665.
(20) Pervushin, K.; Riek, R.; Wider, G.; Wuthrich, K. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 12366–12371.
(21) Van Geet, A. L. *Anal. Chem.* **1970**, *42*, 679–680.
(22) Kuwajima, K.; Kim, P. S.; Baldwin, R. L. *Biopolymers* **1983**, *22*, 59–67.
(23) Roder, H.; Wüthrich, K. *Proteins* **1986**, *1*, 34–42.
(24) McIntosh, L. P.; Griffey, R. H.; Muchmore, D. C.; Nielson, C. P.; Redfield, A. G.; Dahlquist, F. W. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 1244–1248.
(25) Klyachko, N. L.; Levashov, P. A.; Levashov, A. V.; Balny, C. *Biochem. Biophys. Res. Commun.* **1999**, *254*, 685–688.
(26) Rariy, R. V.; Bec, N.; Saldana, J.-L.; Nametkin, S. N.; Mozhaev, V. V.; Klyachko, N. L.; Levashov, A. V.; Balny, C. *FEBS Lett.* **1995**, *364*, 98–100.

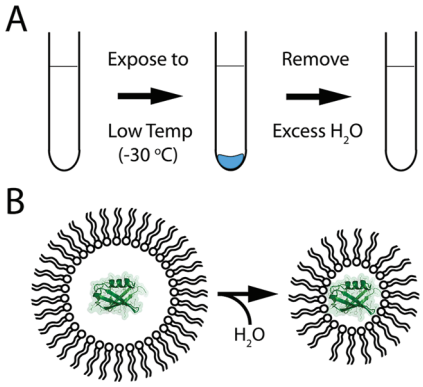

Figure 4. (A) Schematic diagram detailing the process of using water shedding as an extraction method to systematically probe the effects of confinement. Water is shed from the reverse micelle particle after exposure to low temperature and collects in the bottom of the NMR tube. Shed water is removed from the NMR tube by raising the sample temperature and pipetting off the excess water. (B) Diagram showing the relationship between w_0, the size of the reverse micelle, and change in the degree of confinement the protein experiences.

within a reverse micelle sample. Water is shed from the reverse micelles at low temperatures and collects on the bottom of the NMR tube. We have previously determined that water shed from a reverse micelle sample with an original concentration of 1.5 M NaCl, had a effective concentration of $\sim 3 \times 10^{-8}$ M after shedding, as measured by conductance.[4] This water can be easily removed by pipetting to stabilize a lower water loading, as detailed in Figure 4A. Once water is collected at the bottom of the NMR tube it is slowly taken back up into the sample under passive conditions at a rate of about one water loading unit per day.[4] This slow water uptake can be exploited to generate samples with specific intermediate w_0 values. The procedure of removing water from reverse micelles via water shedding is shown schematically in Figure 4. Water or buffer may be subsequently added to adjust the final w_0 value to a precise target value. Alternatively, water shed from reverse micelles may be rapidly and quantitatively returned to the particle by vigorous shaking.

Water loading dictates the size of the reverse micelle, and therefore, the confinement and solvation conditions that the protein experiences also change with water loading, as shown schematically in Figure 4B. In this way, the degree of confinement of an encapsulated protein can be varied both simply and precisely. A wide variety of surfactants and mixtures of surfactants and cosurfactants can also be used, which allows the chemical character of the interior surface of the reverse micelle and effective nature of the confinement to be varied. The range of physical characteristics of the various surfactants can in turn be employed to evaluate the response of the protein to the combination of confinement, desolvation, and widely varying electrostatic environments present in the encapsulated state. Thus, RM-based confinement methods have an important role to play in studies of the influence of excluded volume effects on proteins.

Reverse Micelle Confinement of Ubiquitin. A single reverse micelle sample of encapsulated ubiquitin was used to conduct the confinement studies. The sample was formulated with an initial water loading of 21.3. Exposure to low temperature (−30 °C) led to a reduction in the water loading to 5.7. The influence of confinement was characterized by analysis of chemical shift changes in the amide correlations present in ^{15}N HSQC spectra. The changes in chemical shifts ($\Delta\delta$) were calculated using the following equation:[27−29]

$$\Delta\delta_{\text{combined}} = 0.25\sqrt{[\delta(^1\text{H}_{\text{free}}) - \delta(^1\text{H}_{\text{bound}})]^2 + \{1/5[\delta(^{15}\text{N}_{\text{free}}) - \delta(^{15}\text{N}_{\text{bound}})]\}^2}$$

Residue V17 had the largest $\Delta\delta$ value of 0.10 ppm. Results of the $\Delta\delta$ analysis for the samples with w_0 values of 5.7 and 21.3 are plotted as a function of residue number in Figure 5. Residues containing amide resonances that exhibit the largest chemical shift changes (top 20%) are designated in red on the structural model of ubiquitin shown in the insets for Figure 5. In addition to the $\Delta\delta$ analysis summarized in Figure 5, the raw unprocessed chemical shift differences are included in the Supporting Information.

