Biochimica et Biophysica Acta 1798 (2010) 150–160

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Review

A method for solution NMR structural studies of large integral membrane proteins: Reverse micelle encapsulation

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ARTICLE INFO

Article history:
Received 15 June 2009
Accepted 22 July 2009
Available online 8 August 2009

Keywords:
Reverse micelles
Membrane proteins
Detergents
NMR
Potassium channel
Surfactants

ABSTRACT

The structural study of membrane proteins perhaps represents one of the greatest challenges of the post-genomic era. While membrane proteins comprise over 50% of current and potential drug targets, their structural characterization lags far behind that of soluble proteins. Nuclear magnetic resonance (NMR) offers great potential not only with respect to structural characterization of integral membrane proteins but may also provide the ability to study the details of small ligand interactions. However, the size limitations of solution NMR have restricted comprehensive structural characterization of membrane protein NMR structures to the relatively small β-barrel proteins or helical proteins of relatively simple topology. In an effort to escape the barriers presented by slow molecular reorientation of large integral membrane proteins solubilized by detergent micelles in water, we have adapted the reverse micelle encapsulation strategy originally developed for the study of large soluble proteins by solution NMR methods. Here we review a novel approach to the solubilization of large integral membrane proteins in reverse micelle surfactants dissolved in low viscosity alkane solvents. The procedure is illustrated with a 54 kDa construct of the homotetrameric KcsA potassium channel.

Contents

1. Introduction

A fundamental limitation of solution NMR spectroscopy of large macromolecules is its sensitivity to the effects of slow molecular reorientation. Slow molecular reorientation decreases the efficiency of coherence transfer processes, which form the basis for modern triple resonance NMR experiments, and also broaden the resonance lines of observed signals. These effects become quite severe for proteins larger than about 30 kDa. The traverse optimized spectroscopy approach [1] cleverly avoids many of these spectroscopic issues but does require uniform deuteration of carbons and is limited to selected nuclei. An alternate strategy is to solubilize the protein within the protective water core of reverse micelle particles dissolved in a low

Abbreviations: AOT, sodium bis(2-ethylhexyl)sulfosuccinate; $C_{12}E_4$, *n*-dodecyl tetra ethylene glycol; CTAB, cetyltrimethylammonium bromide; DHAB, dihexadecyldimethylammonium bromide; DM, n-decyl-β-D-maltopyranoside; DPC, dodecylphosphocholine (foscholine); DTAB, dodecyltrimethylammonium bromide; HSQC, heteronuclear single quantum coherence; LDAO, lauryldimethylamine oxide; MCD, main chain directed; NAB, H_N–H_α–H_β; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; T_2, spin–spin or transverse relaxation time constant; TOCSY, total correlation spectroscopy; TROSY, transverse relaxation optimized spectroscopy

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doi:10.1016/j.bbamem.2009.07.027

viscosity fluid [2]. Since its introduction for soluble proteins, solution NMR studies employing reverse micelle encapsulation have proven advantageous in a variety of contexts such as the study of protein cold denaturation [3,4], proteins of marginal stability [5], and membrane proteins [6,7]. Most recently, technical advancements in high pressure equipment [8] and the utilization of the very low viscosity liquid ethane as a bulk solvent [9] have paved the way for the reverse micelle experimental system to warrant serious attention for its ability to overcome the slow tumbling problem of solution NMR applied to larger proteins without the limitations of deuteration.

The use of deuteration and the TROSY-based experiments [1] have already played an important role in extending the size of the biomolecules available to solution NMR studies. However, deuteration requires that the amide protons in a protein peptide backbone be accessible for back-exchange with hydrogen, which is difficult if not impossible to achieve with integral membrane proteins without irreversibly denaturing the protein in the process [10]. Additionally, deuteration of all aliphatic protons severely limits the number of long-range NOE distance restraints available for structure calculations. Here we review the basic elements of preparing samples of fully protonated proteins solubilized in reverse micelles that are suitable for high performance solution NMR with a particular emphasis on our recent adaptation of the methodology for integral membrane proteins.

2. The reverse micelle strategy for solution NMR

To set the stage for describing our recent advances in adapting the reverse micelle encapsulation strategy for solution NMR studies of integral membrane proteins we briefly summarize the state-of-the-art in its application to its original target, soluble proteins. This area has been the subject of extensive reviews and the interested reader is directed there [11,12]. Reverse or inverted micelles often contain hundreds of surfactant molecules with geometric and charge properties that drive the spontaneous organization of a typically spherical particle with an inner water core [13]. About a decade ago, this technology was adapted for high-resolution NMR studies of soluble proteins in low viscosity fluids [2]. In that context, the nature of the sample becomes particularly important. The fundamental motivation was to decrease the molecular reorientation time for the protein relative to free aqueous solution [2]. This is achieved by encapsulating the protein within the protective water core of the reverse micelle and to dissolve the resulting particle in a solvent of sufficiently low viscosity to overcome the "volume penalty" imposed by encapsulation (Fig. 1). A number of technical issues had to be overcome to make this approach viable. It is critically important that each reverse micelle contain only one protein macromolecule. This is generally achieved by limiting the amount of water available. Similarly, as NMR spectroscopy is relatively insensitive, near mM concentrations of protein are required in order to make advanced triple resonance NMR spectroscopy viable. Thus, a library of anionic, cationic and neutral surfactants has been developed to allow the encapsulation of soluble proteins in their native state. These include the anionic, di-acyl chain bis(2-ethylhexyl) sodium succinate (AOT) [2] and a short chain analog [14], the mono-acyl chain, cationic cetyltrimethylammonium bromide (CTAB) [15], the non-ionic *n*-dodecyl tetra ethylene glycol ($C_{12}E_4$) and other members of the (C_nE_m) family of surfactants [16], and the zwitterionic, mono-acyl chain lauryldimethylamine oxide (LDAO) [17] have all been shown in various combinations to be capable of encapsulating proteins with high structural fidelity and with suitable NMR parameters (e.g. concentration, stability, homogeneity, etc.). Though in favorable cases simple physical considerations can lead to identification of optimal encapsulation conditions [16] in general an empirical search is required.

In the alkane solvent–surfactant systems, homogeneous reverse micelle preparations on the order 150 μM are generally achievable. Fortunately, as solutions of encapsulated proteins are relatively non-conductive, modern cryogenically cooled NMR probes perform optimally and the low concentration of protein accessible is more an annoyance rather than a prohibitive barrier to comprehensive study by multidimensional NMR methods [18].

The choice of solvent is critical to the entire strategy, as indicated by Fig. 1. The preparation of solutions of encapsulated proteins dissolved in solvents of sufficiently low viscosity to obtain short

