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# Preparation, characterization, and NMR spectroscopy of encapsulated proteins dissolved in low viscosity fluids

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**Abstract**

Encapsulating a protein in a reverse micelle and dissolving it in a low-viscosity solvent can lower the rotational correlation time of a protein and thereby provides a novel strategy for studying proteins in a variety of contexts. The preparation of the sample is a key element in this approach and is guided by a number of competing parameters. Here we examine the applicability of several strategies for the preparation and characterization of encapsulated proteins dissolved in low viscosity fluids that are suitable for high performance NMR spectroscopy. Ubiquitin is used as a model system to explore various issues such as the homogeneity of the encapsulation, characterization of the hydrodynamic performance of reverse micelles containing protein molecules, and the effective pH of the water environment of the reverse micelle.

## Introduction

Efficient application of modern NMR spectroscopy to study large proteins is often hindered by their long molecular correlation times, which rapidly leads to a significant deterioration in the quality of spectra that can be obtained. To help address this problem, several different methods such as extensive deuteration, transverse relaxation optimized spectroscopy and dissolution of proteins in low viscosity solvents have been developed (Wider and Wüthrich, 1999; Venters et al., 2002). The approach of encapsulating a protein in the protective environment of the water core of a reverse micelle and dissolving the entire assembly in a low viscosity solvent seeks to recover optimal molecular tumbling by taking advantage of the Stokes-Einstein relationship (Wand et al., 1998). Preparations of encapsulated protein dissolved have been shown to be suitable for solution NMR studies in low viscosity alkanes (Wand et al., 1998) and liquid carbon dioxide (Gaemers et al., 1999). The expected gain in transverse relaxation time has been demonstrated to be a function of bulk solvent viscosity (Wand et al., 1998). Importantly, the structure of encapsulated ubiquitin has been shown to be virtually identical to both the free solution and crystalline structures (Babu et al., 2001).

A reverse micelle is a stable assembly formed by water, and dissolved salts, protein, etc., surrounded by surfactant molecules in a low polarity hydrocarbon solvent (Ekwall, 1969). Although there are many different surfactants capable of forming reverse micelles, bis(2-ethylhexyl) sulfosuccinate (AOT) is most widely used for this purpose (Flynn and Wand, 2001). Physical characteristics such as size and aggregation number of empty reverse micelles (reverse micelles without protein) formed in water, AOT, and a hydrocarbon are well characterized and in such systems, the molar ratio of water to surfactant ($W_0$) dictates the size of the empty reverse micelles (Luisi et al., 1988).

Reverse micelles have long been used to study proteins in low water environment (De Gomez-Puyou and Gomez-Puyou, 1998). There are three different procedures for encapsulating water soluble proteins in reverse micelles (Luisi and Magid, 1986; Leser and Luisi, 1990). Protein in bulk aqueous solution may be extracted using a hydrocarbon solution of the sur-

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factant, leading to the so-called liquid-liquid transfer method. Alternatively, dry protein may be encapsulated by a solution of water-filled reverse micelles, leading to the solid-liquid transfer method. Finally, protein hydrated using a precisely defined quantity of water may be injected into the surfactant solution using the injection method.

The sizes of protein-filled (filled) reverse micelles have been determined using analytical ultra centrifugation, small-angle X-ray scattering and small-angle neutron scattering (Levashov et al., 1982; Sheu et al., 1986; Zampieri et al., 1986; Brochette et al., 1988). Based on these results a number of thermodynamic models describing protein uptake into reverse micelles have been developed (Caselli et al., 1988a, b; Rahaman and Hatton, 1991). Clearly, additional experimental results are needed to distinguish the models and to reliably employ them to suggest conditions for creation of protein-filled reverse micelle particle of minimal size. Although efforts to determine the conformation of encapsulated proteins have been made using optical spectroscopy (Nicot and Waks, 1996), detailed structural information on an encapsulated protein has only recently been available (Babu et al., 2001).

Successful application of reverse micelle methods to NMR-based structural studies of larger proteins depends upon several important considerations. The protein must be encapsulated in its native form to millimolar concentrations for studies involving conventional (room temperature) probe technology, although the emerging cold probe technology has been shown to be of great advantage in this context (Flynn et al., 2000). Several critical issues underlie the preparation, characterization and productive use of stable, homogeneous filled reverse micelles of minimal size. While many features of ternary phase diagrams of empty reverse micelles are applicable to the quaternary component filled reverse micelles, many are not. Here, we identify several important parameters affecting the efficiency of protein encapsulation and the resulting size of the filled reverse micelles. We also describe efficient methods for characterizing the hydrodynamic behavior and homogeneity of protein-loaded reverse micelles.

## Materials and methods

### *Chemical shift referencing in reverse micelles*

Chemical shifts of ubiquitin and other molecules in reverse micelles were referenced to internal sodium 3-(trimethylsilyl)-1-propanesulfonate (DSS). A reverse micelle sample in $d_{12}$-n-pentane was prepared using $^{13}C$, $^{15}N$ ubiquitin with 75 mM DSS included in the aqueous buffer. The final concentration of DSS in the NMR sample was 1 mM, i.e., $\sim$ 1 molecule of DSS per reverse micelle. The spectrometer was locked on a deuterium resonance present in the solvent. The methyl peak of DSS in the reverse micelles was referenced to 0.00 ppm for $^{1}H$. The $^{13}C$ and $^{15}N$ chemical shifts were indirectly referenced using the zero-point frequency ($\Xi$) ratios 0.251449530 for carbon and 0.101329118 for nitrogen (Wishart et al., 1995). To identify the difference in resonance frequencies of DSS upon encapsulation, a glass capillary tube containing 10 mM DSS in 99.9% $D_2O$ was used as an external standard in a sample containing $^{15}N$ ubiquitin/AOT/n-pentane with DSS as an internal standard. For this case, the spectrometer was locked on $D_2O$ present in the capillary. Due to the magnetic susceptibility difference between water and pentane, the methyl resonance of DSS in the reverse micelles is shifted downfield by 0.70 ppm relative to DSS in $D_2O$ (Figure 1).

