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ARTICLE

Use of reverse micelles in membrane protein structural biology

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Abstract Membrane protein structural biology is a rapidly developing field with fundamental importance for elucidating key biological and biophysical processes including signal transduction, intercellular communication, and cellular transport. In addition to the intrinsic interest in this area of research, structural studies of membrane proteins have direct significance on the development of therapeutics that impact human health in diverse and important ways. In this article we demonstrate the potential of investigating the structure of membrane proteins using the reverse micelle forming surfactant dioctyl sulfosuccinate (AOT) in application to the prototypical model ion channel gramicidin A. Reverse micelles are surfactant based nanoparticles which have been employed to investigate fundamental physical properties of biomolecules. The results of this solution NMR based study indicate that the AOT reverse micelle system is capable of refolding and stabilizing relatively high concentrations of the native conformation of gramicidin A. Importantly, pulsed-field-gradient NMR diffusion and NOESY experiments reveal stable gramicidin A homodimer interactions that bridge reverse micelle particles. The spectroscopic benefit of reverse micelle-membrane protein solubilization is also explored, and significant enhancement over commonly used micelle based mimetic systems is demonstrated. These results establish the effectiveness of reverse micelle based studies of membrane proteins, and illustrate that membrane proteins solubilized by reverse micelles are compatible with high resolution solution NMR techniques.

Keywords Membrane protein · Gramicidin A · Encapsulation · Reverse micelle · PFG NMR Diffusion

Introduction

Membrane proteins are crucial in virtually all aspects of biology. Apart from key biological functions, damaged membrane proteins are implicated in many diseases and are important drug targets (Sanders and Myers 2004). Balanced against their importance, membrane proteins are generally challenging targets due to a number of factors including: large particle size, difficulty in overexpression, aggregation, and low solubility, all of which lead to the fact that structural studies of these proteins are vastly underrepresented relative to water soluble proteins (Torres et al. 2003). Current structural studies of membrane proteins utilize conventional micelle-forming surfactants to refold and stabilize the native protein conformation (le Maire et al. 2000; Fernandez and Wuthrich 2003). Although the potential for reverse micelles (RMs) to serve as membrane mimetics has been recognized, practical implications have yet to be demonstrated (Wirz and Rosenbusch 1984; Darszon and Shoshani 1992). The methods and results described here establish a solid basis for realizing the potential application of RM encapsulation techniques to studies of membrane proteins.

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RMs are nanoparticles composed of surfactant molecules organized around an aqueous core when dissolved in apolar solvents. RMs have previously been shown to have important industrial and biophysical applications. More recently, benefits to structural biology and biophysical studies of encapsulated proteins have been explored, including the investigation of confinement effects, low temperature unfolding, and enhanced spectroscopic resolution (Wand et al. 1998; Babu et al. 2004; Van Horn et al. 2005; Simorellis and Flynn 2006).

AOT is a relatively short, branched amphiphilic molecule that is the predominant RM forming surfactant. AOT RMs have been shown to encapsulate a wide range of water soluble proteins of various shape, size, and electrostatic character (Luisi, 1985; Flynn, 2004). RMs are well known for their ability to host (encapsulate) water soluble proteins, providing an environment that stabilizes the native fold (Babu et al. 2001). On the other hand, no systematic effort has been invested in developing applications of RMs for studies of membrane proteins. As discussed below, such a new method would be predicted to have a significant impact on the field.

Micelles in aqueous solution at room temperature tumble relatively slowly, leading to rapid transverse relaxation rates for proton-bound nuclei (^{13}C, ^{15}N) in the proteins that are solubilized within them. This rapid transverse relaxation in turn limits the sensitivity and resolution of the NMR experiment. The application of RM based systems in studies of membrane proteins has important practical benefits, one of which is that the particle can be solubilized in low viscosity solvents, which decreases the correlation time (τ_c) and thus the rate of transverse NMR relaxation, resulting in an enhancement of both resolution and sensitivity of solution NMR experiments (Wand et al. 1998). AOT reverse micelles are smaller than their SDS counterparts, and more importantly, can be reconstituted in alkane solvents that have viscosities much lower than water. Low viscosity enhanced tumbling of RM based particles is thus predicted to improve opportunities for detailed characterization of structure and site-specific dynamics of membrane proteins, the latter being relatively unexplored, due to the large effective particle size and resulting slow tumbling. This direct approach makes use of the favorable hydrodynamical properties of the apolar solvent, and complements the spectroscopic approach to the problem of rapid transverse relaxation that has been realized in transverse relaxation optimized spectroscopy (TROSY) (Pervushin et al. 1997).

