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Fast Local Backbone Dynamics of Encapsulated Ubiquitin

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Reverse micelles are thermodynamically stable assemblies of surfactant molecules organized around an aqueous core (nanopool) that spontaneously form transparent solutions in low polarity liquids. The predominant reverse micelle-forming surfactant is sodium bis-(2-ethylhexyl) sulfosuccinate (AOT), which generates particles with excellent protein and water-hosting characteristics. The most important reverse micelle physical parameter is water loading, w_0, which is defined as the molar ratio of water to surfactant, $[H_2O]/[AOT]$. The radius of the AOT reverse micelle particle can vary over a wide range, depending on w_0, and allows encapsulation of complex molecules within the water core. Reverse micelles are a branch of soft nanotechnology that have been extensively investigated since the 1980s as a source of potential nanodevices for a range of applications, including chromatography, separation science, reaction processes, green chemistry, and biophysics.[1−3]

Encapsulation within reverse micelles provides a unique confined environment to study proteins that expands experimental control limits on temperature, salt, and pH.[4−6] In addition, encapsulation provides a platform for examining the influence of confinement on protein stability that can serve as a model of the crowded in vivo cellular environment. Theoretical predictions indicate that encapsulation can significantly decrease the ΔG for folding, and complementary experimental results confirm that proteins confined within sol−gel silica glass matrices and within reverse micelles both experience an increase in stability.[7−9]

Encapsulation has recently been shown to be an effective complement to established methods in NMR-based structural biology and biophysics.[4,10−12] Biochemical studies have demonstrated that encapsulated proteins retain biological function, and NMR-based structural studies of human ubiquitin have verified that encapsulated proteins also retain native structure.[3,9,11] Here, we describe the results of the first studies aimed at investigating the influence of encapsulation on the backbone dynamics in the well-studied and representative protein ubiquitin. Ubiquitin is a 76 amino acid protein that plays a key role in the protein degradation pathway. Ubiquitin is also involved in transcriptional activation, repression, and viral budding.[13,14] This protein has been extremely well-characterized, and both its structure and its biophysical properties are well-known.

The importance of examining molecular motion in proteins derives from the fundamental principle that function often depends on transitions from the ground state to states of higher energy. These higher energy states may be accessed via conformational fluctuations, for example, dynamics, and analysis of NMR relaxation studies provide a detailed, site-specific portrait of dynamics. The use of NMR relaxation as a probe of internal dynamics is a well-established component of NMR methodology, validated by numerous experimental demonstrations that verify the utility of the approach.[15,16] NMR relaxation studies can directly identify areas of protein rigidity and flexibility, which are important in identifying both stability and conformational exchange. Backbone dynamics in polypeptides are most commonly analyzed based on the model-free analysis of measured ^{15}N T_1, T_2, and NOE values.[17] In the simple model-free approach, dynamics are characterized in terms of three parameters: a time-constant for global reorientation of the entire molecule (τ_m), a time-constant for relatively fast internal (local) motion (τ_e), and an order parameter that is related to the amplitude of the fluctuation (S^2). The order parameter is the salient feature of the analysis because it provides an intuitive view of dynamics and also connects the motion with thermodynamical considerations.[18−20] Model-free analysis is most naturally compatible with circumstances in which local motion is rapid relative to the global tumbling correlation time, for example, picoseconds. Conversely, the T_1/T_2 ratio is weakly dependent upon the influence of rapid local motion, and, thus, a statistical consideration of the ratio provides a basis for identifying contribution of longer time scale motions (μs−ms) to relaxation.[21]

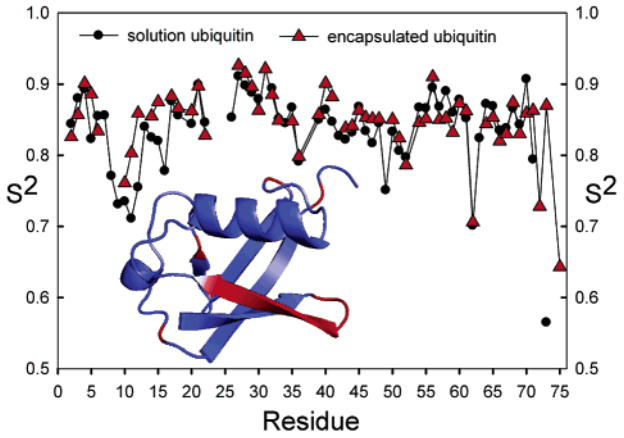

Figure 1. Comparison of order parameters (S^2) for ubiquitin in free and encapsulated states. S^2 ranges from 0 to 1, indicating, respectively, no restriction and complete restriction of N−H bond vector motion. Inset shows low solution S^2 values of ubiquitin colored in red.