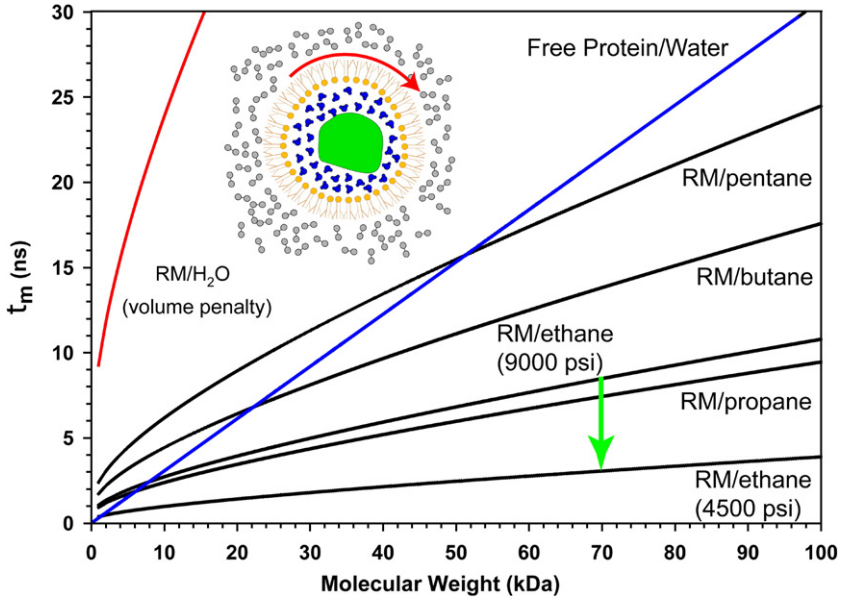

Fig. 1. The potential of protein encapsulation for improved hydrodynamic performance of the protein. Theoretical analysis of the anticipated dependence of the molecular reorientation time (τ_m) of a spherical soluble protein encapsulated within the water core of an AOT reverse micelle. The solid blue line represents the tumbling time for a protein of a given molecular weight in free aqueous solution. The curved red line represents the tumbling time for a protein of a given molecular weight encapsulated in an AOT reverse micelle (theoretically) dissolved in water. This indicates the "volume penalty" for encapsulation that needs to be overcome by use of solvents of low viscosity. The remaining curves correspond to the molecular reorientation times anticipated for encapsulated proteins dissolved in various short chain alkanes. The two curves for ethane represent the situation without (upper curve) and with (lower curve) additives (such as hexanol) present to lower the pressure required for suitable solutions of reverse micelles prepared in this solvent (see [9]).

molecular reorientation times has been restricted to the short chain alkanes [2], liquid carbon dioxide [19] and supercritical xenon [20]. In principle, the short chain alkanes offer the most promise for achieving solutions of lowest viscosity and benefit from having a large library of proven surfactant systems. A liquid at room temperature and pressure, pentane has a viscosity about one fourth that of water and is suitable for initial screening, optimization of conditions, and most experimental studies for proteins less than 25 kDa. Preparations of encapsulated proteins solubilized at NMR concentrations in liquid butane and propane require only modestly elevated pressure to maintain and can be studied in commercially available glass NMR tubes [2]. However, it is only with the recent development of specialized apparatus that it has become possible to undertake the preparation of solutions of encapsulated proteins dissolved in liquid ethane [8] at the pressures required for long-term viability as high performance solution NMR samples [9].

Some surfactants work best in a mixture and this turns out to be a critical feature for the approach described below for the encapsulation of an integral membrane protein. Co-surfactants are often actually required for the formation of small spherical reverse micelles. Hexanol is an example and is required for surfactants that are not soluble in organic solvents, such as most mono-acyl chain surfactants, which have the shape of an inverted cone compared to the cylindrical DHAB and the conical AOT. The volume percent hexanol in the bulk solvent depends on the length of the acyl chain and the properties of the headgroup but is generally surprisingly large. For example, the 16 carbon chain CTAB at 100 mM will require 8% v/v hexanol in the bulk solvent. The shorter, 12 carbon chain, zwitterionic 100 mM LDAO requires 4% v/v in bulk solvent. Importantly, the presence of hexanol does not appreciably increase the apparent viscosity of the preparation (Peterson & Wand, unpublished results).

An important requirement of the reverse micelle encapsulation strategy is often the need to minimize the size of the particle. This is most often achieved by limiting the amount of water present. The introduction of protein to the reverse micelle surfactant system often involves the introduction of the vast majority of the final water content. The water content of reverse micelle preparations is often expressed as a "water loading" which corresponds to the molar ratio of surfactant(s) to water. There are three techniques for the encapsulation of a protein into reverse micelles: bulk phase transfer, the injection method, and solubilization of lyophilized protein [21]. Each technique has its advantages and disadvantages, and depends both on the compatibility of the protein and the experimental goals (i.e. for most NMR studies, the smallest encapsulation assembly achievable is the most desired). Using the phase transfer scheme, reverse micelles are pre-formed in an alkane solvent, with just enough buffer to form empty reverse micelles. The protein is introduced from a concentrated aqueous phase. This approach does not provide a good mechanism for control of the water loading. Subsequent addition of surfactant can be used to reduce the average water loading by partitioning water to empty (non-protein containing) reverse micelles [21]. The injection method involves the addition of a highly concentrated protein solution into the slurry of surfactants and alkane solvent to limit the amount of water introduced [15]. Use of lyophilized protein allows for more precise control of the concentration of both protein and water but is often too harsh for many proteins.

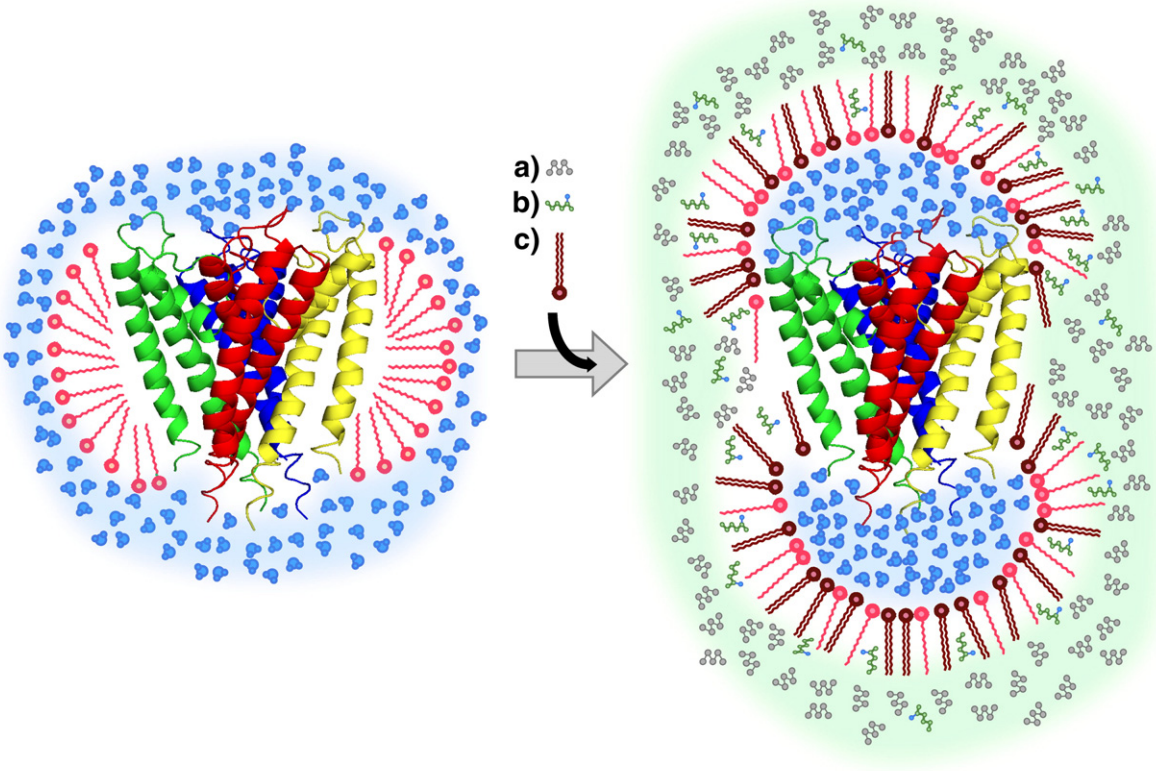

Fig. 2. Conversion from aqueous micelle to reverse micelle solubilized membrane protein. In bulk water a hybrid surfactant such as CTAB solubilizes the hydrophobic transmembrane domains of a membrane protein. Upon insertion of the membrane protein into a bulk alkane solvent (a), the hybrid surfactant serves two purposes: the conventional solubilization of hydrophobic domains, as well as the formation of a water-filled pocket to solubilize and extra- and intracellular regions of the protein. The use of certain co-surfactants such as hexanol (b) is necessary for the transition of some detergents to this hybrid state. This condition promotes control over the process. In other cases additional co-surfactants (c) may be added for the role of charge-balancing of surfactant headgroups and/or different tail regions, such as the double-chained (DHAB). The ribbon representation of $KcsA_{\Delta C35}$ [25] was generated using PyMol [43]. Reproduced from [6] with permission.