The antibiotic gramicidin A (gA) is a prototypical membrane protein ion channel that has been well studied by a variety of spectroscopic and structural techniques (Salom et al. 1992; Ketchem et al. 1997; Burkhart et al. 1998; Townsley et al. 2001; Andersen et al. 2005). The unique sequence of alternating L- and D- chirality apparently renders gramicidin sensitive to the environment in which it is placed, and gA adopts a wide range of conformations. Two major folding motifs have been identified for gramicidin: (i) the single stranded helical dimer or *the channel form* (Arseniev et al. 1985a), and (ii) the double stranded intertwined helix or *the nonchannel form* (Arseniev et al. 1985b; Golovanov et al. 1991). The structure of gA has previously been investigated in a number of crystalline forms (Ramachandran and Chandrasekaran 1972; Veatch et al. 1974; Koeppe et al. 1978; Koeppe et al. 1979; Langs 1988; Wallace and Ravikumar 1988; Langs et al. 1991; Wallace 1992), by NMR in the solution state (Urry et al. 1983; Arseniev et al. 1985a–c; Arseniev et al. 1986a, b; Golovanov et al. 1991; Townsley et al. 2001), and NMR in the solid state (Ketchem et al. 1993, 1996, 1997; Mai et al. 1993; Tian et al. 1996; Cross 1997; Kovacs et al. 1999). Depending on the preparation conditions, gA can adopt either a double-stranded or single-stranded dimer of either left- or right handedness. The protein adopts the double-helical conformation in organic solvents; as confirmed by crystallographic investigations, whereas it is found as the single-stranded dimer in its native membrane-bound form (Wallace 1990); as established by NMR studies. It is now generally accepted (Andersen et al. 2005) that the functionally active (ion conducting) form of gA consists of a right-handed single-stranded dimer wherein the N-terminal end of the monomers form the dimer interface. The structure of gA has previously been studied by solution NMR methods in in both sodium dodecyl sulfate (SDS) micelles and by for solid-state NMR methods in reconstituted lipid bilayers. These studies confirm that gA adopts the native $\beta^{6.3}$–helical conformation when properly solubilized in a model surfactant system (Townsley et al. 2001).

Gramicidin A has served as the primary model system for structural and computational methods targeting membrane proteins for many years. Thus, the ability of AOT reverse micelles to solubilize gA at relatively high concentrations and with spectral resolution greater than the previous SDS structural study represents an important next step and establishes a new method for studying gA in particular and the potential to be applied to membrane proteins in general.

Materials and methods

Sample preparation

AOT reverse micelle standards (gA absent) were prepared in 650 µl n-pentane (EMD OmniSolv spectroscopic grade or 98% deuterated pentane from Cambridge Isotope Laboratories) with 100 mM NaAOT (Sigma-Aldrich ultra grade 99%) and 8.2 µl ddH_2O. Preparation of the simple water-filled AOT reverse micelles followed previous established

procedures (Babu et al. 2003). Briefly, the water is pipetted into a solution of AOT in n-pentane, which has been pre-loaded into a vial or NMR tube. The sample is then vigorously shaken by hand for several minutes, which results in an extremely stable nanodispersion possessing no turbidity. Water loading, w_0, defined as the molar ratio of water to surfactant, is a fundamental parameter of RMs which dictates size and other physical characteristics of the aggregate particle. For the RM-solubilized gA sample described above, the w_0 was 6.1, as measured based on the relative intensities of water and AOT resonances derived from one-dimensional ^{1}H-1D NMR spectrum.