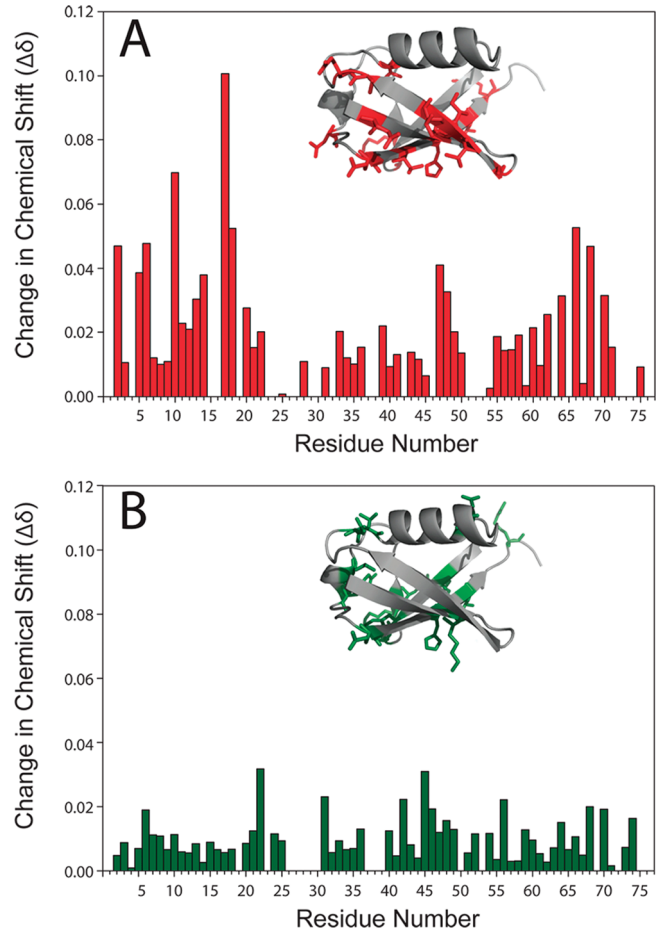

Figure 5. Comparison of reverse micelle confinement and macromolecular crowding effects on ubiquitin. Figure 5A details changes in chemical shifts of the ^{15}N HSQC spectra of a reverse micelle sample in which w_0 was changed from 21.3 to 5.7 ($\Delta w_0 \sim 16$) via water shedding. Figure 5B compares the changes in chemical shifts from an uncrowded ubiquitin sample to a 1:2 molar ratio of ubiquitin to BSA sample. Inset models in parts A and B map the most significant chemical shift perturbation on to the structure of ubiquitin.

(27) Grzesiek, S.; Bax, A.; Clore, G. M.; Gronenborn, A. M.; Hu, J. S.; Kaufman, J.; Palmer, I.; Stahl, S. J.; Wingfield, P. T. *Nat. Struct. Biol.* **1996**, *3*, 340–345.
(28) Cheever, M. L.; Sato, T. K.; de Beer, T.; Kutateladze, T. G.; Emr, S. D.; Overduin, M. *Nat. Cell Biol.* **2001**, *3*, 613–618.
(29) Fisher, R. D.; Wang, B.; Alam, S. L.; Higginson, D. S.; Robinson, H.; Sundquist, W. I.; Hill, C. P. *J. Biol. Chem.* **2003**, *278*, 28976–28984.

The most prominent area of perturbation is along a single surface of the protein which is both electropositive and hydrophobic; however, there is also some general perturbation in the loop regions (see Figure 5). This region of the protein surface, the I44 face, has previously been identified in numerous studies as a promiscuous binding face of the protein. The I44 surface binds many different families of proteins, including UBAs (ubiquitin-associated domains), UIMs (ubiquitin-interacting motifs), CUE (coupling of ubiquitin to ER degradation), and NZFs (Nlp14 zinc fingers).[29−35]

BSA-Crowding Effects on Ubiquitin. BSA is commonly employed as a crowding agent because it is relatively inert, commercially available, and soluble at high concentrations.[13,36] ^{15}N HSQC spectra of ubiquitin crowded by either 1:1 or 1:2 molar ratios of ubiquitin to BSA were compared to a spectrum of free solution ubiquitin using similar criteria employed for the reverse micelle confinement studies. The 1:1 and 1:2 molar samples of ubiquitin to BSA represent total protein concentrations of about 238 and 469 mg/mL, respectively. Under these circumstances, we note the presence of highly localized changes in the chemical shifts of NMR resonances in ubiquitin, indicating that some regions of the protein are more susceptible to excluded volume effects than others. Mapping the larger shifts onto the structure of ubiquitin suggests that there are local perturbations caused by the elevated concentrations of BSA. The location of the change in chemical shifts on the protein surface corresponds very well to the I44 binding face of ubiquitin. For the BSA-crowded ubiquitin sample, residues T22, Q31, R42, F45, and L56 experience the largest chemical shift perturbation in the 1:2 molar ubiquitin:BSA sample. A comparison of the results obtained from the 1:2 ubiquitin:BSA macromolecular crowding experiments and those obtained for the reverse micelle confinement experiments is shown in Figure 5B.