3. Encapsulation of membrane proteins

The central barrier to implementation of the reverse micelle encapsulation approach is simply the question of how to introduce an integral membrane protein into the protective assembly of a reverse micelle for its subsequent dissolution into alkane solvent. Our initial foray into using reverse micelle surfactants to solubilize integral membrane proteins was guided by Montal et al. [22]. In that study protein solubilized with its natural phospholipid membrane was injected at very low μM concentrations into heavy organic solutions of reverse micelle surfactant [23]. Low angle X-ray scattering studies of such complexes indicated a dumbbell like structure that is schematically illustrated in Fig. 2 [24]. We have termed this the "shower cap" model. We followed up on the direct injection approach with the aim of adapting it to light (low viscosity) fluids at NMR concentrations (mM). It proved not to be viable for several large integral membrane proteins. The principle difficulty is that at high protein concentrations the amount of either natural lipids or aqueous detergents present effectively ruins the reverse micelle surfactant phase diagram and renders formation of a well-behaved solution difficult if not impossible to achieve for sufficient concentrations of proteins. Though ^{15}N-HSQC spectra consistent with structured protein were obtained with preparations at very low concentrations, we were unable to reach the concentrations necessary for high quality triple resonance spectroscopy. Stripping the protein of carrier lipid or aqueous detergent resulted in unfolded protein that could not be refolded in the context of the reverse micelle surfactant mixture (unpublished results). Flynn has taken this approach for a peptide oligomer where refolding is apparently not an issue [7]. It would seem however that this strategy is not generally applicable to integral membrane proteins of significant size and topological complexity since they do not remain folded under such conditions and refolding in non-native environments can be problematic.

The key insight was to realize that the necessity of traditional aqueous detergents could be circumvented by employing a special kind of surfactant: one that can act as an aqueous detergent and as a reverse micelle surfactant. Examples include lauryldimethylamine oxide (LDAO), dodecyltrimethylammonium bromide (DTAB), and cetyltrimethylammonium bromide (CTAB). Importantly, this duality is sometimes conferred by the addition of a co-surfactant. For example, CTAB requires the addition of hexanol as a co-surfactant to generate stable reverse micelle assemblies in alkane solvents. In some cases accessory surfactants such as double-chained dihexadecyldimethylammonium bromide (DHAB) or sodium bis(2-ethylhexyl)sulfosuccinate (AOT) may be employed. When placed into an organic solvent the detergent micelle is 'flipped' into a reverse micelle around the soluble-domains of the protein, while its hydrophobic tails continue to protect the protein's transmembrane domain. We have developed this approach with the use

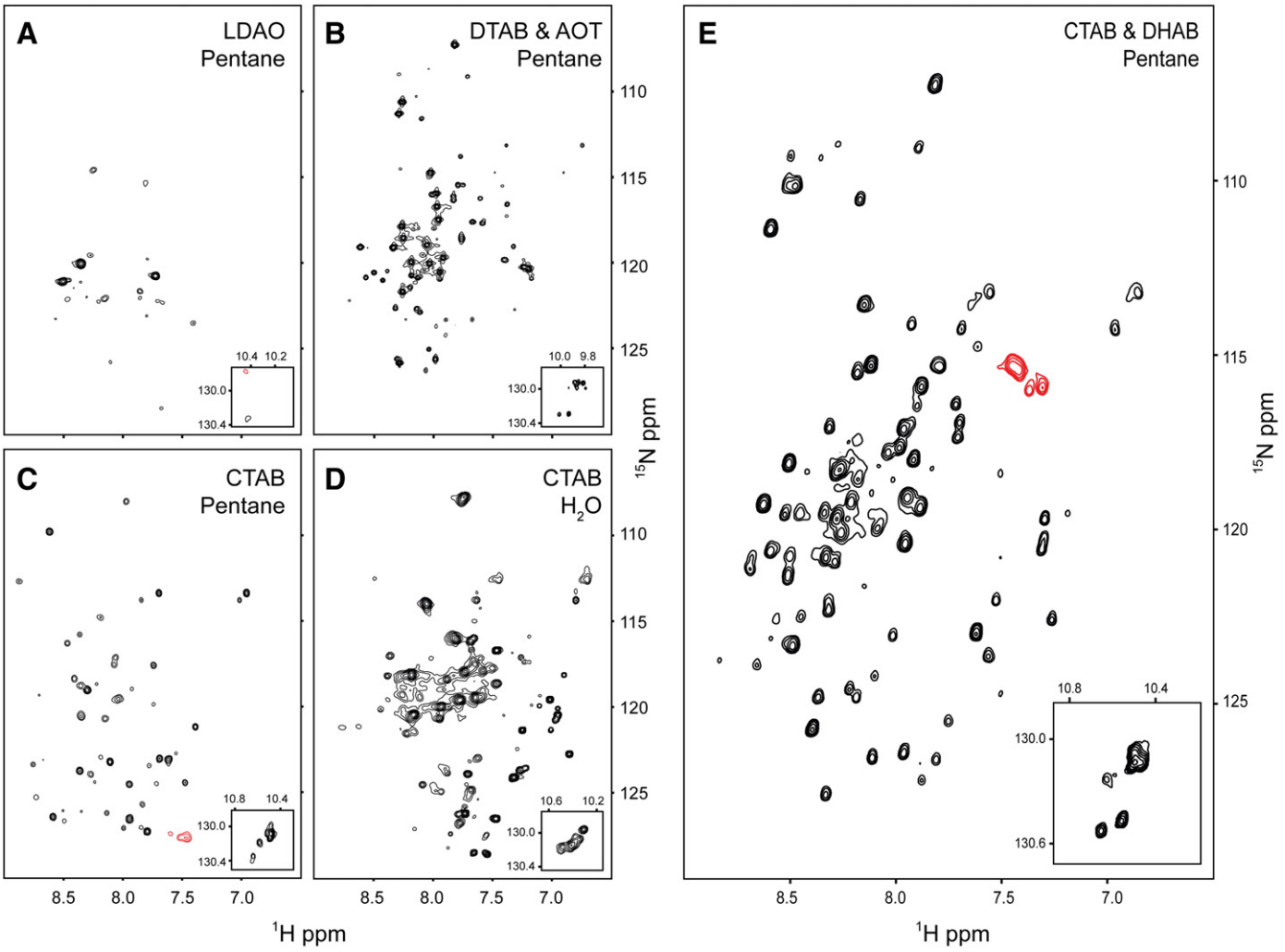

Fig. 3. Reverse micelle surfactant screen for solubilization of $KcsA_{\Delta C35}$. ^{15}N-HSQC spectra of $KcsA_{\Delta C35}$ solubilized in aqueous and reverse micelle surfactant systems. Detergents were tested as aqueous samples first before preparation as reverse micelles. All samples were ~0.15 mM in $KcsA_{\Delta C35}$ solubilized in pentane, and run at 25 °C. Panel A: 200 mM LDAO reverse micelles in pentane with 320 mM hexanol. B: 195 mM AOT and 195 mM DTAB. C: 200 mM CTAB reverse micelles in pentane with 800 mM hexanol. D: 125 mM CTAB and 125 mM DHAB with 390 mM hexanol. Insets show the spectral region containing the tryptophan indole N–H correlations. Reproduced from [6] with permission.

of a 54 kDa construct of the large homotetrameric KcsA potassium channel, with the basic idea illustrated in Fig. 2.