### *Measuring translational diffusion*

Translational diffusion coefficients ($D_s$) were measured using a modified version of the longitudinal encode-decode (LED) experiment (Gibbs and Johnson, 1991; Altieri et al., 1995). Intense solvent lines from pentane were suppressed by a WET (Water suppression Enhanced through $T_1$ effects) pulse sequence (Ogg et al., 1994) as implemented in Varian's 'ProteinPack' package (Patt, 1992; Smallcombe et al., 1995). Experimental details are described in Figure 2. The $D_s$ of ubiquitin in reverse micelles was measured using a $^{15}N$-filtered longitudinal encode-decode pulse sequence (LED-$^{15}N$-HSQC). The pulse sequence was constructed by replacing what would normally be the initial 90° (x-phase) $^{1}H$ pulse in the sensitivity enhanced $^{15}N$-HSQC experiment (Kay et al., 1992a) with elements of the standard $^{1}H$ LED (Altieri et al., 1995) experiment. The experiment was verified using $^{15}N$ labeled ubiquitin in water and found to agree with data recorded using the $^{1}H$ LED experiment. The $D_s$ was determined by fitting the integrals of the interested

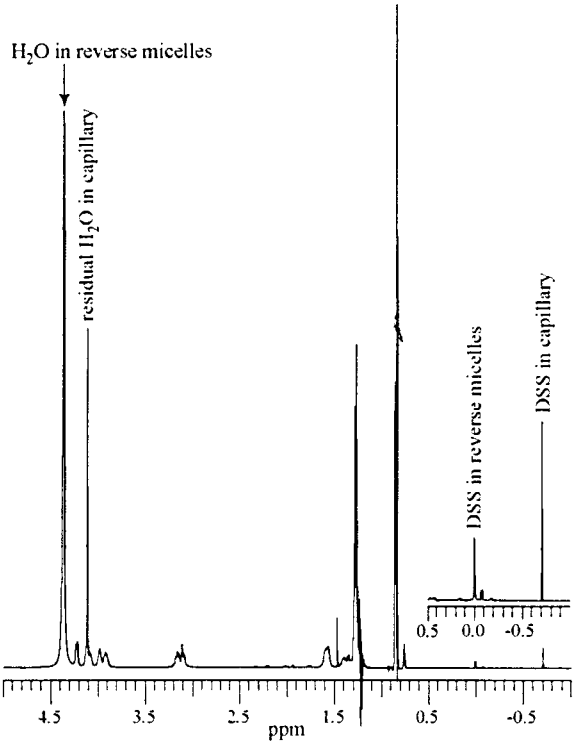


*Figure 1.* Proton 1D spectrum of ubiquitin encapsulated in AOT/pentane reverse micelles. The concentration of $^{15}$N labeled ubiquitin in 75 mM AOT/pentane reverse micelles was 0.46 mM. The $W_0$ was 10 and the buffer used for encapsulating ubiquitin was 50 mM sodium acetate (pH 5.0) containing 75 mM DSS and 250 mM NaCl. A glass capillary with 10 mM DSS in 99.9% $D_2O$ was used as an external standard. A WET sequence was used to suppress the two intense solvent lines from pentane.

signals against gradient strengths to the following equation using Sigma Plot 4.0 (SPSS Inc, IL):

$$I(G) = I(0)\exp\left[-(\gamma\delta G)^2(\Delta - \delta/3)D_s\right],$$

wherein $\gamma$ is the $^1$H gyromagnetic ratio, G represents the z-gradient strength in Gauss/cm, $D_s$ is the translational diffusion coefficient in cm$^2$ s$^{-1}$ and $\delta$ and $\Delta$ are the gradient length and diffusion interval in seconds.

*Survey of sample preparation conditions*

A single lot of $^{15}$N labeled recombinant human ubiquitin was reconstituted in water to 6 mg/ml. The pH was adjusted to 5.0 and the protein dialyzed extensively (4 × 5 L) against water over 3 days. This lot was split into 4 mg aliquots, re-lyophilized in eppendorf tubes and stored at −20 °C until needed.

Typically, the lyophilized protein (4 mg) was hydrated with distilled $H_2O$ or other aqueous solution as required (13.5 μL to give a $W_0$ of approximately 10). In a separate vial, one milliliter of AOT in pentane was prepared. The protein solution was pipetted into the vial of AOT/pentane and vortexed until the solution became clear. The $W_0$ in the final sample was calculated from the 1D data using the peak areas of water and one of the AOT resonances. To study the effect of sample composition, the concentrations of the components were changed individually while maintaining the sample preparation method. Samples were equilibrated at room temperature for approximately one week before NMR data collection. The concentration of ubiquitin in reverse micelle preparations was determined by absorbance at 280 nm using an extinction coefficient of 1400 M$^{-1}$ cm$^{-1}$. Although ubiquitin has a relatively low extinction coefficient at this wavelength, the AOT surfactant has virtually no absorbance in the near UV. To prevent sample evaporation, screw cap NMR tubes (Wilmad) fitted with PTFE seals were used.

