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Communication

NMR spectroscopy of proteins encapsulated in a positively charged surfactant

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Abstract

Traditionally, large proteins, aggregation-prone proteins, and membrane proteins have been difficult to examine by modern multinuclear and multidimensional solution NMR spectroscopy. A major limitation presented by these protein systems is that their slow molecular reorientation compromises many aspects of the more powerful solution NMR methods. Several approaches have emerged to deal with the various spectroscopic difficulties arising from slow molecular reorientation. One of these takes the approach of actively seeking to increase the effective rate of molecular reorientation by encapsulating the protein of interest within the protective shell of a reverse micelle and dissolving the resulting particle in a low viscosity fluid. Since the encapsulation is largely driven by electrostatic interactions, the preparation of samples of acidic proteins suitable for NMR spectroscopy has been problematic owing to the paucity of suitable cationic surfactants. Here, it is shown that the cationic surfactant CTAB may be used to prepare samples of encapsulated anionic proteins dissolved in low viscosity solvents. In a more subtle application, it is further shown that this surfactant can be employed to encapsulate a highly basic protein, which is completely denatured upon encapsulation using an anionic surfactant.

Keywords: NMR spectroscopy; Reverse micelle; Protein encapsulation; Liquid propane; Molecular reorientation

1. Introduction

The sequencing of the genomes of several organisms, including that of *Homo sapiens*, has propelled a massive effort to structurally characterize, either by crystallography or nuclear magnetic resonance (NMR) spectroscopy, the proteins that are coded by this genetic material. Traditionally, large proteins, aggregation-prone proteins, and membrane proteins have been difficult to examine by modern multinuclear and multidimensional NMR spectroscopy. A major limitation presented by these kinds of protein systems is that their slow molecular reorientation compromises many aspects of the more powerful solution NMR methods. Several approaches have emerged to deal with the various spectroscopic difficulties arising from slow molecular reorientation that, to name a few, include extensive deuteration [1–4], transverse optimized relaxation spectroscopy (TROSY) [5–7], and advances in magnetization transfer [8]. An additional approach actively seeks to increase the effective rate of molecular reorientation by encapsulating the protein of interest within the protective shell of a reverse micelle, and dissolving the resulting particle in a low viscosity fluid [9]. Encapsulation of proteins at micromolar concentrations in high density organic solvents was extensively investigated in the 1980s as a means to solubilize enzymes for the purpose of large-scale catalysis of reactions [10]. In the context of solution NMR, homogeneous preparations of protein at significantly higher concentrations in

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relatively low viscosity fluids are required [11]. These requirements have necessitated the development of new encapsulation strategies and apparatus [12–15]. In addition to the ability to adjust the effective correlation time of an encapsulated protein by simply varying the bulk solvent [9], the encapsulation of proteins has also been found to be useful in NMR-based studies of protein cold denaturation [16] and the force-folding and stabilization of metastable proteins via a confined space effect [17]. The latter may prove to be especially important as it appears that significant fractions of small soluble proteins of the proteomes of a variety of species are unfolded under standard NMR sample conditions [18,19], suggesting that they are intrinsically unstable and are forced-folded by the stabilizing excluded volume effect present in the cellular milieu [20].

The preparation of solutions of encapsulated proteins dissolved in solvents of sufficiently low viscosity to obtain short molecular reorientation times has thus far been restricted to the short chain alkanes [9] and liquid carbon dioxide [21]. Previous reports of suitable solutions prepared in liquid propane have focused on the use of the negatively charged surfactant, bis(2-ethylhexyl) sodium succinate (AOT) [9,12,13]. Protein transfer into the water core of a reverse micelle is apparently dominated by electrostatics. To allow for the encapsulation of proteins with low isoelectric points (p*I*) in neutral buffers, an alternative, positively charged surfactant is needed. There has been a previous report of the solubilization of protein at micromolar concentrations in microemulsions of cetyltrimethylammonium bromide (CTAB) and hexanol in the heavy organic solvent octane [22,23]. Here, we report the use of CTAB/hexanol reverse micelles for the encapsulation of both acidic and basic proteins in low viscosity short chain alkane solvents at concentrations suitable for high resolution NMR studies. The model proteins used in these studies were *Cyanobacterium anabaena* flavodoxin C55A mutant, a 19 kDa protein with a predicted p*I* of ~4.2, and enhanced green fluorescent protein (EGFP), a 54 kDa homodimer with a predicted p*I* of ~5.6. There have been no previous reports of CTAB reverse micelle formation (empty or protein containing) in short chain alkanes such as butane or propane, which is necessary for high resolution NMR spectroscopy of these systems. Finally, we show that the cationic CTAB can also be used to encapsulate the highly basic protein cytochrome *c* that is essentially denatured in reverse micelles of the anionic surfactant AOT due to an electrostatic interaction that cannot be successfully ameliorated by high ionic strength buffers.