The construct employed here, $KcsA_{\Delta C35}$, consists of the putative N-terminal helix, both transmembrane helices, the pore helix, and the selectivity filter and is the same used in the original crystal structure of the potassium channel [25]. The 35 residue C-terminal cytoplasmic domain of KcsA is removed by proteolysis along with the His-tag used in the initial affinity purification of the protein. KcsA, which will only refold in a lipid environment [26] or from denaturing conditions in a detergent environment [27], successfully undergoes both the dialysis and lyophilization steps while maintaining the native fold. Prior to testing a protein directly in reverse micelle conditions, the consecutive steps of dialysis, lyophilization and resuspension into buffer were assessed by circular dichroism.

As suggested above, attempts to solubilize KcsA in reverse micelle surfactants directly from lipid or traditional aqueous detergent preparations proved fruitless. The feasibility of employing a "dual use" or "hybrid" surfactant capable of forming both aqueous micelles and reverse micelles in alkane solvents was then explored. The surfactants CTAB and DTAB proved useful in the aqueous solubilization of KcsA while AOT, largely due to its relatively low solubility in water did not. The aqueous detergent LDAO, used for X-ray crystallographic studies of KcsA [25], functions as a reverse micelle surfactant but gave a poor ^{15}N-HSQC spectrum (Fig. 3). Using these basic leads, encapsulation conditions were optimized empirically by varying protein concentration, water loading, co-surfactant and surfactant concentrations and so on. The strategy simply relies on a fundamental property of proteins that arises from the thermodynamic hypothesis for protein folding and stability. This view comes from the response of the protein energy landscape to perturbations from chemical denaturants [28], very low temperature [4] and very high hydrostatic pressure [29] as assayed by the "native state" hydrogen exchange method. As the protein is destabilized, the free energy gap between (partially) unfolded states and between the unfolded states and the native state is compressed (Fig. 4). This results in extensive line broadening in the NMR spectrum as the various non-native states interconvert. The approach is to refine encapsulation conditions with the criterion of more "native-like" ^{15}N-HSQC spectra as the guide. Native-like spectra are characterized by an appropriate number of correlations (cross peaks), uniformity of line shape and intensity, appropriate chemical shift dispersion and so on.

It was empirically determined that the di-acyl chain CTAB analogue, DHAB, when added as co-surfactant with CTAB in an equimolar ratio resulted in the best ^{15}N-HSQC spectrum. This can be rationalized by noting that the expanded volume of the surfactant tail would further stabilize KcsA [30]. Presumably because of the greater volume occupied by the hydrocarbon tails of DHAB, less hexanol is required in this mixture. After identification of the CTAB:DHAB:hexanol surfactant system as the most optimal for KcsA, other solubilization conditions were refined. For example, the extra- and intracellular regions of an integral membrane protein are presumably the only regions that require solubilization; the water loading reflects that required for two small proteins. Indeed, for KcsA a water loading of 6 was found to give the most native-like ^{15}N-HSQC spectrum.

In addition to the search for reverse micelle solubilization conditions that optimally support the structural integrity of the protein, one also has to carefully consider the effect of a given set of encapsulation conditions upon the NMR performance of the protein. An important example of this is the effect of reverse micelle surfactant concentration on the effective viscosity of the solution. While higher levels of surfactant will support higher protein concentration it can also lead to degradation in the relaxation properties of the protein. A balance between absolute protein concentration and optimal relaxation properties must be reached [21]. In the case of KcsA, the improvement in ^{15}N spin–spin (T_2) relaxation times of backbone amide sites at low surfactant concentration is significant (Fig. 5). Temperature is another parameter that provides a potential opportunity to significantly improve the relaxation properties of large macromolecular assemblies. However, in contrast to water, the temperature dependence of the bulk viscosity of liquid alkanes is relatively small. In addition, the long term stability of the KcsA reverse micelle preparation is greatly shortened at significantly elevated temperatures. Modest improvements in relaxation times are seen on going from 15° to 35 °C (Fig. 6). At 25 °C bulk pentane has a viscosity of ~200 μPa s, a 3.5-fold

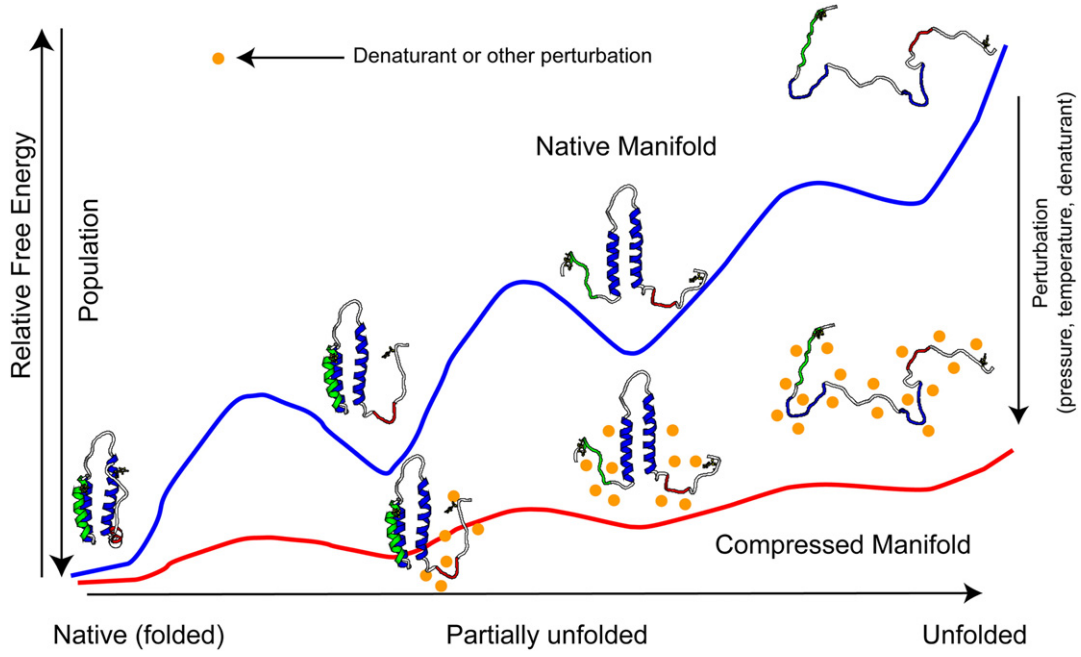

Fig. 4. Refinement principle for membrane protein encapsulation. Schematic representation of the effect of surfactant (denaturant) or other perturbation on the free energy landscape of a protein. For a destabilizing perturbation, the free energy landscape is compressed i.e. the free energy gap between the folded native state and all partially unfolded states is reduced. This causes both averaging effects in ^{15}N-HSQC spectra and an increased distribution of populations. The net result is significant line broadening and/or loss of signals.

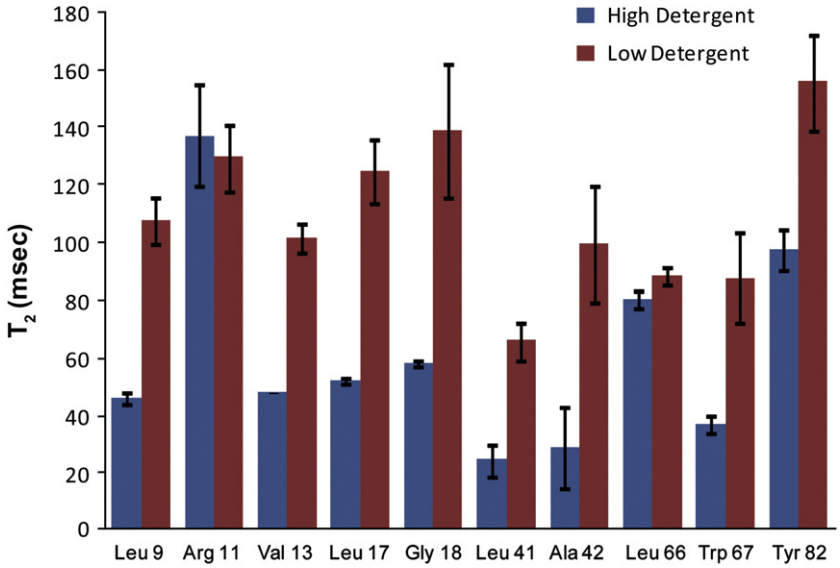

Fig. 5. Effect of reverse micelle surfactant concentration on the NMR relaxation properties of $KcsA_{\Delta C35}$. A comparison of amide $^{15}NT_2$ relaxation times for $KcsA_{\Delta C35}$ reverse micelle samples in pentane at 25 °C. The high detergent sample contained 390 mM total surfactant of a 1:1 molar ratio of CTAB:DHAB, and 560 mM hexanol. The low detergent sample contained 150 mM total surfactant of a 1:1 molar ratio of CTAB:DHAB and 400 mM hexanol. ^{15}N T_2 relaxation was measured using the pulse sequence of Farrow et al. [44] and collected at 600 MHz (^1H) on a Varian Inova NMR spectrometer.

