Validation of Protein Structure from Preparations of Encapsulated Proteins Dissolved in Low Viscosity Fluids

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Application of modern NMR methods to large proteins is often hindered by their long molecular reorientation correlation time which leads to unfavorable relaxation properties. Various approaches such as extensive deuteration[1] and transverse relaxation optimized spectroscopy[2] have been developed to achieve more optimal relaxation behavior. Despite the success of these and other methods, additional approaches to the problem are desirable. Recently, we have introduced a method that actively seeks to reduce the effective tumbling time of a protein.[3] This is achieved by the encapsulation of the protein in the protective environment of the water core of a reverse micelle[4] and dissolving the entire assembly in a low viscosity fluid.[3] In principle, sufficient reduction of solvent viscosity will allow a protein to effectively tumble as a much smaller protein would in water. This was demonstrated for the protein ubiquitin encapsulated in reverse micelles dissolved in short chain alkane solvents,[3] and subsequently for pancreatic trypsin inhibitor solubilized in liquid CO_2.[5] Importantly, it was shown that the effective spin−spin relaxation rate of encapsulated ubiquitin in reverse micelles decreased in a roughly linear fashion with the decrease in bulk solvent viscosity.[3]

A great deal of effort has been put forth over the past several decades in the development of reverse-micelle-forming solvents and surfactants and in the characterization of the reverse micelles and the molecules encapsulated within them.[6] Remarkably however, no comprehensive structural information has been obtained for an encapsulated protein. The absence of such information raises the issue of whether reverse micelle encapsulation is a viable tool for the determination of biologically relevant protein structures. To evaluate whether an encapsulated protein adopts its native (i.e. free solution) structure we have determined the structure of human ubiquitin encapsulated in reverse micelles. We show here that the structure of encapsulated ubiquitin is virtually identical to both the free solution and crystal structures.

$^{13}C/^{15}N$-enriched recombinant human ubiquitin[7] was solvated in 50 mM sodium acetate (pH 5.0) containing 250 mM NaCl (8 mg protein/27 μL buffer). This protein solution was injected into 75 mM bis(2-ethylhexyl) sulfosuccinate (AOT) in *n*-pentane or in *n*-butane.[8] The effective concentration of ubiquitin in the final solution was 0.3 mM, and the molar ratio of water to AOT was 10. Samples prepared in butane/AOT employed standard 8 mm Wilmad pressure-vacuum NMR tubes, pressurized to 50 psi under N_2. Samples prepared in pentane/AOT employed standard 5 mm and 8 mm sample tubes. Preparations of encapsulated ubiquitin in either solvent are stable for several months. NMR data were recorded at 20 °C on Varian Inova spectrometers operating at 600 and 750 MHz (^1H). NMR data were processed using Felix and analyzed using XEASY.[9]

Backbone assignments were obtained using the HNCACB[10] and CBCA(CO)NH[11] experiments. The bulk of the side-chain carbon assignments were obtained from a CC(CO)NH[12] spectrum collected at 750 MHz. The assignments were completed and reinforced using HCCH-TOCSY[13] and ^{13}C-HSQC[14] spectra. The experimental details and complete resonance assignments will be published elsewhere.

Distance restraints were derived from NOESY ^{15}N-HSQC[15] and NOESY ^{13}C-HSQC[16] spectra acquired with mixing times of 90 ms. Upper distance restraints were derived from NOE peak volumes using CALIBA.[17] Dihedral angle (ϕ) restraints were calculated from the HNHA quantitative *J* correlation experiment.[18]

Qualitative inspection of short- and medium-range NOEs revealed that all secondary structure elements present in the free solution[19,20] and crystal structures[21] of human ubiquitin are conserved in ubiquitin encapsulated in reverse micelles (Supporting Information). Structure calculations were carried out using a distance restraint set based on 1805 unique NOEs (873 intraresidue, 336 short-range, 239 medium-range, and 357 long-range). Upper-distance restraints were corrected for floating stereospecific assignments, as required. Removal of fixed distance restraints and restraints which would not be violated in any conformation led to 1291 final restraints used in the calculation. This corresponds to more than 16 restraints per residue. The distribution of distance restraints across the primary sequence is presented in the Supporting Information. In addition to the distance restraints, 63 ϕ torsion angle and 23 H-bond restraints were imposed.