Fast local backbone dynamics for ubiquitin encapsulated within AOT reverse micelles were determined using analysis of ^{15}N NMR relaxation conducted at both 50.68 and 60.78 MHz. A plot of S^2 versus residue for encapsulated and free solution forms of ubiquitin is shown in Figure 1; solution ubiquitin S^2 values were obtained from a recent study conducted by Bax and co-workers.[21] Comparison of the data reveals that the trend of backbone dynamics for encapsulated ubiquitin follows that of solution ubiquitin. In most regions, encapsulated ubiquitin exhibits only slightly higher order parameters, indicating minor rigidification of the polypeptide backbone. Significant differences in the order parameters for the free and encapsulated forms are found in the residues L8 through G16 (residues 7 and 9 removed from analysis). This region involves the loop between β-strands 1 and 2, as well as most of β-strand 2. Five additional residues exhibit significantly increased S^2 values in the encapsulated state relative to the solution state: Q40, Q41, L56, L71, and L73. Residues Q40 and Q41 lie at the beginning of β-sheet 3 and residue L56 lies at the beginning of the short 3_{10}-

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helix. Residue L71 is the last residue in the terminal β-sheet 5, and L73 is part of the unstructured C-terminus. In addition, residues R72 and G75 exhibit relatively low S^2 values when encapsulated, but were not characterized in the free-solution study. The common feature of residues that experience significant increase in S^2 upon encapsulation is their location either in loop regions, at the intersection between loop regions and regular secondary structure motifs, or in the unstructured C-terminal region of the molecule. Confinement thus appears to restrict the amplitude of backbone dynamics in regions that typically possess relatively high structural flexibility. These observations are consistent with previous experimental and theoretical results that conclude that confinement generates an increase of structural stability.

In addition to fast motion dynamics, the influence of encapsulation on slower motions was also investigated. Studies conducted by Bax and co-workers on the free-solution form of ubiquitin indicate that residues E18, I23, N25, and I36 undergo significant conformational exchange on the μs−ms time scale, based on analysis of the ^{15}N T_1/T_2 ratios of peptide amide groups in the protein.[21] Additionally, residues 8−11, 62, and 73−76 were identified as having low NOE values, which are indicative of motions occurring on a time scale of 10^{-10} s to 10^{-9} s. More recently, Palmer and co-workers identified significant μs−ms conformational exchange in residues I23, N25, T55, and V70 based on rotating analysis of rotating frame $R_{1\rho}$ measurements of the peptide amide groups.[22,23] Our results for encapsulated ubiquitin indicate that residues T7, I23, N25, and K27 exhibit significant conformation exchange on the μs−ms time scale using the same protocol employed in the studies of Bax and co-workers.[21] Comparison of our results with ^{15}N relaxation analysis from two previous comprehensive investigations of dynamics in ubiquitin thus indicates that residues I23 and N25 are universally identified as exhibiting dynamical motion on the μs−ms time scale. It thus appears that the dynamic behavior on both the fast and the μs−ms time scales is native in the encapsulated state, although ubiquitin is modestly stabilized in the reverse micelle.

Data recorded for the present study employed 5 mg of ubiquitin in 100 mM AOT, 1M NaCl, and 50 mM NaOAc, pH 5 at a w_0 of 22, which represents conditions that simultaneously optimize nativelike hydration conditions as well as encapsulation efficiency. AOT has proven to be the most effective general surfactant for encapsulation of polypeptides; however, previous studies have established that reconstitution conditions can influence the stability of the encapsulated proteins.[4]

A number of groups have investigated the influence of confinement on the thermodynamic stability of proteins.[7,8,24,25] Consensus results suggest that substantial thermal stability can be achieved for confined proteins, for example, for α-lactalbumin, an increase in the T_m of up to 32 °C has been observed.[8] The combination of encapsulation technology and NMR relaxation analysis represents an important complement to such studies, providing precise site specific details of structural fluctuation occurring on a broad time scale range. Ubiquitin was selected as a first study example because its structure has been well-studied in both the solution and the encapsulated state, and NMR relaxation experiments have also been conducted in solution, giving a baseline of comparison for backbone dynamics. We have shown that the dynamics of ubiquitin in a reverse micelle are generally similar to results obtained in solution state, with slight decreases in the amplitude of fast local motion in regions of the molecule that are generally associated with increased structural flexibility. Importantly, reverse micelles, suitably reconstituted, do not induce non-native physical properties that might in principle result from interactions between the protein and the surfactant. Results presented here enhance our understanding of protein dynamics under crowded conditions, which is an area that has thus far largely been overlooked in biophysical studies, and also provides the foundation for future site-specific studies of the influence of confinement.

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Supporting Information Available: Protocol for the preparation of isotopically enriched ubiquitin and for the encapsulation of the proteins. Tables of ^{15}N T_1, T_2, NOE, S^2, and τ_e measurements are also reported. This material is available free of charge via the Internet at http://pubs.acs.org.

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