improvement over water at the same temperature. This provided for triple resonance data of sufficient quality to allow for the backbone assignments of nearly 60% of the amide cross peaks for $KcsA_{\Delta C35}$, however the assignments were sharply skewed towards the sharper (longer T_2) resonances in the spectra (see below).

A final variable in the refinement of sample conditions was the use of the very low viscosity solvent liquid ethane. The use of ethane typically entails significantly elevated pressure, requiring specialized apparatus [8] for both preparation of the sample and its use in the NMR spectrometer [9]. Such apparatus is now commercially available (Daedalus Innovations, LLC). Backbone amide ^{15}N T_2 relaxation times of KcsA reverse micelles prepared in liquid ethane have generally superior transverse relaxation properties than the corresponding sample in pentane (Fig. 7).

Sample consistency can be problematic for membrane proteins and their associated lipids and detergents; the same protein can have vastly different spectra in different detergents [31]. It is important that membrane proteins be cleanly exchanged from the extraction detergent to the reverse micelle surfactant system to provide a homogenous sample. For many His-tagged or other adhesion tagged membrane proteins, the detergent can be exchanged while the protein is attached to the affinity matrix [32]. With KcsA, after cell lysis, extraction using n-decyl-β-D-maltopyranoside (DM), and ultracentifugation, the supernatant containing the full-length protein is run over a nickel affinity column. After high salt and low imidazole washes containing DM, the protein is washed extensively in-place with buffer containing CTAB. The resin is then repacked into a column, washed with additional CTAB-containing buffer, and eluted with a high concentration imidazole solution

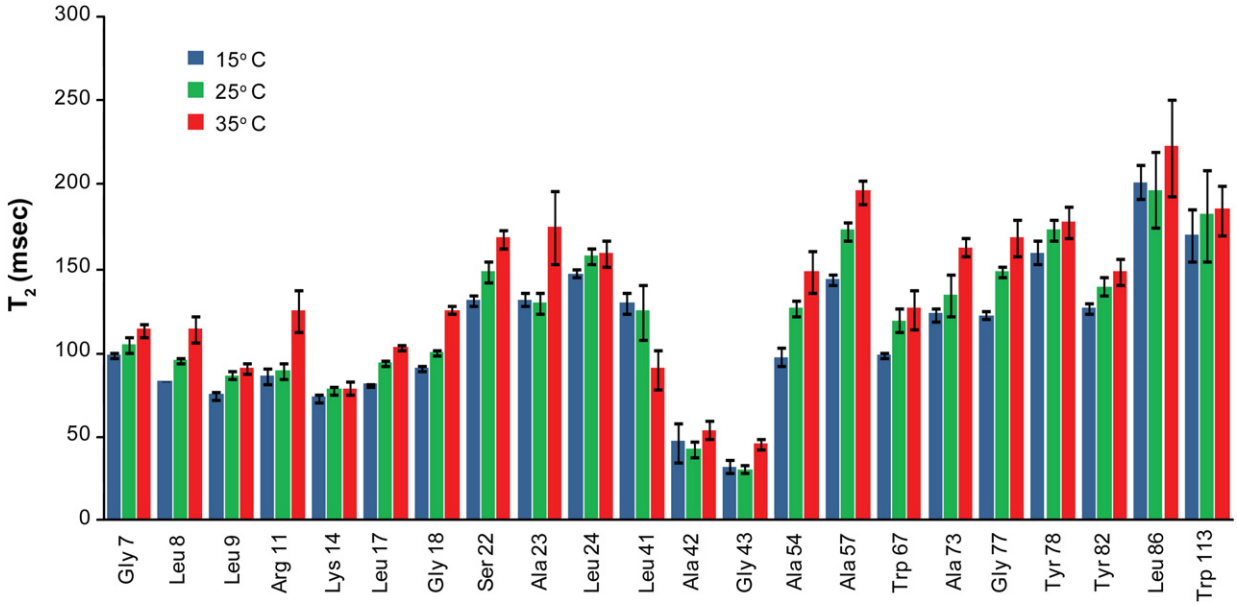

Fig. 6. Effect of temperature on the NMR relaxation properties of the $KcsA_{\Delta C35}$ encapsulated in reverse micelles. A comparison of amide ^{15}N T_2 relaxation times for $KcsA_{\Delta C35}$ reverse micelle samples in bulk solvent pentane at 15, 25, and 35 °C. The sample contained 194 mM total surfactant of a 1:1 molar ratio of CTAB:DHAB and 340 mM hexanol. ^{15}N T_2 relaxation was measured using the pulse sequence of Farrow et al. [44] and collected at 500 MHz (^1H) on a Varian Inova NMR spectrometer.

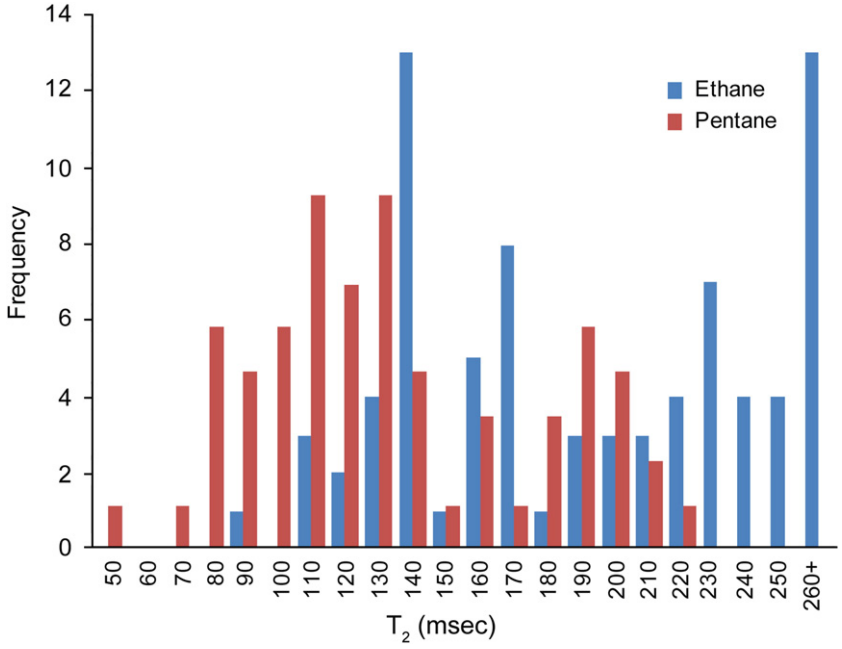

Fig. 7. Comparison of NMR relaxation properties of $KcsA_{\Delta C35}$ in reverse micelles dissolved in pentane and ethane. A comparison of the distributions of amide ^{15}N T_2 relaxation times for $KcsA_{\Delta C35}$ reverse micelles in pentane (red) and ethane (blue) at 600 MHz (^1H). The distribution clearly shows an improvement in transverse relaxation properties of residues in ethane. Higher T_2 values in both samples (>150 ms) are attributed to residues in more mobile regions of KcsA, such as loop regions and the N-terminal helix. The pentane sample contained 150 mM total surfactant at a 1:1 molar ratio of CTAB:DHAB with 400 mM hexanol. The ethane sample contained 180 mM total surfactant at a 1:1 molar ratio of CTAB:DHAB with 800 mM hexanol and included 5% v/v of pentane. The sample was pressurized to 4500 psi (300 bar). ^{15}N T_2 relaxation was measured using the pulse sequence of Farrow et al. [44] and collected at 600 MHz (^1H) on a Varian Inova NMR spectrometer.

containing CTAB. It should be noted that extraction of the membrane protein with the milder DM gave a better protein yield than extraction using CTAB alone. Generally this approach has yielded reasonably consistent spectra in the bulk solvent ethane (Fig. 8).