Discussion

Comparison of BSA-Crowding and RM Confinement Studies of Ubiquitin. A comparison of changes in the chemical shifts of resonances in ubiquitin that arise under the influence of excluded volume, either by reverse micelle confinement or BSA crowding, indicates that the protein is locally susceptible to perturbation under confining environments. The perturbation occurs along the biologically relevant I44 face of the protein, which binds various types of proteins in different families. The similarity of perturbation between reverse micelle confinement and BSA crowding is demonstrated in Figure 6. The data presented herein supports the generality of use of reverse micelle confinement as a valuable tool to investigate the influence of excluded volume on the stability of proteins.

While the major effects of excluded volume, as determined by ^{15}N HSQC experiments, is independent of whether reverse micelle confinement or BSA crowding is used, there are some subtle differences. We note that there are more resonances with significant shifts ($\Delta\delta$) in reverse micelle confinement than we observe with BSA crowding. The most direct and probable explanation for greater changes in chemical shifts in the reverse micelle system is that the level of confinement is greater. The less effective crowding of the protein by BSA is due to the relatively large size of BSA, which limits the ability of the crowding agent to pack around the smaller probe protein molecule, ubiquitin. The quantitative differences in the measured $\Delta\delta$ values arise from differences in the average electronic environment experienced by each amide ^{1}H/^{15}N pair, as described in the following paragraph.

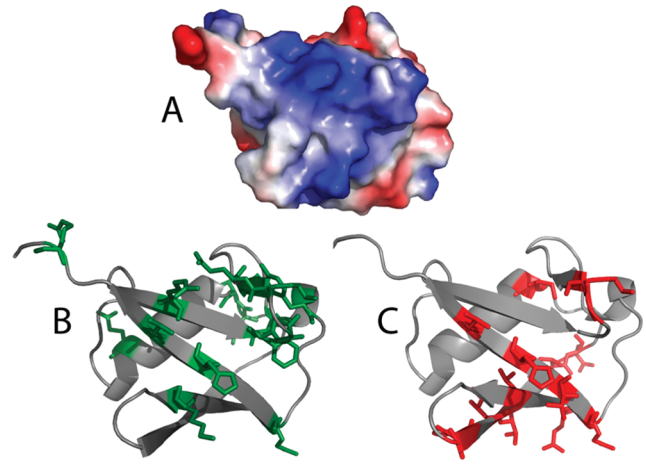

Figure 6. Comparison of the electrostatic surface of ubiquitin with resonances that are perturbed by excluded volume effects arising from either BSA crowding (B) or reverse micelle confinement (C).

The observed chemical shift, δ, is a manifestation of chemical shielding, σ. The convention in solution NMR studies of course is to report a chemical shift relative to a reference compound (TMS, DSS etc.), e.g., $\delta \propto \sigma_{ref} - \sigma$, and the chemical shielding scales the magnetic field present at the nucleus by the factor $1 - \sigma$. The applied and effective local magnetic fields may not be aligned; in which case the chemical shielding may be represented mathematically as a second rank tensor, and thus, the chemical shift is spatially anisotropic. In isotropic liquids only an average or isotropic shift is observed, which is proportional to the simple average of the three principal components of the shielding tensor. In addition to the averaging of the principal shielding components, the individual values of the shielding components may be different for the individual molecules in the sample, and these differences may be time dependent. Chemical shielding (and thus the chemical shift) can be decomposed into local (through bond) substituent effects and remote effects. Local substituent effects can change the local electronic structure around the probe nucleus, e.g., electron-withdrawing and -donating groups can decrease or increase local electron density, leading to changes in the individual components of the chemical shift tensor (and thus to changes in the chemical shift anisotropy). In addition, the chemical shift tensor could be influenced both by interaction with remote parts of the same molecule (as in remote residues of a protein brought together by the folded state of the molecule), by specific and nonspecific interactions with other molecules including the solvent, and by specific and nonspecific interactions with the sample container, e.g., the interior surface of the reverse micelle. These remote effects arise due to the variations in the magnetic susceptibilities

(30) Ponting, C. P. *Biochem. J.* **2000**, *351*, 527–535.
(31) Alam, S. L.; Sun, J.; Payne, M.; Welch, B. D.; Blake, B. K.; Davis, D. R.; Meyer, H. H.; Emr, S. D.; Sundquist, W. I. *EMBO J.* **2004**, *23*, 1411–1421.
(32) Wang, Q.; Goh, A. M.; Howley, P. M.; Walters, K. J. *Biochemistry* **2003**, *42*, 13529–13535.
(33) Wang, B.; Alam, S. L.; Meyer, H. H.; Payne, M.; Stemmler, T. L.; Davis, D. R.; Sundquist, W. I. *J. Biol. Chem.* **2003**, *278*, 20225–20234.
(34) Beal, R.; Deveraux, Q.; Xia, G.; Rechsteiner, M.; Pickart, C. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 861–866.
(35) Verma, R.; Peters, N. R.; D'Onofrio, M.; Tochtrop, G. P.; Sakamoto, K. M.; Varadan, R.; Zhang, M.; Coffino, P.; Fushman, D.; Deshaies, R. J.; King, R. W. *Science* **2004**, *306*, 117–120.
(36) Rivas, G.; Fernandez, J. A.; Minton, A. P. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 3150–3155.

of the chemical groups, from polarization of bonds due to the presence of electric fields, and from van der Waals interactions.[37]