2. Results

Solutions of encapsulated flavodoxin were prepared in pentane by the direct injection method. Twenty-seven microliters of 9.3 mM solution of flavodoxin in 50 mM potassium phosphate, pH 5.8, was injected into 0.8 mL of 150 mM CTAB in a pentane/hexanol mixture (8% hexanol v/v). The molar ratio of water to CTAB (W_0) was 12.5. CTAB/hexanol/alkane mixtures are stable to temperatures as low as −10 °C. Encapsulated flavodoxin was prepared in propane using an evaporation method. This approach involved preparing a standard sample in deuteropentane with 10% hexanol (v/v) by the injection method, evaporating 75% of the solvent, placing the remaining sample in a 5 mm Wilmad pressure–vacuum NMR tube, and then adding propane. Propane samples were pressurized to 200 psi, which is sufficiently above the liquefaction pressure to maintain sample stability in the presence of small leaks. The resulting samples were then characterized by standard ^{15}N HSQC spectra obtained at 600 MHz (^{1}H) on a Varian Inova-class NMR spectrometer equipped with a triple resonance *z*-axis pulsed field gradient NMR probe cooled to cryogenic temperatures. Fig. 1 shows a ^{15}N HSQC spectrum for flavodoxin encapsulated in CTAB reverse micelles dissolved in propane.

The flavodoxin spectrum in CTAB reverse micelles dissolved in propane is closely similar to spectra obtained in pentane and for flavodoxin in free aqueous solution [24], indicating that flavodoxin was successfully encapsulated in CTAB reverse micelles without significant structural rearrangement. Slight differences in chemical shift presumably arise from the electrostatic effects of encapsulation, as was seen for ubiquitin [25]. Comparison of spectra collected at the same protein concentration and with the same acquisition parameters indicated that the signal/noise for the pentane reverse micelle sample is approximately 35% better than that of the corresponding water sample (50 mM potassium phosphate, pH 5.8). In the 3:1 propane/pentane (v/v) sample, the signal/noise is approximately 50% better than in the corresponding water sample. The predicted viscosity of the propane/pentane mixture is such that the tumbling time of flavodoxin in a CTAB reverse micelle of the water loading used is roughly equivalent to that of free flavodoxin in water. Thus, the increased S/N of the propane sample relative to the aqueous sample largely reflects the advantages of lower conductivity of the former in the context of the performance of cryogenically cooled NMR probes [26].

To explore the general applicability of the CTAB/hexanol system for proteins of low isoelectric point, we prepared solutions of enhanced green fluorescent protein (EGFP) in a similar fashion using pentane as the bulk solvent. Wild-type GFP from *Aequorea victoria* has been crystallized as both a monomer [27] and a dimer [28], and exists as a dimer in free aqueous solution at concentrations above 100 μM [29]. EGFP differs from wild-type GFP by four amino acids (insertion of V at position two, and F64L, S65T, H231L). Translational diffusion