4. Resonance assignments of encapsulated $KcsA_{\Delta C35}$

The task of assigning the backbone and sidechain resonances for a protein encapsulated in reverse micelles followed much the same strategy employed for soluble proteins and utilized fully protonated protein and the non-TROSY triple resonance experiments [33]. The sample optimizations described above ensure that the shortest molecular reorientation correlation time is achieved for the membrane protein in reverse micelles. Use of deuterated solvents and surfactants and the relatively reduced water content (4 M) of the sample allowed for spectra relatively free of artifactual signals.

The combination of the high helical content, sequence redundancy, and the general homogeneous environment of the transmembrane domains in KcsA contribute to excessive spectral crowding and considerable degeneracy, particularly in the carbon spectrum of the

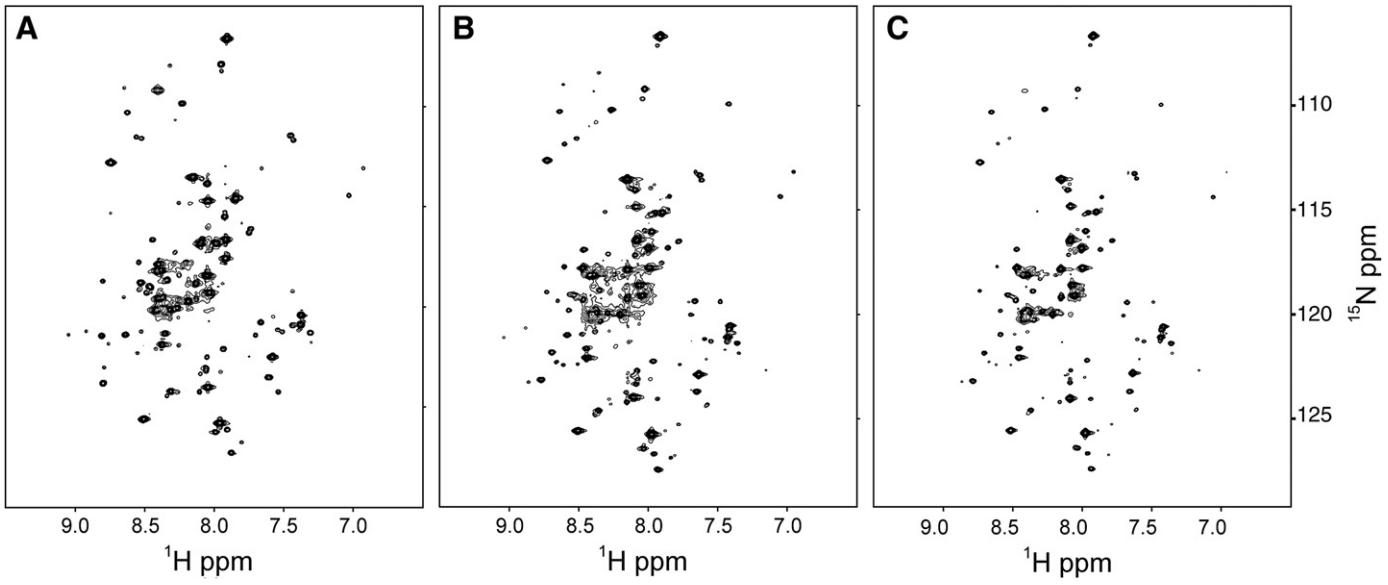

Fig. 8. Reproducibility of preparations of $KcsA_{\Delta C35}$ in reverse micelles dissolved in ethane. ^{15}N-labeled $KcsA_{\Delta C35}$ (A), $^{13}C^{15}N$-labeled $KcsA_{\Delta C35}$ (B), and $^{13}C^{15}N$-labeled $KcsA_{\Delta C35}$ (C), all from different preparations. Each sample contains ~200 mM of 1:1 CTAB:DHAB. and 0.225 mM $KcsA_{\Delta C35}$ monomer concentration. The bulk solvent is ethane at 4000–4500 psi, with an additional 5% v/v of pentane, and 800 mM hexanol. The theoretical viscosity of the sample at 4500 psi is 86.9 μPa s, calculated from the molar fractions of ethane and pentane at 4500 psi and taking into account the compressibility of pentane at high pressure.