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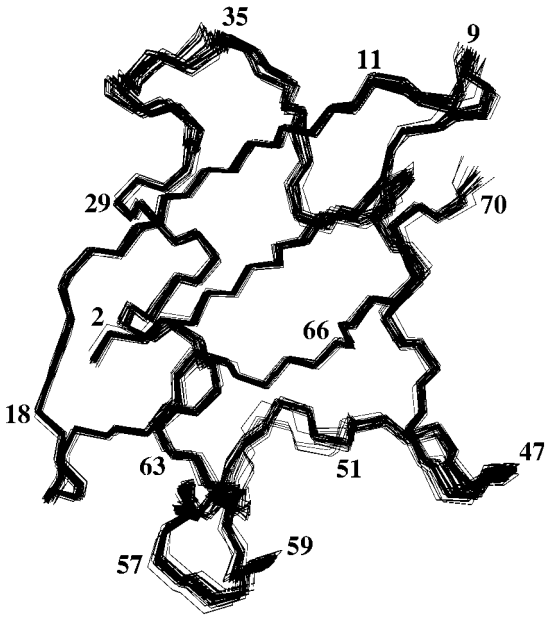

Figure 1. Structure of encapsulated human ubiquitin. The family of 32 structures were superimposed on backbone atoms for residues 2−70. Residues 71 through 76 are disordered and are not shown. The structures have been deposited in the PDB under code 1G6J.

Structures were calculated by simulated annealing using torsion angle dynamics (TAD) in the program DYANA[22] starting from random conformers. Each structure was subjected to 4000 TAD steps at high temperature (eight target function units), followed by slow cooling during 16000 TAD steps to the final temperature (0 target function units). This refinement was completed with 1000 steps of conjugate gradient minimization. The planarity of peptide bonds was fixed during the calculation. The final family consisted of 32 structures having the lowest-target function values out of 100 total structures calculated. Structure visualization and analysis were performed using MOLMOL.[23]

The final family of structures has an average rmsd of 0.26 ± 0.05 Å for backbone atoms and 0.77 ± 0.04 Å for heavy atoms to the mean structure (residues 2−70) (Figure 1). The target function values ranged from 0.05 to 0.23 Å^2 with an average of 0.16 ± 0.05 Å^2. Structural statistics are summarized in Table 1 and indicate that the structure is determined to high precision. Residues 9, 10, 35, 36, 52, and 53 have slightly higher local rmsds in comparison to the average. These regions are suggested to be flexible according to generalized order parameters obtained for N−H vectors for ubiquitin in free solution.[24] Ramachandran plot analysis using the Procheck[25] criteria indicates that 87% of residues fall in the "most favored" and 13% in "additional allowed" regions when glycines and prolines are excluded.

Table 1. Structural Statistics for the Family of 32 Structures

average DYANA target function (Å^2)	0.16 ± 0.05
backbone rmsd to the mean structure (Å)[a]	0.26 ± 0.05
heavy atom rmsd to the mean structure (Å)[a]	0.77 ± 0.04
average of maximum upper restraint violations (Å)	0.17 ± 0.07
average of maximum van der Waals violations (Å)	0.10 ± 0.02
average of maximum dihedral angle violations (°)	2.3 ± 1.1
average sum of upper restraint violations (Å)	1.20 ± 0.2
average sum of van der Waals violations (Å)	0.80 ± 0.2
average sum of dihedral angle violations (°)	11.45 ± 0.2

[a] rmsds were calculated for residues 2−70.

Table 2. Comparison of Crystalline, Free Solution, and Reverse Micelle Encapsulated Human Ubiquitin Structures

comparison[a]	backbone rmsd (Å)	heavy atom rmsd (Å)
crystal vs rev. micelle	0.77 ± 0.03	1.63 ± 0.05
free solution vs rev. micelle	0.79 ± 0.04	1.57 ± 0.07
crystal vs free solution	0.35 ± 0.01	1.15 ± 0.05

[a] rmsds were calculated for residues 2−70.

To identify potential differences, the family of structures for encapsulated ubiquitin was compared to the crystal structure[21] and to the structure determined in free solution[20] (Supporting Information). These quantitative comparisons are summarized in Table 2. Superpositions were generated using residues 2 through 70, that is, omitting the C-terminus that is known to be unstructured in solution[19,24] and to have large thermal motion in the crystal.[21] Earlier work[26] and the precision of the model determined here (0.26 Å) suggests that the structure obtained for encapsulated ubiquitin and that obtained for the protein in free solution[20] and in the crystal[21] are in quantitative agreement. Minor variations in the structure of encapsulated ubiquitin can be identified in the first reverse turn between residues 8 and 10, in the region between residues 32 and 34 and near residue 62 (Supporting Information). Most of these differences probably arise as a result of the relative scarcity of NOEs derived for these regions. One apparent exception, however, involves residues 62−64 where short distance interactions (NOEs) are seen in the structure of the encapsulated protein but are not observed in the free solution structure. These distance restraints cause a minor localized variance (<1.3 Å) for residue 62. The origin of these minor variations is unclear.

These structural comparisons demonstrate that the structure of ubiquitin remains largely undisturbed upon encapsulation. The results presented here for ubiquitin therefore represent a significant first step in the validation of the reverse micelle approach as a tool for determining structures of proteins using standard triple-resonance based solution NMR methods.

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Supporting Information Available: Figures summarizing short and intermediate range NOEs, the distribution of distance restraints across the protein sequence, superposition of crystal, free solution and reverse micelle structures and the average backbone rmsds across the protein sequence (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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