The observed chemical shift changes that occur with crowding and confinement are the result of several possible effects, subject to tensor and time and ensemble averaging. Changes in the average structure that arise from changes in excluded volume will contribute to chemical shift changes, as can changes in the local dynamics within the protein. Modulation of the local electric field around the amide groups, which might occur as a protein (ubiquitin) undergoes a weak transient interaction with the surface of the crowding protein (BSA) or the surface of the reverse micelle, may also contribute to changes in the chemical shifts. What is remarkable is that the observed chemical shift changes appear to be independent of the manner in which the excluded volume is changed. The interior surface of the reverse micelle is concave and composed essentially of sulfonate headgroups with relatively small sodium counterions and water molecules. By contrast, the surface of BSA is complex but generally convex (roughly heart shaped), and a detailed inspection of the surface of the very closely related protein HSA suggests a relatively broad spatial distribution of ionizable groups. Note that there is no published structure for BSA; however, the primary sequences of the proteins share nearly 80% primary sequence homology, and we have adopted the common practice of using the HSA structure as a proxy for visualizing the surface of BSA. Although it is possible that effects such as weak RM−ubiquitin or BSA−ubiquitin interactions may contribute to the observed chemical shift changes, such effects must be very similar in the two cases, which seems unlikely given the chemical and physical differences mentioned above.

Importantly, a previous study of fast motion dynamics of ubiquitin in the encapsulated state has revealed that residues are differentially influenced by confinement, with H−N bond vectors that have the lowest order parameters experiencing the largest increase in restriction.[5] In addition to the analysis of fast local motion, a comparative analysis of chemical exchange was also conducted. Two previous studies of chemical exchange in the ^{15}N NMR relaxation conducted on ubiquitin by Bax and co-workers and by Palmer and co-workers formed the basis of our comparative analysis.[38,39] The results generated by the Bax and Palmer groups do not agree exactly, but interestingly, consensus residues that are identified as having chemical exchange contributions to relaxation are the same ones identified in the analysis of relaxation for encapsulated ubiquitin. This comparative examination of chemical exchange demonstrates that encapsulation does not significantly promote chemical exchange on the microsecond to millisecond time scale.

Results from the current study establish that resonances with the largest chemical shift perturbation are correlated with residues that experience larger effects on local backbone dynamics. Thus, while a variety of effects may contribute to the chemical shift changes induced by crowding or confinement, a significant component appears to arise due to changes in local motion that are in turn due to the imposed excluded volume effects.

The results presented in this paper provide an important new comparative analysis of crowding effects and encapsulation. Chemical shift perturbation analysis has previously been used to identify changes in proteins that result from changes in the solution conditions and, importantly, also to map residues in proteins that bind small molecules (SAR by NMR), peptides, or other proteins. Results indicate that the influence of macromolecular crowding produces local effects (assayed at the level of amide H and N pairs) that are very similar to those produced by reverse micelle encapsulation. Although the quantitative magnitudes of the perturbation vary somewhat between crowding and encapsulation-based confinement, the general trends are clear.

One potential concern of reverse micelle based systems as a platform for investigating confinement is that the interior surface of the reverse micelle may interact with the surface of the encapsulated protein. Indeed, previous studies have revealed RM−protein interactions, both in cases in which the interior RM and protein surfaces are complementary, e.g., highly basic proteins using AOT, and also a case in which a reconstitution buffer of low ionic strength was employed at low w_0.[4,40−44] We observe that, although the external surface of BSA and the interior surface of AOT are chemically distinct, the influence of both are surprisingly similar, and thus, properly reconstituted RM systems represent an excellent model system.

A comparison of the results presented here with those recently obtained using in-cell overexpression methods and microinjection techniques reveals the importance of continued development of the encapsulation approach.[45−55] Although promising, the overexpression approach has been shown to be complicated in some cases by protein export from cells.[45,46,56] It is currently unclear how general this phenomenon may be. Wagner and coworkers have recently applied a microinjection approach to introduce purified and concentrated protein into oocytes from *Xenopus laevis*.[54,55] This system represents a genuine eukaryotic environment with significant advantages for investigating the