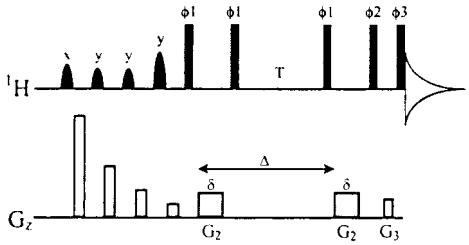


*Figure 2.* The modified WET-LED experiment used for measuring $D_s$. Selective pulses employ the SEDUCE-1 shape (Mccoy and Mueller, 1992), generated using 500 steps. The shape was convoluted to generate a frequency shifted laminar shape (Patt, 1992) which is selective for the two intense pentane solvent lines. The duration of the individual pulses were 6 ms, and tip angles of 98.2°, 80.0°, 75.0° and 152.2° were used (Smallcombe et al., 1995). Gradient pulses between the selective pulses were applied for 2 ms each with field strengths of 32, 16, 8, and 4 G/cm, respectively, followed by 2 ms delay prior to each successive seduce pulse (Smallcombe et al., 1995). The phases of the pulses in the LED portion of the experiment were identical to those described earlier (Altieri et al., 1995). $\phi1$ = 8(x), 8(−x), 8(y), 8(−y); $\phi2$ = 4(x), 8(−x), 4(x), 4(y), 8(−y), 4(y); $\phi3$ = x, y, −x, −y, 2(−x, −y, x, y), x, y, −x, −y, y, −x, −y, x, 2(−y, x, y, −x), y, −x, −y, x; $\phi_{rec}$ = 2(x, y, −x, −y), 2(−x, −y, x, y), 2(y, −x, −y, x), 2(−y, x, y, −x). The gradient G3 was applied for 500 μs with amplitude of 2.7 G/cm. The gradient G2 was applied for 6 ms and the strength was varied from 0.2 G/cm to 27 G/cm in increments of 0.2 G/cm. The gradient recovery time after G2 and G3 were 250 μs. The recycle delay was 1 s and the acquisition time was 1.8 s. The diffusion interval, T, was set to 100 ms.

*Determination of $^{15}N$ transverse relaxation times*

The transverse relaxation of $^{15}N$ nuclei was measured using a modified version of the $^{15}N$-HSQC experiment (Farrow et al., 1994). Relaxation delays of 8, 16, 24, 32, 48, 64, 80, 104 and 8 ms (to provide for error estimation) were used, together with a recycle delay of 2.0 s. Cross correlation effects during transverse $^{15}N$ relaxation were eliminated by applying $^{1}H$ 180° pulses centered in the CPMG module (Kay et al., 1992b; Palmer et al., 1992). Transverse $^{15}N$ relaxation time constants ($T_2$) were determined by fitting cross-peak intensities to a single exponential model.

## Results and discussion

*Spectroscopic challenges*

Application of modern triple resonance and total correlation spectroscopy to preparations of encapsulated proteins dissolved in low viscosity solvents presents many challenges. Because of the need to maintain low bulk solvent viscosity and to achieve long term stability, total AOT surfactant concentrations are limited and result in effective reverse micelle concentrations significantly below 1.0 mM. In addition to presenting an obvious issue of signal-to-noise, the presence of multiple, relatively intense and J-coupled solvent and surfactant resonances further compromises the quality of spectra that can be obtained. For example, a typical ubiquitin sample prepared in butane will have $\sim$ 0.5 mM protein, 75 mM AOT, 750 mM $H_2O$ and 10 M alkane solvent giving a dynamic range of $\sim 10^5$ between single resonances of the protein and the methyl resonances of the solvent. Fortunately, deuterated alkane solvents are available albeit often with only 98% isotopic enrichment. Use of complex deuterated solvents does complicate spectrometer locking but allows for acquisition of high quality multidimensional NMR data.

Because of incomplete deuteration of solvent and the unusual length of data acquisition time required to obtain sufficient signal-to-noise, the residual solvent signals generate appreciable correlated noise ('$t_1$ noise') in NOESY spectra. This results in a significant reduction in the number and quality of NOEs involving methyl resonances that can be identified and adequately quantitated. A representative $^{13}C$-slice from the 3D NOESY-$^{13}C$-HSQC is shown in Figure 3 to illustrate these points.

*Comparison of chemical shifts of ubiquitin in free solution and in reverse micelles*

Essentially complete assignments for encapsulated ubiquitin have been obtained and are deposited in the BioMagResBank under accession number 5387. Backbone chemical shifts for ubiquitin in water were generated from data recorded in aqueous solution (50 mM sodium $d_3$-acetate buffer, pH 5.0, 250 mM NaCl and 0.5 mM DSS) based on the previously published assignments (Wang et al., 1995, 1996).

Backbone chemical shifts of ubiquitin in reverse micelles were compared to those for ubiquitin in free aqueous solution. The histogram of chemical shift differences plotted against the amino acid sequence revealed that the residues 7–9, 45–49 and 71–75 have significant chemical shift deviations (Figure 4). The residues with higher chemical shift deviations seem to be in flexible regions as judged from lower generalized order parameters for N-H vectors in water (Schneider et al., 1992). Furthermore, these residues are located at the surface on one face of the protein where three (out of 4) arginines in the sequence are also located. The deviations may be the result of electrostatic interaction of the positive charge of this region of protein interacting with the negatively charged head group surface formed by the AOT surfactant, but changes in dynamic chemical shift averaging cannot be ruled out. There is, however, no evidence of significant structural changes giving rise to these localized and relatively minor chemical shift differences between encapsulated ubiquitin and ubiquitin in free solution.

Importantly, the very high correlation observed for carbon chemical shifts of residues implies that the chemical shift index of backbone carbons of proteins in reverse micelles can be used to qualitatively identify secondary structures of proteins in reverse micelles as it is done in aqueous solutions (Wishart and Sykes, 1994) since the structure of ubiquitin is virtually unchanged by encapsulation (Babu et al., 2001).

*The effective pH of encapsulated protein*

The question of pH is a critical issue for any study of protein structure and function. There is a controversy in the literature regarding whether or not the pH of the water pool inside reverse micelles can be changed (Smith and Luisi, 1980; Hasegawa, 2001). Under the condition of limited water in reverse micelles, the buffering capacity of protein must be taken into account.