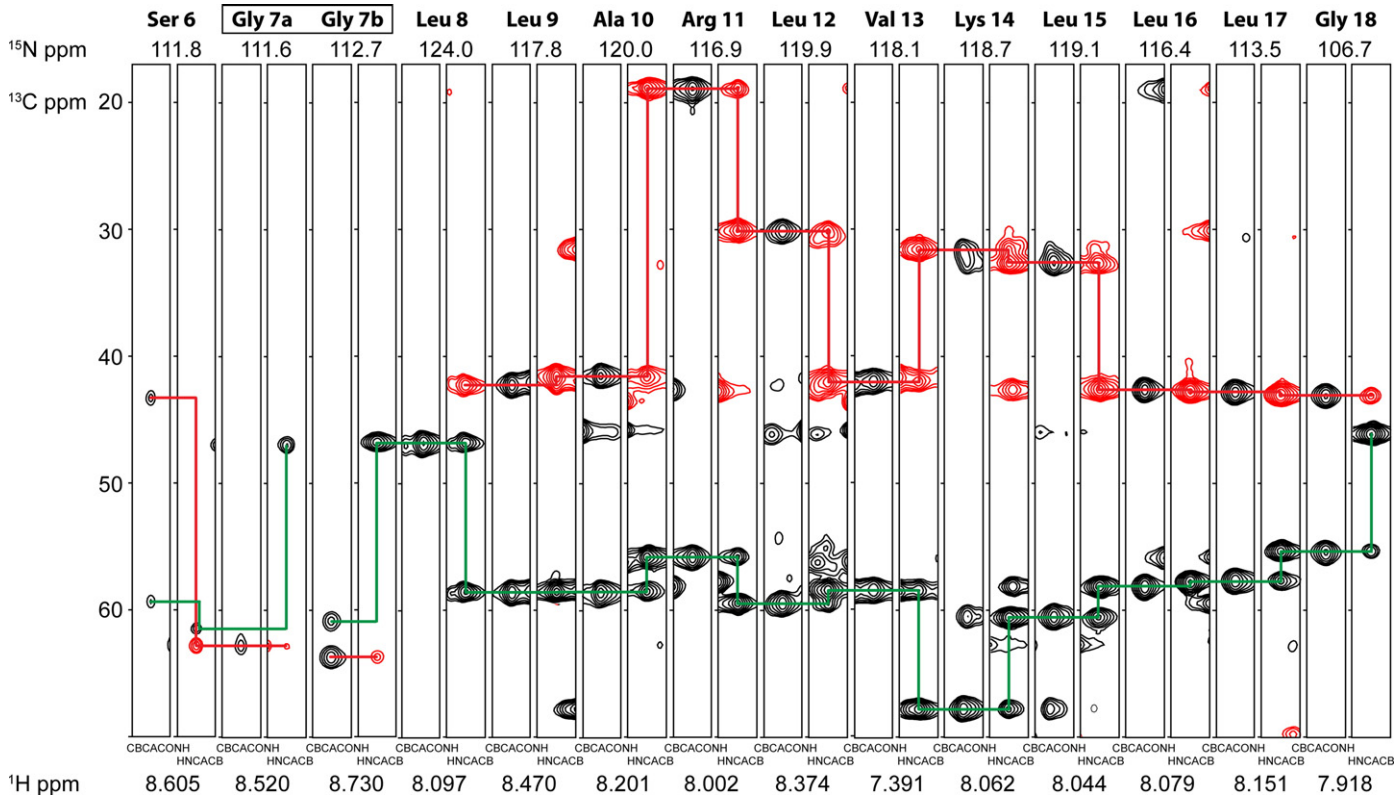

Fig. 9. Triple resonance assignment of the N-terminal helix of $KcsA_{\Delta C35}$ in reverse micelles dissolved in ethane. Slices of the HNCACB and CBCACONH spectra of $KcsA_{\Delta C35}$ in reverse micelles dissolved in ethane showing the connectivities for N-terminal helix from G18 to S6. The opposite sign HNCACB peaks are shown in red. The C_α connectivity is shown by a solid green line and C_β connectivity is shown by a solid red line.

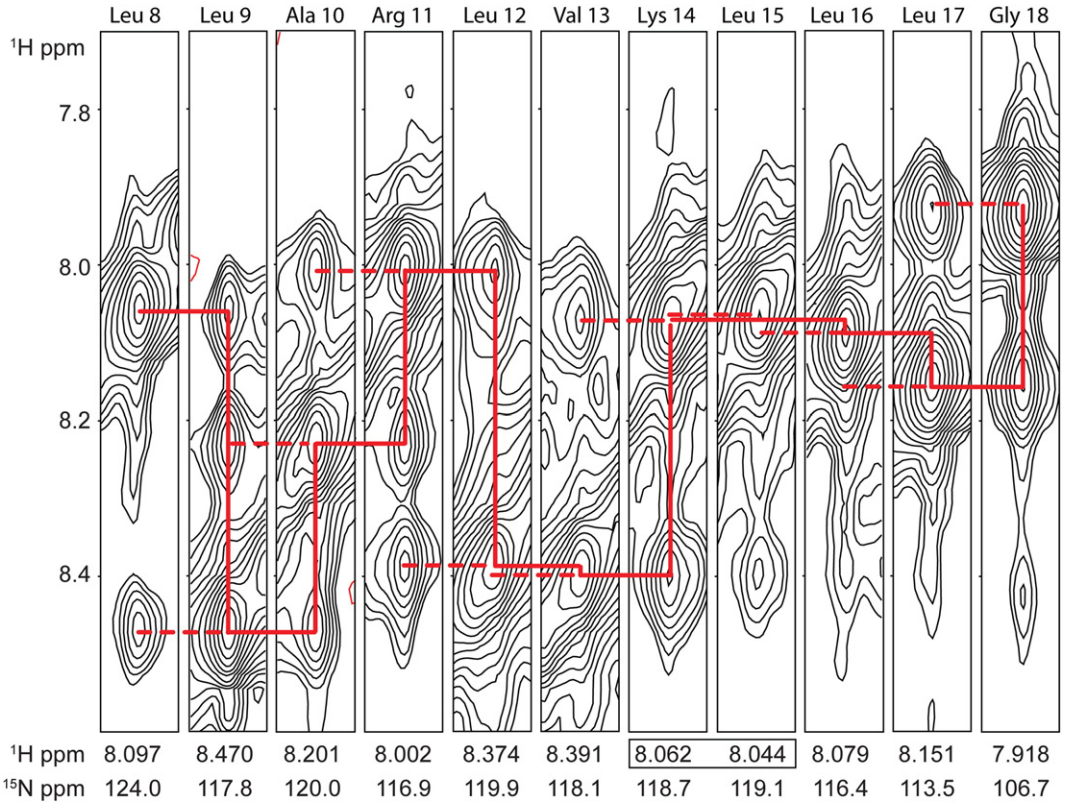

Fig. 10. Main chain directed NOE connectivity of the N-terminal helix of $KcsA_{\Delta C35}$ in reverse micelles dissolved in ethane. Slices of the ^{15}N NOESY-HSQC spectrum of $KcsA_{\Delta C35}$ in reverse micelles dissolved in ethane showing the amide HN–amide HN sequential connectivities for the N-terminal helix from G18 to L8. A 90 ms mixing time was used.

protein. These factors made the establishment of sequential connections based on traditional through-bond correlation experiments quite difficult. An inventory of the HNCO spectrum gave an essentially complete count of amide correlations. The standard suite of experiments was utilized and the HNCACB/CBCA(CO)NH and HNCA/HN(CO)CA pairs formed the foundation for the triple resonance based assignment. To help resolve the extensive chemical shift degeneracy in the carbon spectrum, we turned to the main chain directed (MCD) assignment strategy originally developed to disentangle the complexities of two-dimensional ^{1}H–^{1}H spectra of proteins [34,35]. The MCD procedure is based on hierarchical pattern recognition in NOESY spectra using the intra-residue H_N–H_α–H_β (NAB) sets that are relatively easily identified [36]. Here, the latter so-called NAB sets were identified in HC(CCO)NH-TOCSY spectra and emphasize the advantage of being able to employ fully protonated protein. Helical MCD patterns were then located in ^{15}N-NOESY-HSQC spectra to resolve ambiguities in triple resonance backbone connectivities.

To illustrate the application of the assignment strategy outlined above for KcsA in reverse micelles, we describe the identification of resonances in the N-terminal sequence of the $KcsA_{\Delta C35}$ construct used here. This region of the protein structure was disordered in the crystal structure [25]. Strip plots of the HNCACB and CBCACONH spectra for the N-terminal helix of KcsA are shown in Fig. 9. The highlighted box indicates two resonances for G7, illustrating an issue of conformational exchange that is encountered for some residues of the protein. One of the resonances for G7 follows from L8, and though it is clearly a G(i)–S(i − 1), it does not correlate with the lone S(i)–L(i − 1) pair in KcsA. Instead, a nearby G resonance matches cleanly to the resonance for S6, continuing the string of assignments. The assignments of the N-terminal helix of KcsA suggested by the triple resonance connectivities were guided and confirmed by analysis of the MCD NOE patterns in the ^{15}N-NOESY-HSQC spectrum. The corresponding amide NH–amide NH NOE connectivities are shown in Fig. 10.

The wave-like pattern of the chemical shifts of sequential amide H_N is indicative of an amphipathic helix lying along a water–membrane interface, which is consistent with earlier solution NMR studies of KcsA in DPC micelles [32] and EPR studies in liposomes [37]. A TALOS [38] analysis of the N, C_α, C_β, C′, and H_α chemical shifts of the N-terminal helix in KcsA in reverse micelles also indicates backbone phi and psi torsion angles typical of α-helical structure.

5. Structural integrity of encapsulated $KcsA_{\Delta C35}$

Though the resonance assignments are on-going, a sufficient number of C_α and C_β resonances of KcsA in reverse micelles have been assigned to allow identification of regions of helical secondary structure elements [39,40]. The major helical domains of KcsA (N-terminal helix and transmembrane helices 1 and 2), as well as the attendant turns and loops have been determined. The pattern of secondary structure elements found in KcsA in reverse micelles is consistent with other solution NMR efforts in aqueous KcsA micelles [10,32], solid state NMR of KcsA in bilayers [41] and, aside from the N-terminal helix discussed above, the crystal structure [25].