(37) Sitkoff, D.; Case, D. A. *Prog. NMR Spectrosc.* **1998**, *32*, 165–190.
(38) Massi, F.; Grey, M. J.; Palmer, A. G., III *Protein Sci.* **2005**, *14*, 735–742.
(39) Tjandra, N.; Feller, S. E.; Richard, W.; Pastor, R. W.; Bax, A. *J. Am. Chem. Soc.* **1995**, *117*, 12562–12566. Tjandra, N.; Feller, S. E.; Richard, W.; Pastor, R. W. *J. Am. Chem. Soc.* **1995**, *117*, 12562–12566.
(40) Melo, E. P.; Baptista, R. P.; Cabral, J. M. S. *J. Mol. Catal. B-Enzym.* **2003**, *22*, 299–306.
(41) Melo, E. P.; Costa, S. M. B.; Joaquim, M. S.; Cabral, P. J.; Peterson, S. B. *Phys. Lipids* **2003**, *124*, 37–47.
(42) Peterson, R. W.; Pomentum, M. S.; Shi, Z.; Wand, A. J. *Protein Sci.* **2005**, *14*, 2919–2921.
(43) Shi, Z.; Peterson, R. W.; Wand, A. J. *Langmuir* **2005**, *21*, 10632–10637.
(44) Shioi, A.; Harada, M.; Takahashi, H.; Adashi, M. *Langmuir* **1997**, *13*, 609–613.
(45) Bryant, J. E.; Lecomte, J. T. J.; Lee, A. L.; Young, G. B.; Pielak, G. J. *Biochemistry* **2005**, *44*, 9275–9279.
(46) Bryant, J. E.; Lecomte, J. T. J.; Lee, A. L.; Young, G. B.; Pielak, G. J. *Biochemistry* **2006**, *45*, 10085–10091.
(47) Dedmon, M. M.; Patel, C. N.; Young, G. B.; Pielak, G. J. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, 12681–12684.
(48) Serber, Z.; Corsini, L.; Durst, F.; Doetsch, V. *Method Enzymol.* **2005**, *394*, 17–41.
(49) Serber, Z.; Doetsch, V. *Biochemistry* **2001**, *40*, 14217–14323.
(50) Serber, Z.; Keatinge-Clay, A. T.; Ledwidge, R.; Kelly, A. E.; Miller, S. M.; Doetsch, V. *J. Am. Chem. Soc.* **2001**, *123*, 2446–2447.
(51) Serber, Z.; Ledwidge, R.; Miller, S. M.; Doetsch, V. *J. Am. Chem. Soc.* **2001**, *123*, 8895–8901.
(52) Serber, Z.; Straub, W.; Corsini, L.; Nomura, A.; Shimba, N.; Craik, C.; Ortiz de Montellano, P.; Dötsch, V. *J. Am. Chem. Soc.* **2004**, *126*, 7119–7125.
(53) Sakai, T.; Tochio, H.; Tenno, T.; Ito, Y.; Kokubo, T.; Hiroaki, H.; Shirakawa, M. *J. Biomol. NMR* **2006**, *36*, 179–188.
(54) Selenko, P.; Serber, Z.; Gadea, B.; Ruderman, J.; Wagner, G. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 11904–11909.
(55) Selenko, P.; Wagner, G. *Nat. Methods* **2006**, *3*, 80–81.
(56) Bryant, J. E.; Lecomte, J. T.; Lee, A. L.; Young, G. B.; Pielak, G. J. *Biochemistry* **2007**, *46*, 8206.

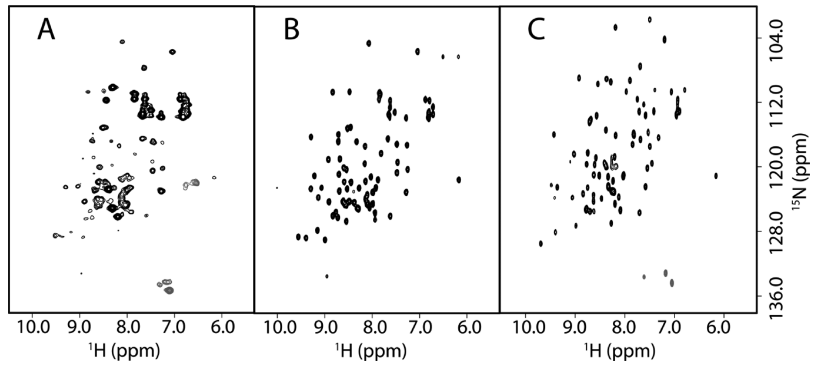

Figure 7. ^{15}N HSQC spectra of ubiquitin (A) crowded using BSA, (B) free in solution, and (C) confined within reverse micelles. All samples contain 5 mg of protein. The spectra are plotted at different contour levels: the level of spectrum A is 10 times lower than that of spectrum B, which is two times higher than spectrum C.

influence of a complex environment on protein stability. Nevertheless, production of these samples is extremely challenging, and as with the in-cell studies, the lifetimes of the samples are relatively limited. On the other hand, encapsulation of proteins within reverse micelles has been shown to be compatible with proteins having a wide variety of sizes and chemical properties, with practical advantages such as relatively long sample lifetime and significantly superior spectroscopic resolution and sensitivity.

Benefits of Reverse Micelle Confinement over Crowded Solutions. The results of crowding experiments are generally difficult to investigate at atomic detail, and NMR is one of a few techniques that can provide the required high-resolution perspective. Nevertheless, at biologically relevant concentrations of crowding agents (ca. 300−400 mg/mL), the protein rotational correlation time (τ_c) is increased to values that result in rapid transverse relaxation, which in turn limits the resolution and sensitivity of the NMR experiment.[1,8] While advances in transverse relaxation-optimized spectroscopy (TROSY) have helped to overcome these limitations, they remain a significant challenge.[20] A comparison of ^{15}N HSQC spectra recorded for ubiquitin under the conditions of BSA-mediated crowding with encapsulation-based confinement (Figure 7) illustrates the problems associated with NMR-based crowding studies, as well as the spectroscopic advantages of employing reverse micelle confinement studies. It should be noted that the spectra in Figure 7 are plotted at different levels. The spectrum of the ubiquitin standard is plotted at a level that is twice as high as that used for the spectrum of the reverse micelle confined ubiquitin sample, which is itself plotted at a level that is 10-fold higher than that used for the spectrum from the BSA-crowded ubiquitin sample. It should also be mentioned that it was necessary to record the BSA crowded sample at a temperature elevated by 10 °C relative to the conditions used for the uncrowded and reverse micelle encapsulated ubiquitin samples, e.g., 30 °C versus 20 °C, to enhance sensitivity and resolution of the resulting spectrum by lowering the viscosity of the solution, and in turn increasing the tumbling rate.