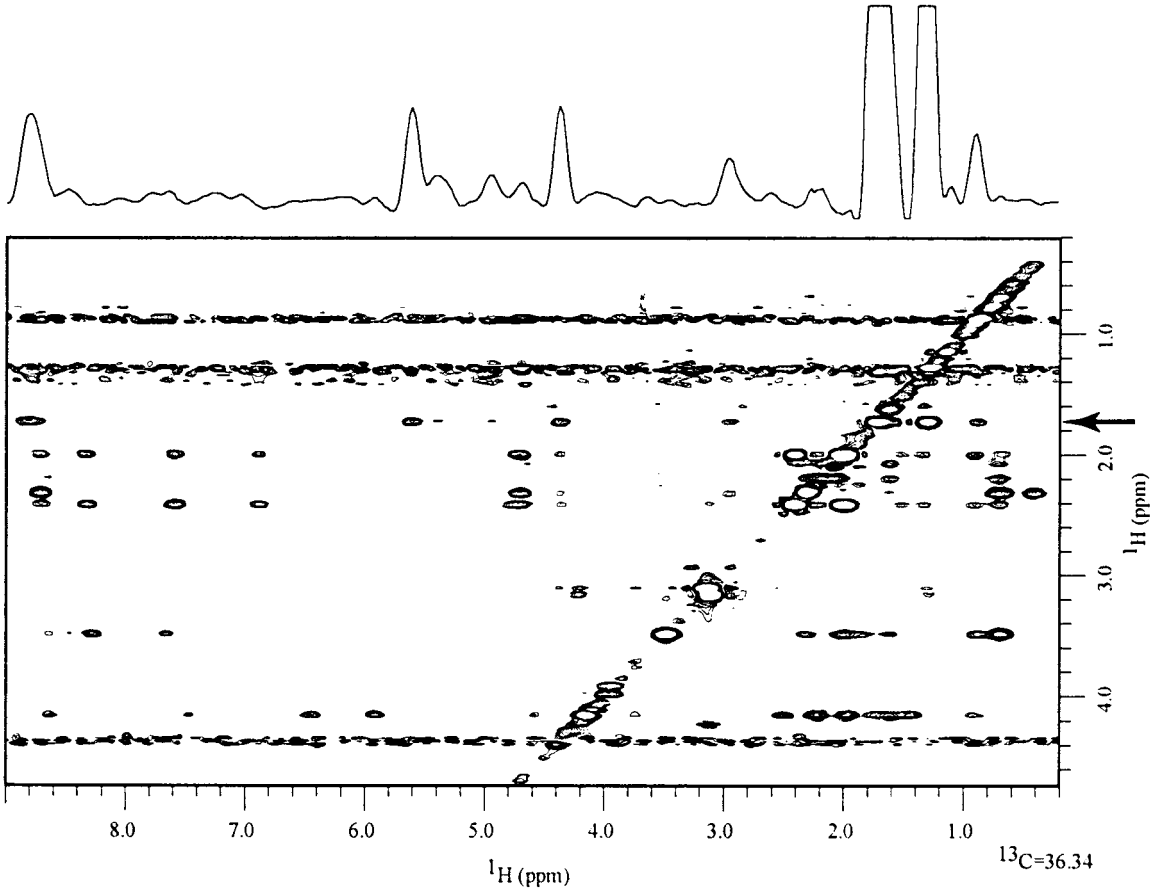


*Figure 3.* Representative slice of a 3D $^{13}$C-resolved NOESY spectrum illustrating the effects of residual solvent resonances. The streaks between 0.8 and 1.4 ppm are from residual solvent resonances and the streak at ~ 4.4 ppm is from water in reverse micelles. The 1D spectrum shown was selected at the position indicated by the arrow.

When the pH of an aqueous preparation of ubiquitin was adjusted to 5.0 prior to lyophilization, there was no change in the $^{15}$N-HSQC spectra of ubiquitin in reverse micelles prepared using water or phosphate buffer (pH 8.0) as the encapsulation solution (Figure 5A, B). This implies that the pH of the water pool cannot be changed simply by dissolving the protein in an encapsulation buffer at the appropriate pH. A simple calculation of buffer to protein molar ratio reveals the buffering capacity of the water pool in reverse micelles is very low. However, when the pH of the protein was adjusted to 8.8 prior to lyophilization and the reverse micelles were made with pure water, the cross peaks for arginine $H^{\varepsilon}$ are absent (Figure 5C), which demonstrates conclusively that the pH of the protein and the water pool has been raised towards the pKa of the side chain (Figures 5A, C).

An empirical relation has been derived relating the optimum pH for protein (25 μM) transfer into reverse micelles, the size and the pI of the protein based on liquid-liquid transfer studies (Wolbert et al., 1989). According to the expression, the pH for protein encapsulation should always be lower than the iso-electric point of the protein when AOT (containing a negative head group) is used as a surfactant. However, the ability to quantitatively transfer ubiquitin equilibrated to pH values (8.8) higher than its iso-electric point (6.5) calls this simple rule into question when using the injection method to encapsulate proteins.

Under the condition of limited water, it would appear that the protein determines the effective pH of the water pool. Furthermore, the complete absence of multiple resonances arising from individual charged states of titratable side chains requires that the water pools of individual reverse micelles be averaged on a