6. Functional competence of encapsulated $KcsA_{\Delta C35}$

A central issue in structural studies of an integral membrane protein solubilized in reverse micelle surfactants is whether the protein

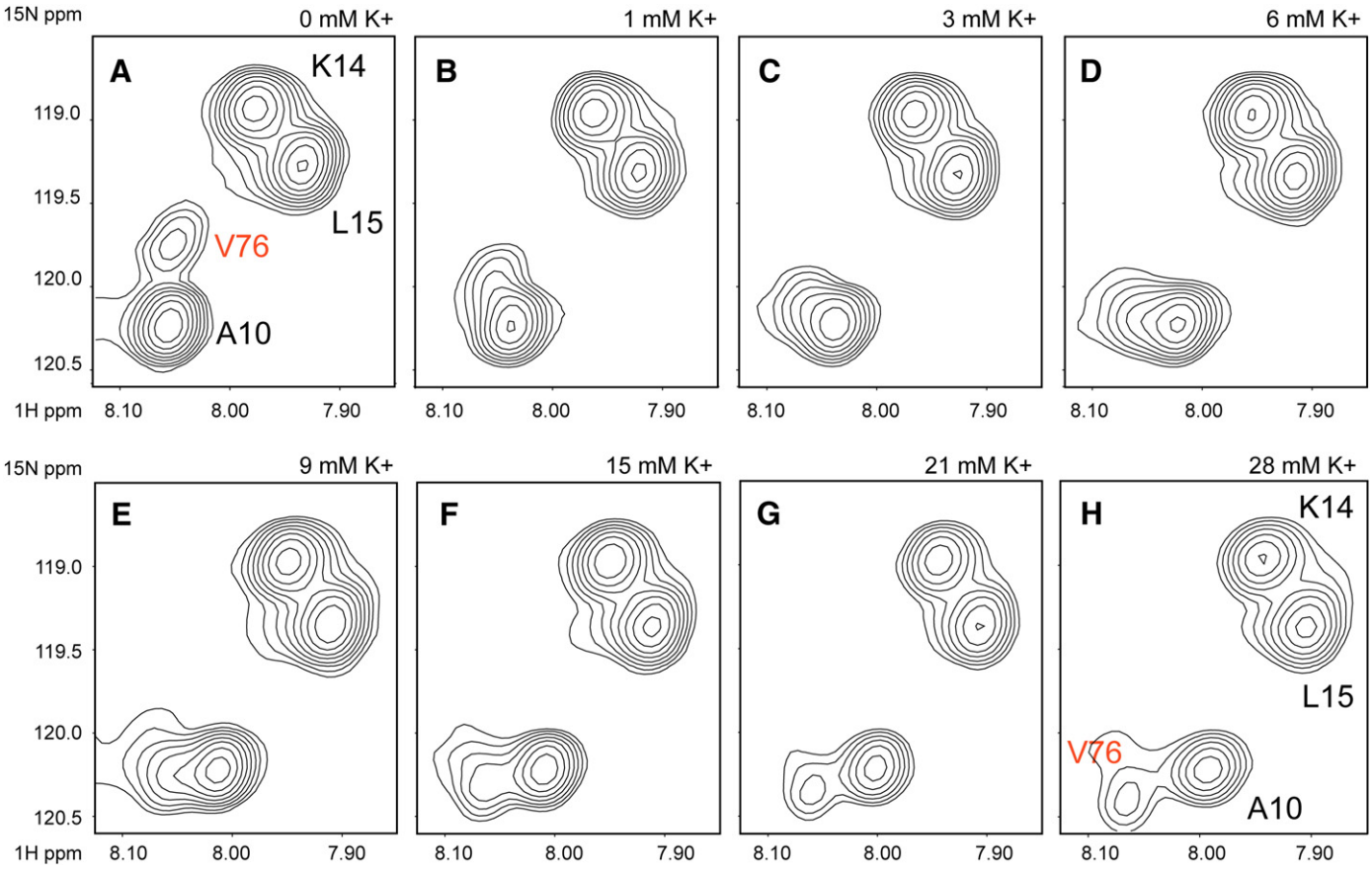

Fig. 11. K^+ titration experiments. The selectivity filter residue V76 undergoes a significant change in its chemical shift relative to neighboring, non-selectivity filter residues in the spectra:(A) 0 mM K^+, (B) 1 mM K^+, (C) 3 mM K^+, (D) 6 mM K^+, (E) 9 mM K^+, (F) 15 mM K^+, (G) 21 mM K^+, and (H) 28 mM K^+ titrated into a sample. The titration was carried out using the original sample buffer 50 mM Bis–Tris, 50 mM NaCl, and 50 mM DSS (for reference) at pH 7.0. Potassium was added in ~0.5 μL increments of 50 mM Bis–Tris, pH 7.0 with varying concentrations of KCl to achieve the desired potassium level in the sample. The water loading of the reverse micelle sample was increased by 3 over the full course of the titration, an amount that did not result in changes to the overall spectra. Samples were prepared in pentane. Spectra were obtained at 600 MHz (^{1}H) and 25 °C.

maintains its functional state. The ability of the KcsA channel in reverse micelles to selectively coordinate potassium was therefore examined by titration of the channel with potassium in a competing background of sodium. Since the KcsA selectivity filter uses carbonyls from each monomer of the homo tetrameric KcsA to serve as surrogate water molecules for passing potassium ions [42], this assay also confirms the quaternary structure of the channel. Fig. 11 shows chemical shift changes of V76, a residue located in the selectivity filter. Chemical shift changes were also observed for the selectivity filter residues Y78, G77, T75, T74, and nearby residues A73, T72, and E71. These residues demonstrate fast exchange kinetics for the titration of potassium ions into the channel. The existence of conformers for the latter two residues, T72 and E71, represents the presence of slow exchange on the chemical shift timescale in the flap region of the filter. As discussed previously [6], this slow exchange may represent either the channels transition from a low- to high-potassium occupancy state, or the exchange between the binding sites 1,3 and 2,4 occupancy states of potassium ions in the filters four ion-binding sites.

7. Conclusions

Technical advancements in the reverse micelle NMR technology have made the encapsulation of macromolecules in very low viscosity solvents routine. Here we have demonstrated an approach for transferring integral membrane proteins from an aqueous detergent solubilized state to the reverse micelle system that maintains the structural integrity of the protein. This involves the use of a hybrid surfactant that allows the transfer without the need to strip the protein of contaminating lipids or aqueous detergents that would severely compromise the reverse micelle phase diagram. We have presented here a general strategy for the refinement of encapsulation conditions for integral membrane proteins that parallels the screening method used for aqueous membrane protein detergent systems. Additionally we have identified some of the key variables that play a role in the optimization of experimental conditions. The balance of protein concentration, and the attendant surfactant concentration, versus the transverse relaxation properties of the protein emerges as a central concern. The improved relaxation properties of proteins encapsulated in reverse micelles in ethane have allowed triple resonance spectra of good quality to be obtained without the need for extensive deuteration. This allows for traditional main chain directed ^{1}H–^{1}H NOE analysis to be employed to resolve ambiguities in the highly degenerate triple resonance spectra of large alpha helical integral membrane proteins such as KcsA. Most importantly we have shown the preservation not only of secondary, tertiary, and quaternary structure of KcsA in reverse micelles, but the additional properties of functionality by proper coordination of potassium ions.

Acknowledgements

This work was supported by the Mathers Foundation and the National Institutes of Health (NIH) grant GM 085120. J.M.K. is the recipient of a National Science Foundation Graduate Research Fellowship (2004016452) and is an NIH predoctoral trainee (GM 08275). We thank Dr. Ronald Peterson for helpful discussion. A.J.W. declares a competing financial interest as a member of Daedalus Innovations, LLC, a manufacturer of reverse micelle apparatus.

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