Reverse micelle confinement studies have two major experimental benefits relative to the analogous macromolecular crowding experiments. First, there is greater inherent flexibility with reverse-micelle-based systems, which arises from the fact that the w_0 can be adjusted over a wide range and that the physical properties of the confining surface can be tuned by varying the composition of the surfactant mixture. Second, a single sample may be used to probe a range of confinement, thus minimizing concerns related to effort and reproducibility of the samples, which is a key challenge in studies of this type.

Reverse micelle structures result from a remarkable balance of plasticity and flexibility. In addition, there are a number of readily available, chemically diverse reverse-micelle-forming surfactants. This has very significant implications for reverse micelle confinement studies, since one can use cationic, anionic, nonionic, or mixtures of reverse micelle forming surfactants to tune the confinement that best approximates a particular in vivo condition.

Cellular conditions are comprised of a myriad of various biomolecules of various shapes and sizes, which include proteins, nucleic acids, lipids, and a host of small organic and inorganic molecules. The size and geometric diversity of the in vivo population supports a high degree of confinement. In contrast, BSA is relatively large (∼66 kDa) and homogeneous, which inherently limits the degree of crowding that can occur. For example, the 1:2 molar ratio of ubiquitin to BSA sample contains approximately 469 mg/mL of BSA and ubiquitin, which superficially might be considered to represent a concentration that is slightly greater than the typical in vivo situation. Although crowding is most commonly discussed in casual concentration units such as mg/mL, a more precise and useful definition can be made in terms of excluded volume effects. The interior volume of the cell is typically occupied by macromolecules at about 20−30%.[8] In the 1:2 ubiquitin:BSA crowded sample, only about 8.5% of the volume is occupied by BSA. Moreover, under the conditions which we used to study the BSA crowding effects on ubiquitin, with a ubiquitin concentration of ∼0.75 mM, there are only two BSA molecules per molecule of ubiquitin, which is generally thought to be much more dilute crowding than the protein would encounter in the cell. Reverse micelle confinement, on the other hand, is a much more effective probe of excluded volume, due to the shape complementarity of the interior surface of the reverse micelle and the exterior surface of the protein. Under the limits of BSA crowding used in this study, simple calculations reveal that the distance between protein surfaces is about 80 Å, which is larger than the diameter of a reverse micelle particle. Thus, reverse micelles can provide greater confinement than can practically be obtained using crowding agents composed of a single large protein because they provide a chamber that is complementary to the shape of most single-domain proteins and globular multimeric protein complexes.

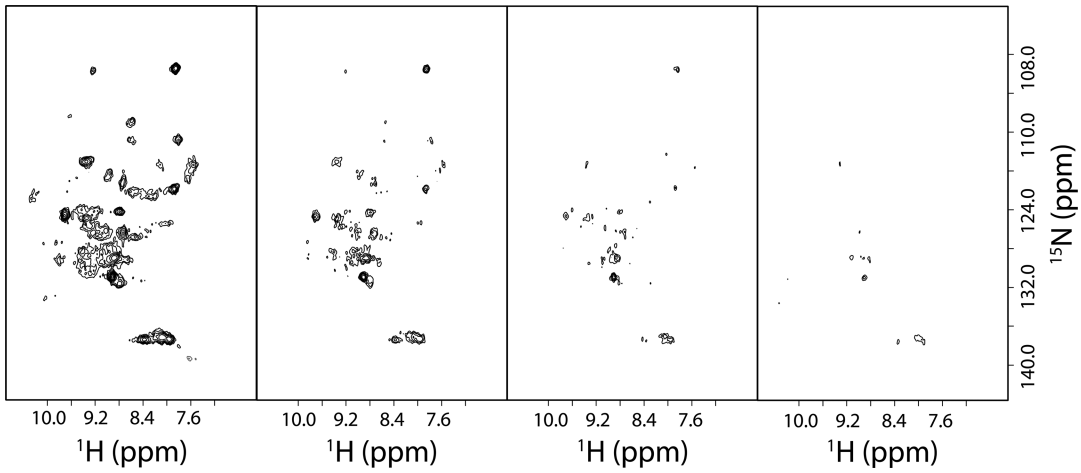

Figure 8. ^{15}N HSQC experiments of encapsulated ubiquitin at −30 °C. Each experiment was taken in 3 h increments for a total of 12 h with measured $w_0 = 4.29$, 3.48, 3.33, and 3.31, respectively. This series of data shows the protein shedding from samples prepared using buffers of low ionic strength. The sample was composed of 100 mM AOT, 5 mg ubiquitin, and ddH_2O core (no salt) in 650 μL of *n*-pentane-d_{12}.