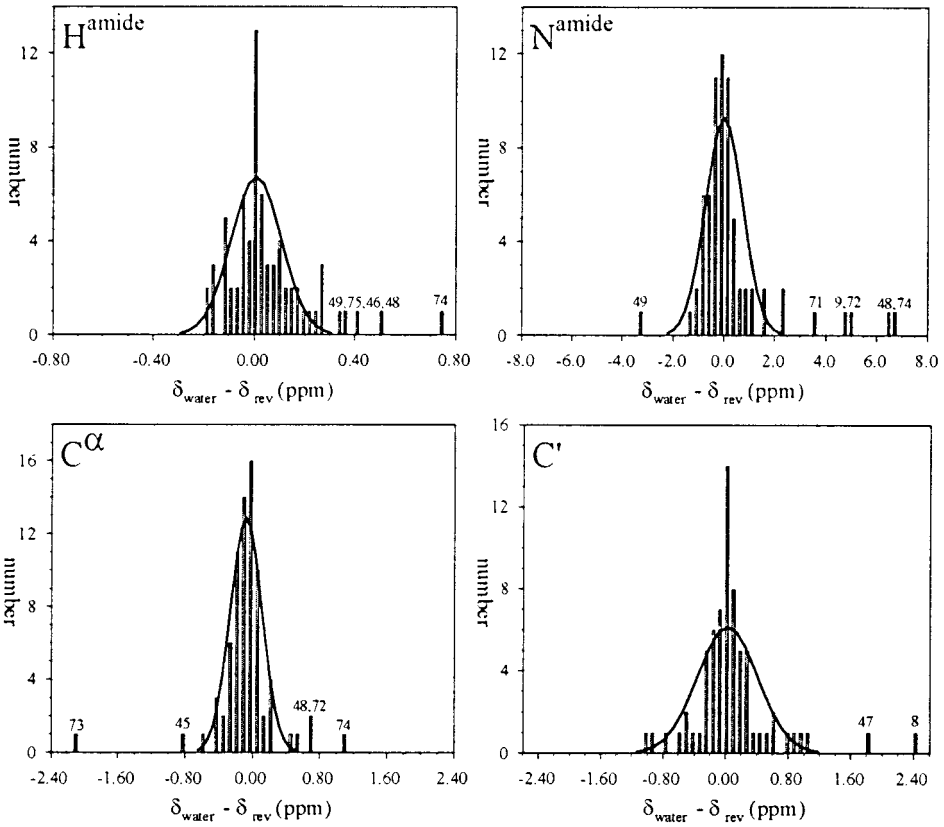


*Figure 4.* Histograms of chemical shift deviations calculated between encapsulated and free solution ubiquitin. For visual comparison, fitted Gaussian curves for amide proton, amide nitrogen, alpha carbon and carbonyl carbon chemical shifts with the standard deviation of 0.10, 0.73, 0.19, and 0.39 ppm respectively are shown. The residues with deviation outside the normal distribution are labeled.

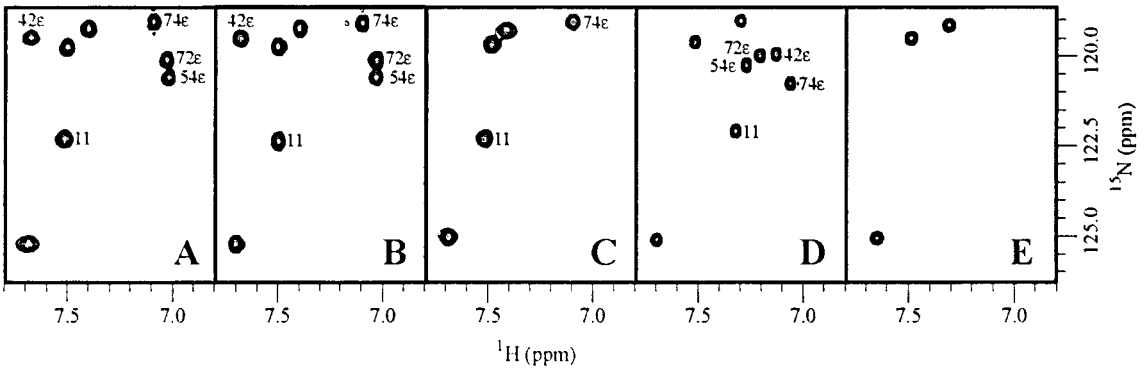


*Figure 5.* Expanded spectral regions of the $^{15}$N-HSQC spectra of ubiquitin in aqueous and reverse micelle solutions at various pH values. The cross peaks for side-chain arginine $H^{\varepsilon}$ and the backbone Lys11 are indicated. The arginine side-chain assignments for ubiquitin in water were derived from previously published data (Weber et al., 1987; Wand et al., 1996). In all reverse micelle samples, the concentration of AOT was 75 mM in pentane and the $W_0$ was 10. (A) The pH of the protein was adjusted to 5.0 prior to lyophilization, then hydrated in pure water and encapsulated. (B) Protein prepared as in part A, but hydrated in 75 mM sodium phosphate, 50 mM sodium acetate buffer, pH 8.0 prior to encapsulation. (C) The pH of the protein was adjusted to 8.8 prior to lyophilization, then hydrated in pure water and encapsulated. (D) Ubiquitin dissolved in 50 mM sodium $d_3$-acetate buffer, pH 5.0, 250 mM NaCl and 0.5 mM DSS. (E) Ubiquitin dissolved in pure water at pH 8.8.

time scale that is rapid with respect to the chemical shift.

*Effect of salt on encapsulation efficiency*

There have been numerous reports on the effects of ionic strength in the use of passive liquid-liquid phase transfer to encapsulate proteins in reverse micelles (Leser and Luisi, 1990). Generally, these studies have been carried out at relatively low protein concentrations. To study the effect of salt on protein encapsulation at the near mM concentrations employed here, ubiquitin was dissolved in a fixed volume of aqueous solution ($W_0 = 10$) with increasing concentrations of salt. This protein solution was actively mixed (vortexed) with a fixed volume of 75 mM AOT in pentane. The results summarized in Table 1 indicate that inclusion of NaCl up to 0.5 M in the encapsulation buffer has only minor effects on the encapsulation efficiency, and that encapsulation efficiency is significantly affected only at very high concentrations of NaCl.

The experimentally determined $W_0$ for the reverse micelle sample prepared with 1 M NaCl was 5.4 and is substantially lower than the target amount, i.e., only approximately half of the water molecules provided were encapsulated. The presence of high salt appears to prevent the effective encapsulation of water into AOT/pentane solution (Leser and Luisi, 1990) and leads to a lower concentration of ubiquitin in reverse micelles. Apart from the change in signal to noise ratio due to lower protein concentrations, there was no change in the chemical shifts of resonances present in the $^{15}$N-HSQC spectra of ubiquitin in these samples. These data indicate that the structure of ubiquitin in reverse micelles is independent of the salt concentration in the encapsulation buffer, but nevertheless the protein encapsulation efficiency into reverse micelles does strongly depend on salt concentration in the encapsulation buffer.