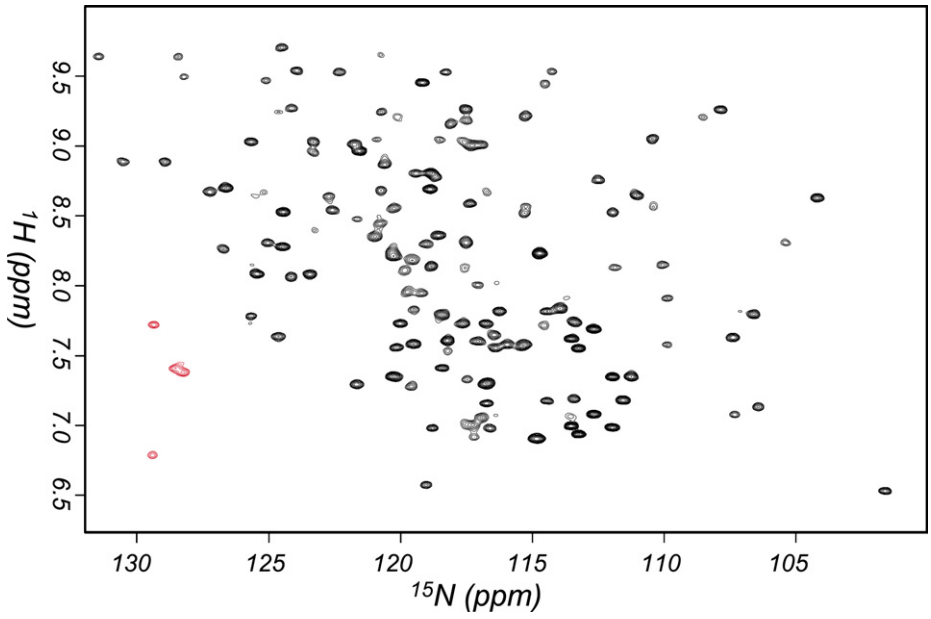

Fig. 1. ^{15}N HSQC spectrum for flavodoxin in CTAB reverse micelles dissolved in propane/hexanol (8% v/v) with a water loading of 12.5. The sample was 215 μM in flavodoxin. The spectrum was recorded at 25 °C on a Varian Inova spectrometer operating at 600 MHz (^{1}H) and equipped with a cold probe.

measurements for GFPuv, which differs from wild-type by three amino acids (F99S, M153T, and V163A), also support a dimeric form in solution [30]. Backbone assignments [31,32] and dynamics [30] measurements for the GFPuv mutant have also been performed. Encapsulated EGFP dimer was prepared in butane in a manner similar to that used for flavodoxin. The ^{15}N HSQC spectrum obtained for the protein in CTAB/butane/pentane/hexanol reverse micelle is indicative of a well-structured protein and is closely similar to that of protein in free aqueous solution, indicating that the protein was successfully encapsulated without significant structural perturbation (Fig. 2). In butane, the S/N is approximately 40% better than in a corresponding aqueous sample at the same protein concentration (10 mM sodium phosphate and 20 mM NaCl, pH 6.5), again largely reflecting the advantages of lower conductivity of the reverse micelle sample in the context of the performance of cryogenically cooled NMR probes [26].

For both EGFP and flavodoxin, the fidelity and efficiency of encapsulation were quite sensitive to the content of the co-surfactant hexanol. In addition, excess hexanol can significantly degrade the spectroscopic performance of the preparations by increasing the effective viscosity of the solvent. Though the use of a co-surfactant increases the parameter space that needs to be optimized the two examples illustrated here demonstrate the utility and promise of using the cationic surfactant CTAB to encapsulate acidic proteins at pH values above their isoelectric points.

In an interesting contrast, we explored the ability of CTAB to encapsulate a cationic protein, cytochrome *c*, which has an isoelectric point well above 11. Thus at physiological pH or lower, this protein is highly positively charged. Though the protein can be incorporated into anionic AOT reverse micelles, the recombinant protein [33,34] is completely denatured within the reverse micelle as judged by both NMR and UV/Vis spectroscopy (not shown). Attempts to screen the electrostatic interaction by increasing the ionic strength of the aqueous buffer resulted in more native-like UV/Vis spectra but significantly reduced incorporation efficiency such that NMR spectroscopy was no longer feasible. Interestingly, CTAB in pentane/hexanol (9:1 v/v) was able to efficiently encapsulate recombinant cytochrome *c* in 50 mM potassium phosphate buffer (pH 5.5) with essentially complete fidelity (Fig. 3). This can be rationalized by concluding that the preference of the protein-containing aqueous phase for the interior of the reverse micelle is sufficient to overcome electrostatic repulsion between the protein and the surfactant head-groups, and bring the highly water-soluble protein into the reverse micelle core. In this regard, it is interesting to note that the local concentration of the bromide would exceed 4 M in these samples if the ion is uniformly dispersed. However, the very slight chemical shift perturbations seen would strongly suggest that bromide remains tightly associated with its surfactant counterion and is not freely dissolved in the aqueous core of the reverse micelle.

Ultimately, the reverse micelle approach aims to significantly increase the number of proteins amenable to triple resonance NMR spectroscopy. The successful encapsulation of acidic and basic proteins in CTAB is