Gramicidin A (Sigma) samples hosted within reverse micelles were prepared as above except with a final gA concentration of $\sim$1 mM. Gramicidin (ca. 85% gramicidin A) was purchased from Sigma and used without further purification. The gA was delivered to pre-formed reverse micelles as 10.4 µl of 250 mM gramicidin solution in 2,2,2 trifluoroethanol (TFE). The final TFE concentration in the gA/reverse micelle sample was $\sim$1.6%. This small amount of TFE showed no detectible effect on the properties of the sample. The addition of gA to reverse micelles initially caused precipitation; however, under gentle agitation (overnight) the peptide was resolubilized. The water loading (w_0) of the gramicidin solubilizing reverse micelle sample was measured to be 6.0. Note that maximum gramicidin solubility was found to occur between w_0 of 5 and 10 for 100 mM AOT, and for maximal gramicidin solubility it was necessary to pre-form the reverse micelles with the optimal target water loading prior to gA/TFE solution delivery.

The RM-solubilization efforts described here represent an important step in the goal of incorporating this approach in studies of membrane peptides and proteins. Although our protocol is not yet directly applicable to the broad class of membrane proteins, reconstitution of membrane proteins (regardless of the solubilization method) remains a central challenge, so that approaches that are complementary to more established procedures should prove valuable. We note that several research groups have published protocols that could be readily modified to explore the viability of RM solubilization. For example, a study by MacKinnon and coworkers (Valiyaveetil et al. 2002) includes the description of a protocol wherein the potassium ion channel protein KcsA is unfolded in 50% TFE in water, and is thus possibly compatible with the current protocol. Likewise, it has been established that the classic G-protein coupled receptor protein bacteriorhodopsin can be reconstituted from both formic acid in ethanol and TFE, and may also be compatible with the current approach (Huang et al. 1981). Thus while we continue to investigate the potential of RM solubilization applied to more complex membrane proteins, we believe that other groups may wish to integrate RM forming surfactants into their solubilization protocols, and the protocol described here may provide important initial guidance.

All reverse micelle samples were prepared in screw cap NMR tubes (Wilmad Lab Glass) to minimize evaporation of pentane. In order to make use of the NMR spectrometer deuterium field lock for the reverse micelle samples prepared using perprotiopentane, a 1.5 mm O.D. by 100 mm length capillary tube was filled with D_2O (Aldrich 99.9% deuterium) and flame sealed, then added to the sample. Experiments recorded using samples prepared using d_{12}-n-pentane were locked on the pentane solvent.

The preparation of the aqueous gA/sodium dodecyl sulfate (SDS) micelle sample was modeled after the protocol used by Hinton and coworkers (Townsley et al. 2001). 65 µl of 50 mM gramicidin A/TFE solution was added to 585 µl of 250 mM SDS, 10% D_2O, in 100 mM phosphate buffer pH 6.0. The final solution is 10% by volume TFE (650 µl total sample volume).

NMR experiments

All experiments were recorded on a Varian INOVA 500 MHz ^{1}H NMR spectrometer using a Varian broadband indirect triple-axis PFG probe. All experiments were recorded at 20.0 ± 0.1 °C. The temperature was calibrated using the chemical shift difference between the two ^{1}H resonances of neat methanol (Van Geet 1970). One dimensional ^{1}H spectra were used to directly monitor the w_0 of the reverse micelle samples, and for comparison of the amide and aromatic regions of gramicidin A hosted in AOT reverse micelles or SDS micelles. The translational self-diffusion coefficient (D_t) was used to monitor the multimeric state of the reverse micelle particle in the absence and presence of gramicidin A. A WET PFG DSTE LED diffusion experiment (Simorellis and Flynn 2004) was used to measure the translational diffusion coefficient, D_t. This experiment compensates for errors that arise from thermal convection within the NMR sample tube, which is common in low viscosity solvents such as n-pentane, while also providing superior solvent suppression. The diffusion time employed in the experiment was 100 ms, and a PFG pulse length of 3 ms was used. Data were fit to the following expression:

$$I(G_Z) = I_0 \exp\left[-(\gamma\delta G_Z)^2(\Delta)D_t\right]$$

$$\Delta = T + 4\delta/3 + 2\tau$$

wherein $I(G_z)$ is the gradient-dependent intensity, I_0 is the initial signal intensity, G_z is the strength of the applied gradient pulse, δ is the duration of the PFG pulse, T is the delay which together with the length of the PFG pulse (δ)

and a gradient recovery interval (τ) defines the total diffusion time of the experiment. The translation diffusion coefficient was derived from the fit of the experimental data to the expression given above. The selective amide T_2 values were determined using a one-dimensional $^1H90^{\circ}_{x} - \tau - 90^{\circ}_{-x}$ $(1-1)$ echo sequence as described by Bax and coworkers using echo delays of between 1 and 25 ms (2 ms increments) (Sklenar and Bax, 1987; Anglister et al., 1993). ^{1}H-^{1}H NOESY experiments were recorded in phase-sensitive mode using mixing times of 75 ms and 150 ms. The directly detected dimension was recorded using 1024 complex points and a dwell time 153.85 μs, and the indirectly detected dimension was recorded using 512 complex points and a dwell time of 153.85 μs.

Results and discussion

Refolding of gramicidin A into reverse micelles

An important component of membrane protein studies is the ability to reconstitute the protein in a membrane mimetic. Commonly, for proper sample reconstitution the membrane protein must be refolded into a native like state. As such it is important to note that the ^{1}H NMR data shown in Fig. 1 demonstrate that gA can be refolded from a denatured state present in a stock solution of 2,2,2-trifluoroethanol (TFE) using AOT/H_2O/n-pentane reverse micelles. The significant increase in the ^{1}H chemical shift dispersion present in the amide region of the spectrum that occurs with AOT solubilization confirms the effectiveness of the extraction and refolding processes. The process of transferring and refolding gA into a RM was accomplished by dissolving a relatively large amount of gA in a small amount of TFE and mixing the gA/TFE solution with a RM solution (see Materials and Methods section titled sample preparation). Solubilization of gA in AOT RMs is highly dependent on specific details of the protocol, including a key dependence on water loading. Optimal efficiency of gA transfer and refolding to reverse micelles was found for w_0 values between 5 and 10. The resulting gA concentration in 100 mM AOT RM/pentane solution was found to be $\sim$1 mM. It should be noted that gA is not directly soluble in pentane, and in order to maximize the solubility of gA in RMs it is crucial that the RMs be preformed with an optimizing water loading value.

Investigation of the structure of gramicidin A in reverse micelles

In order to investigate the protein fold present in RM solubilized gA samples, we compared the chemical shifts of the amide proton "fingerprint" region of the one-dimensional