*Characterization of the hydrodynamic performance of reverse micelles*

The primary goal of protein encapsulation is to improve the NMR performance of the protein by shortening its effective rotational diffusion time. Rotational diffusion may be directly assessed by use of $^{15}$N-relaxation studies. However, a comprehensive approach using these methods is necessarily instrument intensive and cannot be used in a routine manner to assess the success of a given reverse micelle preparation. Substantial time may be saved by evaluating transverse relaxation times using a two-point approach (see below).

A potentially more efficient method for quantifying hydrodynamic behavior is the use of pulsed-field gradient translational diffusion experiments (Stejskal and Tanner, 1965; Tanner, 1970; Stilbs, 1987; Gibbs and Johnson, 1991). Convection artifacts in measuring diffusion constants, especially acute in low viscosity fluids, can be suppressed by employing double stimulated echo experiments (Jerschow and Muller, 1997). Exchange of small molecules between reverse micelles is rapid (Fletcher and Robinson, 1981; Fletcher et al., 1984) and suggests that monitoring the easily observed $^1$H resonances of surfactant and/or encapsulated water to study diffusion might be misleading. This was tested by measuring $D_s$ of water and AOT resonances in empty and protein containing reverse micelles. The simple Stokes–Sutherland relation predicts a linear dependence of the rate of translational diffusion of a particle on its radius. The relative ratio of translational diffusion constants for empty reverse micelles prepared with water loadings of 10 and 30 (Table 2) is reasonably consistent with that calculated using radii from light scattering measurements (Zulauf and Eicke, 1979). However, the absolute values of the radii do not agree with the values reported in the literature measured using other methods.

A reverse micelle sample with protein may contain both protein filled (filled) and empty reverse micelles and the $D_s$ measured for either water and AOT may not accurately differentiate between empty and filled reverse micelles. The extent to which the $D_s$ of water and AOT measured in samples with protein can be used to evaluate the relative sizes of filled reverse micelles was investigated. The size of filled reverse micelles was determined by both translational diffusion (via $D_s$) and rotational diffusion (via amide $^{15}$N $T_2$) of ubiquitin (see below). Due to the presence of intense solvent and surfactant resonances in the $^1$H spectra, the $D_s$ of ubiquitin in reverse micelles was determined using the LED-$^{15}$N-HSQC. With a $W_0$ of 10, the $D_s$ calculated based on the protein resonances was similar ($2.00 \times 10^{-10}$ m$^2$ s$^{-1}$) to that calculated for AOT and water using WET-LED (Table 2). This suggested that the $D_s$ of water and AOT measured in the presence of protein reflects the size of filled reverse micelles. However, as mentioned earlier, the radii calculated from $D_s$ values for protein containing samples with $W_0$ 10, 15 and 30 (Table 2) do not quantitatively agree with radii calcu-

*Table 1.* Salt dependence of encapsulation efficiency of ubiquitin into AOT/pentane reverse micelles

| Aqueous phase used in reverse micelle preparation | $A_{280}$ | measured $W_0$ |
|---|---|---|
| Water | 0.708 | 9.8 |
| 50 mM sodium acetate (pH 5.0), 75 mM DSS | 0.710 | 9.7 |
| 50 mM sodium acetate (pH 5.0), 75 mM DSS, 0.25 M NaCl | 0.650 | 8.9 |
| 50 mM sodium acetate (pH 5.0), 75 mM DSS, 0.5 M NaCl | 0.603 | 8.3 |
| 50 mM sodium acetate (pH 5.0), 75 mM DSS, 1.0 M NaCl | 0.153 | 5.4 |
| Water (not in reverse micelles)[a] | 0.650 | – |

[a]Ubiquitin dissolved in water.

*Table 2.* Translational diffusion coefficients for $H_2O$ and AOT in reverse micelles[a]

| Sample | $W_0$ | $D_s$ ($m^2\ s^{-1}$) | | $R_t$ (Å)[c] |
|---|---|---|---|---|
| | | $H_2O$ | AOT[b] | |
| $H_2O$/AOT (empty) reverse micelles | 10 | $2.13 \times 10^{-10}$ | $1.99 \times 10^{-10}$ | 45 |
| $H_2O$/AOT (empty) reverse micelles | 30 | $1.15 \times 10^{-10}$ | $8.48 \times 10^{-11}$ | 83 |
| Ubiquitin/$H_2O$/AOT reverse micelles | 10 | $2.03 \times 10^{-10}$ | $1.77 \times 10^{-10}$ | 47 |
| Ubiquitin/$H_2O$/AOT reverse micelles | 15 | $1.70 \times 10^{-10}$ | $1.49 \times 10^{-10}$ | 56 |
| Ubiquitin/$H_2O$/AOT reverse micelles | 30 | $1.05 \times 10^{-10}$ | $8.98 \times 10^{-11}$ | 91 |

[a]All samples contained 75 mM AOT in pentane with varying amount of $H_2O$ depending on $W_0$. Four milligrams of $^{15}N$ labeled ubiquitin was used to make protein containing reverse micelles.
[b]An average of $D_s$ calculated for AOT head group resonances at 4.25 and 3.15 ppm.
[c]The radius ($R_t$) was calculated from the $D_s$ for $H_2O$ using the expression $R_t = \frac{kT}{6\pi\eta D_s}$ at 20 °C assuming a bulk viscosity of 224 μPa s.

lated from amide $^{15}N$ $T_2$ (Table 4). Even though the $D_s$ of water and AOT measured in the presence of protein reflect the increase in filled reverse micelle size on $W_0$ (Table 2) the relative radii estimated from $D_s$ do not agree with the relative radii calculated from amide $^{15}N$ $T_2$ (Tables 2 and 4).