Time-Dependent Effects on Encapsulated Protein. Careful monitoring of ubiquitin and water concentrations by NMR at low temperature reveals two time-dependent changes in the RM−protein system. The investigation of excluded volume effects with reverse micelle encapsulation requires water shedding for optimal control over the degree of confinement. A series of spectra used to monitor the time-dependent loss of water is shown in Figure 3. The degree of water shedding is inversely proportional to the ionic strength of the encapsulated aqueous solution, and water shedding occurs both in the presence and absence of encapsulated protein.[4,6] During efforts to probe the influence of extreme confinement via water shedding, we observed that analogous protein shedding can also occur. The loss of encapsulated protein from reverse micelles, like water shedding, is inversely proportional to the ionic strength of the coencapsulated aqueous buffer. We initially observed that exposure of a low ionic strength sample of encapsulated ubiquitin to low temperature resulted in a time-dependent decrease of resonance intensity from ubiquitin in ^{15}N HSQC experiments (Figure 8). A search for the physical origin of the loss of resonance intensity reveals an intriguing phenomenon. In an effort to establish whether protein was expelled from the RMs and shed to the bottom of the NMR tube (as is the case of encapsulated water), encapsulated ubiquitin was prepared by dissolving the protein in a low ionic strength buffer, and that sample was held at low temperature for several hours. The sample was subsequently warmed to room temperature and the aqueous phase was removed. Every effort was made to minimize agitation of the sample during the removal of the water shed from the reverse micelles. Curiously, no signal was observed in ^{15}N HSQC spectra recorded on the resulting reverse micelle sample nor in the expelled aqueous fraction. Remarkably, following agitation of the sample, signals in the ^{15}N HSQC returned to the previous intensity, suggesting that the protein may adhere to the NMR tube walls as it is shed. It thus appears that exposure of the encapsulated proteins system to low temperature drives two processes, water shedding and an analogous protein shedding. The time scales of the two effects are very distinct, as shown in Figure 3, which confirms that water shedding reaches equilibrium in a matter of minutes, whereas protein shedding occurs on the time scale of hours. The resonance intensities present in ^{15}N HSQC spectra of encapsulated ubiquitin following exposure to −30 °C are charted in Figure 8. It is noteworthy that samples reconstituted using higher ionic strength (e.g., 1 M NaCl) had no tendency to shed protein. This observation is in agreement with the literature reports that confirm the stabilizing effects of ionic strength on the physical properties of reverse micelles.[4,6,57,58]

Impact on Low-Temperature Unfolding Studies. Studies of low-temperature protein unfolding, e.g., cold denaturation, provide an effective platform for investigating the enigmatic but fundamentally important process by which proteins adopt their stable, unique structure. Reverse micelle protein encapsulation uses low-polarity solvents that are not only useful for enhancing NMR experiments, but also, because of the low freezing points (T_f (*n*-pentane) ≈ −130 °C) of these liquids, the systems provide unique access to considerations of low-temperature phenomena.[4,6,7,18]

Expanded studies of low-temperature effects on encapsulated water and proteins have confirmed that water shedding occurs in the presence of protein. This result has important implications for encapsulation-based studies of proteins conducted at low temperatures. Hydration of the encapsulated protein has a fundamental impact on the structure, stability, and function of proteins, and the ability to vary hydration over a wide range, including very low relative levels of hydration, while also maintaining a relatively crowded/confined environment, is an important new biophysical tool. Looked at from another perspective, the combined effects of confinement and loss of hydration must be fully considered when interpreting thermodynamic and kinetic behavior of encapsulated proteins. Water shedding in low ionic strength samples has been shown to control the size and physical properties of the reverse micelle, both of which are issues that should be integrated into any comprehensive examination of physical and/or biochemical process studied in the encapsulated state.

Previous studies have shown that the ionic strength has critical influence on water shedding, which, if unappreciated and left uncompensated, e.g., through the use of buffers of moderate ionic

(57) Kon-No, K.; Kitahara, A. *J. Colloid Interface Sci.* **1970**, *33*, 124–132.
(58) Kon-No, K.; Kitahara, A. *J. Colloid Interface Sci.* **1971**, *41*, 47–51.

strength, can lead to changes in hydration and confinement that can complicate the interpretation of the low-temperature data.[4] Objections have been raised regarding the formulation of reverse micelles using buffers of moderate ionic strength.[18] Specifically, it has been suggested that a putative anion (chloride) binding site, which was discovered by Makhatasde and co-workers and is believed to be present in the C-terminal region of ubiquitin, might lead to stabilization of the encapsulated protein in buffers of high ionic strength.[59] Although the results of this calorimetric study suggested that the melting temperature increased with increasing ionic strength, the presence of a specific binding site could not be confirmed through a site-directed mutagenesis study. Moreover and importantly, the study was conducted at a pH of 2 to emphasize electrostatic interactions, and at this pH the total charge on ubiquitin in solution is approximately +13. Indeed, these circumstances would be anticipated to emphasize any latent influence of anion binding on the thermodynamics of the protein folding. Nevertheless, even under conditions of extreme pH, the dissociation constant, K_D, is estimated to be 0.15 M, consistent with a relatively weak nonspecific interaction. The conclusions of Makhatasde and co-workers present an important contribution to protein biophysics; however they have no significant relevance to published reverse micelle encapsulation studies. The pH of the encapsulation buffer used in our low-temperature study was ~5, in which case the protein has an estimated total charge of between 0 and +1, which should minimize possible anion binding and relieve any potential stabilizing influences. Of much greater potential concern would be uncompensated interactions between the protein surface and the interior surface of the reverse micelle. We are confident, based on the results of dozens of studies, that our reconstitution conditions are optimized to generate results of central importance and broad relevance.