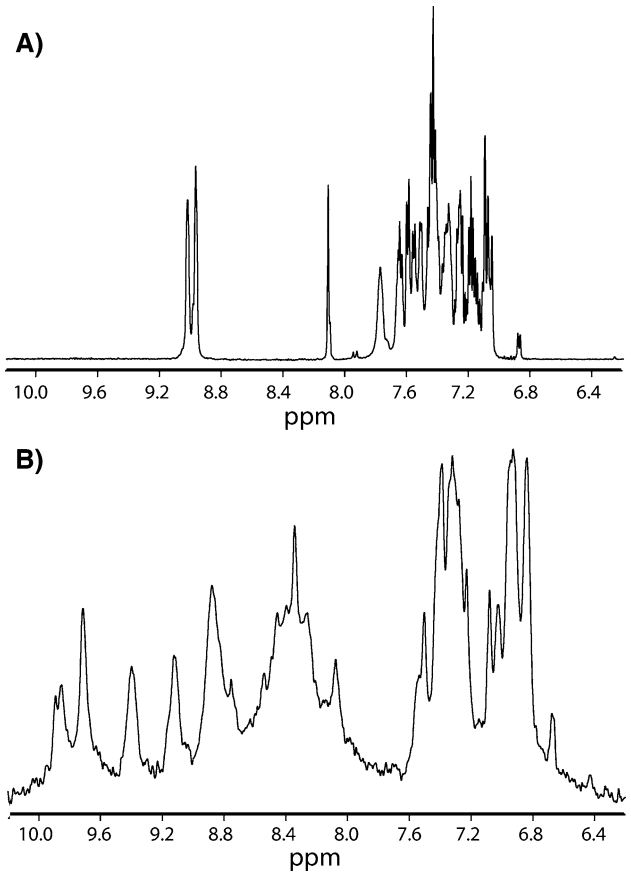

Fig. 1 NMR spectra of gramicidin A. (**a**) ^{1}H NMR Spectrum A is 16 mM gramicidin A in neat TFE. The spectrum shown in (b) is 1 mM gramicidin solubilized in an AOT RM and pentane solution. The ^{1}H NMR spectrum of gramicidin A in AOT shown confirms that the protein can be refolded using reverse micelles. All spectra recorded at 20°C

^{1}H spectrum from RM and SDS solubilized samples. First, the ^{1}H chemical shifts of amide protons of the SDS sample were compared to previously published values and found to be identical (Townsley et al. 2001). Second, a comparison of the ^{1}H chemical shifts of the amide protons in a sample of AOT-solubilized gA with those present in the SDS study revealed identical shifts for the protein in the two environments (see Fig. 2). This straightforward analysis suggests that gA is natively folded in AOT reverse micelles.

The previously published ^{1}H assignments of Hinton and coworkers allowed us to conduct a straightforward semi-quantitative investigation of the structure of gA in RMs (Townsley et al. 2001). Analysis of the observed NOE patterns present in the AOT-solubilized gramicidin NOESY spectrum together with a detailed tabulation of interproton distances derived from the gA structure, indicate that gA is folded with the native β-helical fold (Ketchem et al. 1996; Townsley et al. 2001). In the canonical $\beta^{6.3}$ helical structure, amide protons that are six residues apart lie on the same face of the β helix and less than 5 Å of one another, which is close enough to produce

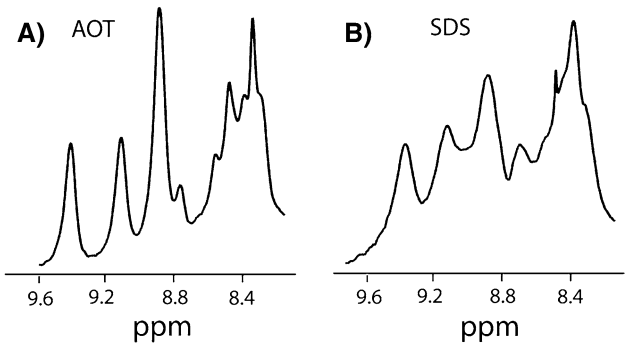

Fig. 2 Comparative analysis of NMR spectra of gramicidin A. The similarity of the chemical shift patterns present in the ^{1}H NMR spectra of of gA in (**a**) AOT reverse micelles and (**b**) SDS micelles suggest that the structure of the protein is very similar in both environments. All spectra recorded at 20°C

an observable NOE (Table 1 in supplementary material). These interresidue H–N NOEs are shown in Fig. 3, thus confirming the presence of the canonical β structure. Coupling the results from the ^{1}H 1D experiments and comparison with previously published data and the NOE data strongly suggest that gA is natively folded in the AOT reverse micelle environment.

Assessment of the reverse micelle architecture hosting gramicidin A

Pulsed field gradient (PFG) diffusion experiments were used to investigate the polymeric state of the RM solubilized gA (Stejskal and Tanner 1965; Simorellis and Flynn 2004). These experiments have emerged as an accurate and efficient method for characterizing the size of RMs. The diffusion experiments provide a time and ensemble average measurement of the hydrodynamic behavior of the particle during the experimental diffusion time, which in our experiments was 100 ms. The results indicate that AOT-solubilized gramicidin diffuses at a significantly slower rate than the average 'empty' RMs as monitored by direct detection of gA and AOT resonances. The translational diffusion coefficient for gramicidin A was $1.91 \times 10^{-6} \pm 0.06$ cm^2/s and the diffusion coefficient for the AOT reverse micelle was $2.55 \times 10^{-6} \pm 0.04$ cm^2/s. We are unaware of any systematic examination of the solubility of TFE in short chain alkane solvents; however, our observations indicate that TFE is sparingly soluble in n-pentane, $<$2% v/v. The measured diffusion coefficient of TFE was $5.19 \times 10^{-6} \pm 0.03$ cm^2/s, which is consistent with the predicted value for free TFE in n-pentane, and which thus excludes the possibility that TFE interacts significantly with the gA-RM particle.