### *Sizes of protein filled reverse micelles from amide $^{15}N$ $T_2$*

To better understand the factors affecting the size of protein filled reverse micelles, the amide $^{15}N$ $T_2$ values of encapsulated ubiquitin in reverse micelles were measured with varying concentrations of all three components in the system ($H_2O$, AOT, and protein). The $T_2$ dependence of ubiquitin on protein concentration, AOT concentration and $W_0$ in reverse micelles is summarized in Table 3. The $T_2$ values are reported for representative residues with high backbone order parameters (Schneider et al., 1992) to compare the global tumbling of encapsulated ubiquitin. Table 3 shows that increasing the amount of protein in reverse micelles from 2 mg to 4 mg leads to a decrease in the measured $T_2$. This result indicates that the concentration of protein molecules alone does not dictate the concentration of reverse micelle particles, and that the water molecules are not shared only among the protein molecules. If this was the case, the increase in protein concentration should have led to a decrease in the filled reverse micelle size due to a lower number of water molecules per protein per particle.

When protein and AOT concentrations are fixed, increasing the $W_0$ decreases the $T_2$ of encapsulated ubiquitin substantially (Table 3). The dependence of filled reverse micelle radius on $W_0$ is summarized in Table 4. The Table 4 shows that an increase in $W_0$ leads to a direct increase in the radius of filled reverse micelles. At $W_0$ 10, the radius of the water pool inside reverse micelles is similar to the hydrodynamic radius of ubiquitin in free aqueous solution (17.0 Å), and the inclusion of ubiquitin does not significantly change the radius of empty reverse micelles. When the $W_0$ is 15 or 30, the radius of filled reverse micelles is unexpectedly smaller than the corresponding radius of empty reverse micelles with the same $W_0$ values. Similar results have been observed previously for cytochrome c in reverse micelles (Brochette et al., 1988; Pileni, 1989).

*Table 3.* $T_2$ (ms) dependence of ubiquitin on protein concentration, AOT concentration and $W_0$ in reverse micelles[a]

| Protein, mg | 2 | 3 | 4 | 4 | 4 | 4 | 4 |
|---|---|---|---|---|---|---|---|
| $W_0$ | 10 | 10 | 10 | 15 | 30 | 10 | 10 |
| [AOT], mM | 75 | 75 | 75 | 75 | 75 | 150 | 300 |
| L15 | $83.3 \pm 3.2$ | $80.2 \pm 3.2$ | $79.1 \pm 2.3$ | $49.0 \pm 1.9$ | $30.0 \pm 3.1$ | $73.9 \pm 1.6$ | $58.5 \pm 2.4$ |
| D39 | $88.6 \pm 1.8$ | $82.8 \pm 1.8$ | $79.7 \pm 1.3$ | $50.3 \pm 1.0$ | $27.5 \pm 1.6$ | $75.0 \pm 0.8$ | $61.3 \pm 1.4$ |
| L43 | $91.5 \pm 5.0$ | $89.3 \pm 7.1$ | $85.8 \pm 4.6$ | $52.7 \pm 3.4$ | $29.6 \pm 4.1$ | $80.2 \pm 3.0$ | $57.4 \pm 3.6$ |
| S57 | $81.2 \pm 2.6$ | $76.7 \pm 2.2$ | $76.2 \pm 1.6$ | $48.6 \pm 1.7$ | $28.4 \pm 3.2$ | $73.7 \pm 1.2$ | $57.8 \pm 1.8$ |
| Q62 | $97.7 \pm 3.6$ | $93.2 \pm 4.6$ | $89.1 \pm 3.0$ | $54.8 \pm 2.1$ | $21.3 \pm 3.6$ | $87.8 \pm 2.0$ | $66.9 \pm 2.7$ |
| T66 | $83.9 \pm 2.8$ | $81.4 \pm 3.4$ | $80.0 \pm 2.3$ | $48.1 \pm 1.9$ | $32.2 \pm 3.5$ | $76.0 \pm 1.7$ | $59.5 \pm 2.4$ |

[a]Backbone $^{15}N$ transverse relaxation times (ms) at 750 MHz ($^1H$).

*Table 4.* Dependence of ubiquitin filled reverse micelle radius on $W_0$[a]

| Sample | $W_0$ | $T_2$ (ms) | $\tau_m$ (ns)[b] | $R_r$ (Å)[c] | $T_2'$ (ms)[d] |
|---|---|---|---|---|---|
| Ubiquitin/$H_2O$/AOT reverse micelles | 10 | 86 | 8.0 | 33 | 85 |
| Ubiquitin/$H_2O$/AOT reverse micelles | 15 | 53 | 13.3 | 39 | 53 |
| Ubiquitin/$H_2O$/AOT reverse micelles | 30 | 30 | 23.8 | 47 | 27 |

[a]The sample (1 ml) had 4 mg $^{15}N$ labeled ubiquitin, 75 mM AOT and amount of water defined by the $W_0$. The backbone $^{15}N$ transverse relaxation time (ms) of L43 at 750 MHz ($^1H$) was used for calculating radii.

[b]The correlation time ($\tau_m$) was estimated using simple model free treatment (Lipari and Szabo, 1982a, b) with chemical shift anisotropy for $^{15}N$ of $-170$ ppm, an H-N distance of 1.04 Å, an order parameter of 0.92 and a $\tau_e$ of 50 ps.

[c]The radius ($R_r$) was calculated using the expression $R_r = \left[\frac{3\tau_m kT}{4\pi\eta}\right]^{1/3}$ at 20 °C assuming a bulk viscosity of 224 μPa s.

[d]The backbone $^{15}N$ transverse relaxation time (ms) of L43 calculated using the first point and the point where the initial intensity has decreased to $\sim 50$ %. For the residues shown in Table 3, the $T_2$ values calculated using the two point fit were 10 to 22% longer than the $T_2$ obtained from the single exponential fit.