Results presented here clarify the apparent discrepancies of previous investigations and chart a clear course for future studies. Specifically, a previous report on the cold denaturation of encapsulated ubiquitin concluded that a locally cooperative unfolding transition occurs exclusively in the region of the protein that lies near residue I44 (the so-called I44 face) at low temperature.[7,18] The work of a number of research groups has previously established that the I44 face represents a promiscuous binding face that has key functional significance.[29−35] The interpretation of local unfolding in encapsulated ubiquitin has been challenged on the basis of a subsequent study of encapsulated ubiquitin examined under different buffer conditions.[4] Here, we describe the careful analysis of the protein-shedding effect, which in turn provides a resolution of the apparent discrepancy found in studies by Wand and co-workers and our previous results. We observe that encapsulation-based low-temperature unfolding studies conducted using low ionic strength buffer conditions are unsuitable for thermodynamic analysis, since such studies occur under nonequilibrium conditions. Specifically, the loss of resonance intensities in spectra of encapsulated ubiquitin dissolved in low ionic strength buffer that are observed at low temperature is dominated by kinetic effects (protein shedding) and thus cannot properly report on the thermodynamic unfolding event. Using reconstitution conditions that stabilize the encapsulated protein against both water- and protein-shedding effects, we have shown that the temperature-dependent NMR data are consistent with a global unfolding event. The globally cooperative low-temperature unfolding event observed for ubiquitin is also supported by at least two other experimental studies of low-temperature denaturation of ubiquitin.[60,61] In addition, a comparative NMR-based study of heat and cold denaturation focusing on yeast frataxin has established that the unfolded states of the protein are the same.[62] Studies of small α/β proteins may hold the best hope for elucidating the mechanism of low-temperature unfolding. The results presented here confirm that, under appropriate conditions, encapsulation is an effective method by which to examine these proteins with the goal of elucidating structural and dynamical details of the cold denatured state.

Conclusions

Studies of excluded volume effects have historically been under-represented due to practical limitations in the methods employed to generate crowding. The data presented here demonstrate that reverse micelles provide a highly effective method for probing the influence of confinement, with time and spatial resolution that is generally greater than other currently available methods. In addition, implementation of the RM-based confinement is relatively simple compared to the use of crowding agents or in-cell approaches.

Two major new insights are established by this paper. The first is that macromolecular crowding and reverse micelle encapsulation influence the probe protein molecule in very similar ways. The use of an isotopically enriched protein allows us to make this comparison on a nearly complete per-residue basis. This result allows for a direct connection between macromolecular crowding and RM confinement, thus linking two vast and important areas of inquiry. A central concern for both crowding and confinement studies is the extent to which the imposed increase in excluded volume reproduces the scale of changes in excluded volume that exist within the cell. Our results represent a significant step forward by establishing that the influence of crowding and confinement appear to be quite general and relatively independent of the specific manner by which excluded volume is changed.

The second major new insight is that while low-temperature studies of water and proteins using reverse micelles are extremely powerful tools, such systems must be carefully monitored to confirm that the system components remain constant with respect to changes in temperature. We previously characterized the physical phenomenon of "water shedding", which involves the time-dependent loss of water from reverse micelles reconstituted from low ionic strength water cores when the system is exposed to low temperature.[4,6] Here, we follow with a more detailed consideration of the influence of low temperature on an encapsulated protein and discover that an analogous "protein shedding" can also occur. A detailed consideration of encapsulation conditions reveals that protein shedding is the likely explanation for apparently differing views on the nature of low-temperature-driven protein denaturation.[4,7,18] The current paper provides a clear strategy for the effective use of RM encapsulation in low-temperature studies of proteins.

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(59) Makhatadze, G. I.; Lopez, M. M.; Richardson, J. M., III; Thomas, S. T. *Protein Sci.* **1998**, *7*, 689–697.

(60) Ibarra-Molero, B.; Makhatadze, G. I.; Sanchez-Ruiz, J. M. *Biochim. Biophys. Acta* **1999**, *1429*, 384–390.

(61) Kitahara, R.; Okuno, A.; Kato, M.; Taniguchi, Y.; Yokoyama, S.; Akasaka, K. *Magn. Reson. Chem.* **2006**, *44*, S108–S113.

(62) Pastor, A.; Martin, S. R.; Politou, A.; Kondapalli, K. C.; Stemmler, T.; Temussi, P. A. *J. Am. Chem. Soc.* **2007**, *129*, 5374–5375.

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Supporting Information Available: Tables of chemical shifts of amide ^{1}H and ^{15}N resonances for ubiquitin (1) free in solution, (2) crowded using BSA in a 2:1 BSA:ubiquitin molar ratio as the macromolecular crowding agent, and (3 and 4) encapsulated using water loading values of 5.7 and 21.3 (see Materials and Methods for additional details). This material is available free of charge via the Internet at http://pubs.acs.org.

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