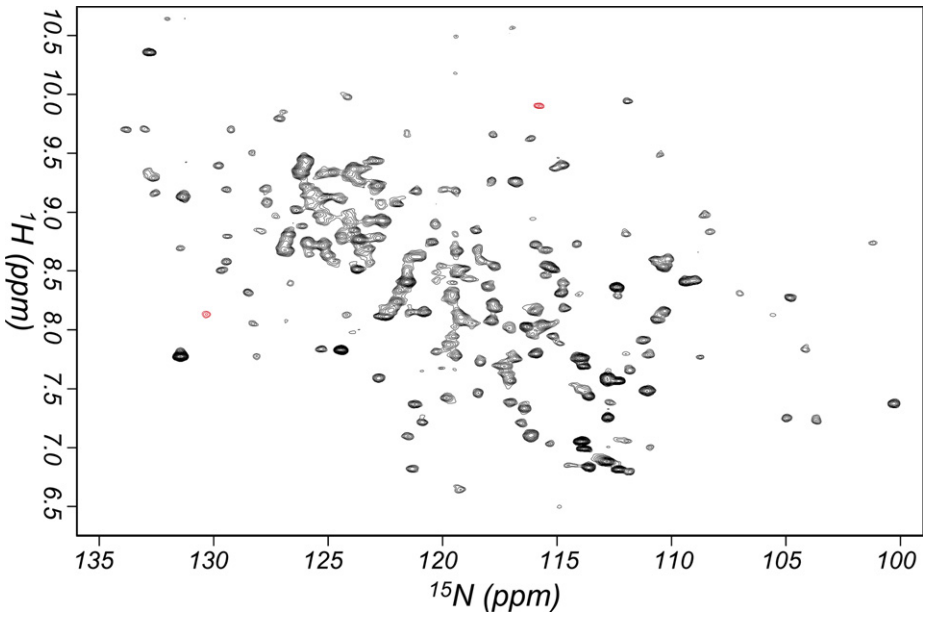

Fig. 2. ^{15}N HSQC spectrum for enhanced green fluorescent protein in CTAB reverse micelles dissolved in butane/hexanol (8% v/v) with a water loading of 12.5. The sample was 180 μM in EGFP dimer. The spectrum was recorded at 25 °C on a Varian Inova spectrometer operating at 600 MHz (^1H) and equipped with a cold probe.

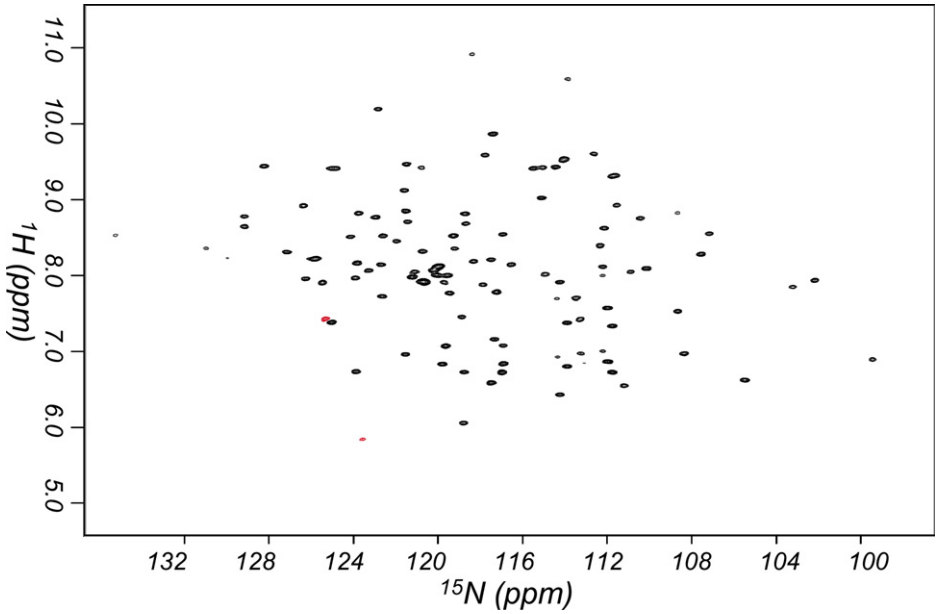

Fig. 3. ^{15}N HSQC spectrum for recombinant horse heart oxidized cytochrome *c* in CTAB reverse micelles dissolved in pentane/hexanol (10% v/v) with a water loading of 15. The sample was 200 μM in cytochrome *c*. The spectrum was recorded at 25 °C on a Varian Inova spectrometer operating at 600 MHz (^1H) and equipped with a cold probe.

a major step in the development of this technique. The improvement in spectrum quality in moving to lower-viscosity solvents was demonstrated by dissolving CTAB reverse micelles in solvent mixtures containing propane and butane. This solvent system was shown to be especially advantageous in the context of cold NMR probe technology. Future studies with this surfactant system will focus on extending these techniques to larger acidic proteins and to dissolving the reverse micelles in solvent mixtures containing ethane.

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