These results imply that a simple relationship between the hydrodynamics of a sphere to its volume and the bulk solvent viscosity is not applicable here. This is likely due to at least two features of the reverse micelle system. On the one hand, the traditional approach is to assume the interaction between the particle and the solvent to be in the so-called 'stick boundary' limit where solvent is stationary at the particle surface, i.e., travels with the particle. Here we have a relatively non-interacting solvent that may in fact be removed from the stick boundary limit towards the slipped boundary condition. This would in itself explain the apparent discrepancy between the translational and rotational diffusion constants obtained for the reverse micelles. It would, however, not be sufficient to explain the apparent discrepancy of the ratios of translational and rotational diffusion constants as a function of water loading, which indicates a differential scaling of translational and rotational diffusion with a change in particle radius. The most probable origin of this apparently anomalous behavior is the effect of excluded volume. The particles are of sufficient size and concentration to occupy a significant fraction of the solution volume. If one considers the cage formed by reverse micelles around one another as a sphere, then we have a system consisting of two spherical surfaces separated by a fluid. This physical system has been examined in detail previously (Broersma, 1959) where it was shown that translational diffusion is significantly more sensitive to changes in the relative volume of the inner sphere than is rotational diffusion in the range of geometries applicable here, regardless of whether the fluid interaction with either surface is in the stick or slip boundary limit. Taken together, these observations suggest that rotational diffusion cannot be simply predicted from translational diffusion in these systems.

*Table 5.* $T_2$ (ms) dependence of ubiquitin on $W_0$ with the fixed concentration of $H_2O$ at 600 MHz[a]

| Residue | Measured $W_0$ | | |
|---|---|---|---|
| | 18.5 | 8.3 | 6.9 |
| L15 | $40.5 \pm 2.2$ | $95.6 \pm 1.4$ | $93.4 \pm 4.4$ |
| D39 | $34.0 \pm 0.9$ | $95.3 \pm 0.9$ | $78.0 \pm 1.9$ |
| L43 | $36.6 \pm 2.3$ | $95.2 \pm 2.1$ | $88.7 \pm 6.2$ |
| S57 | $32.1 \pm 1.5$ | $91.3 \pm 1.0$ | $83.1 \pm 2.8$ |
| Q62 | $37.3 \pm 1.6$ | $114.9 \pm 2.0$ | $111.8 \pm 7.5$ |
| T66 | $32.9 \pm 1.6$ | $95.9 \pm 1.3$ | $85.8 \pm 3.5$ |

[a]All data were collected on a same sample (4 mg $^{15}N$ ubiquitin, 37.5 mM AOT in pentane, $W_0 = 18.5$) by adding solid AOT to change the $W_0$ from 18.5 to 8.3 and later to 6.9.

Finally, as a compromise between accuracy and efficiency, we compare the $T_2$ relaxation times calculated from two relaxation time points (Table 4). This corresponds to roughly 3 h in experiment time and also provides a satisfactorily accurate measure of the true value obtained when a comprehensive relaxation decay profile is employed.

*Decreasing the filled reverse micelle size by adding solid AOT*

Decreasing the reverse micelle particle size should improve the spectroscopic performance of such preparations and therefore efforts were made to reduce the $W_0$ value in the hopes of decreasing the filled reverse micelle size. The data in the Table 5 demonstrates that at a fixed total concentration of water, decreasing the $W_0$ from 18.5 to 8.3 achieved through the addition of AOT increased the $T_2$ of encapsulated ubiquitin. This suggests that it is possible to decrease the size of filled reverse micelles by decreasing $W_0$ simply by adding solid AOT. The added AOT forces the reverse micelles to re-equilibrate the water pool, resulting in a net decrease in the particle radius. Such re-equilibration occurs rapidly. However, when the $W_0$ falls below 8.3, the $T_2$ of encapsulated ubiquitin decreases, possibly due to the aggregation of reverse micelles (Table 5). Finally, when the $W_0$ was decreased to 5.0 through the further addition of AOT, no protein signal could be detected in the $^{15}N$-HSQC experiment even though the reverse micelle solution appeared clear. This leads to the conclusion that there is a lower limit on $W_0$ achievable by adding solid AOT while maintaining a high performance solution.

*Implications for large proteins in reverse micelles*

The ubiquitin filled reverse micelle particle with $W_0$ of 10 has an approximate radius of 33 Å (Table 4). When ubiquitin is inserted into the water pool of empty reverse micelle, the calculated radius of water pool and protein (9.0 Å, from structure) combined increases to 18.3 Å. Since the protein in the water pool is protected by AOT (12 Å), the theoretically calculated radius of filled reverse micelle particle is 30.3 Å. This agrees well with the experimentally measured radius of 33 Å (Table 4). This means that the contribution from the water layer (9.3 Å) surrounding the protein dominates the overall particle size in the case of smaller proteins such as ubiquitin.

Comprehensive examination of conditions confirm that stable ubiquitin reverse micelle with the minimum radius is formed when the $W_0$ is nearly 10. The water pool radius (17.5 Å) of empty reverse micelles with $W_0$ of 10 is close to the apparent hydrodynamic radius of ubiquitin measured in water. The hydrodynamic radius of the protein thus appears to define the minimum radius for filled reverse micelles, and the hydrodynamic radius might be used to choose the appropriate $W_0$ for the encapsulation of a given protein. This prediction remains to be verified for other larger proteins. Importantly, in this regard, an empirical relationship has recently been derived linking the measured hydrodynamic radius of protein and the number of residues (Wilkins et al., 1999). The hydrodynamic radius of protein is found to have a non-linear dependence on number of residues (Wilkins et al., 1999), suggesting that the contribution from water layer surrounding the protein for large proteins may be relatively small.

## Conclusions

Complete resonance assignments for encapsulated ubiquitin in reverse micelles have been obtained and its structure determined with high precision confirming the general effectiveness of encapsulation. Adjusting the pH of the protein prior to encapsulation is required to change the pH of protein in reverse micelles. Higher salt concentrations in the encapsulation buffer lower the encapsulation efficiency of protein when the injection method is used to prepare reverse micelles. The $D_s$ of water and AOT determined in the presence of protein do not always reflect the size of filled reverse micelles. The size of the filled reverse micelles can be determined from the amide $^{15}N$ $T_2$ of

protein. Increase in $W_0$ increases the radius of filled reverse micelles. Finally, decreasing the $W_0$ with the addition of solid AOT can minimize the size of filled reverse micelles and represents an important strategic improvement in sample preparation.

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