The diffusion coefficients of RMs both with and without gA were measured by monitoring the 1′ proton resonance

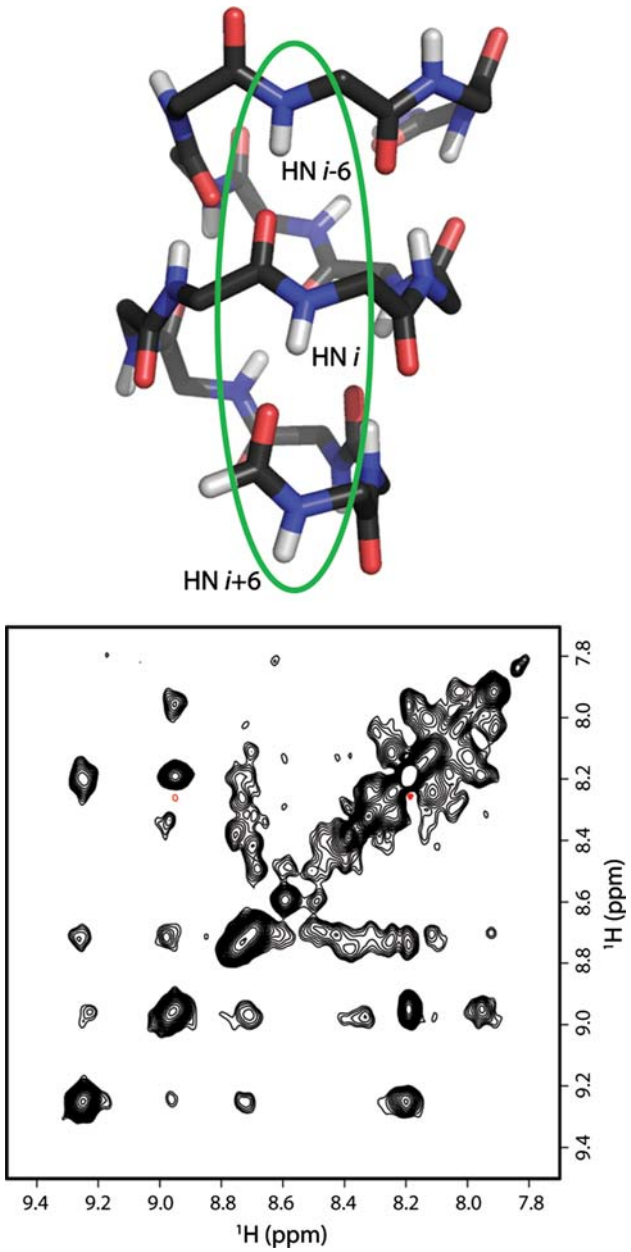

Fig. 3 Structural model of gA and NOESY spectrum. Top: Stick model representation of the peptide backbone of gramicidin A detailing the $\beta^{6.3}$ helical fold. Bottom: Amide region of the 150 ms ^{1}H-^{1}H NOESY spectrum showing intrachain backbone amide correlations used to confirm the global fold. Data recorded at 20°C

of AOT. The D_t for gA was measured directly by monitoring the aromatic proton resonances of tryptophan residues present in gA. The diffusion characteristics of TFE were determined by monitoring the alcohol proton resonance. The RM solubilized peptide has a diffusion coefficient that is smaller than the average RM particle, consistent with a larger average particle size. Importantly, a control experiment with a RM sample prepared in the absence of gA establishes that the average micelle size is

not affected by addition of the small volume of TFE used to solubilize gA. The ratio of diffusion coefficients can be used to estimate the oligomerization state of monomers (Teller et al. 1979; Altieri et al. 1995). For a dimer of two rigid spherical particles, the ratio of $D_{t\text{-}dimer}$:$D_{t\text{-}monomer}$ is expected to be 0.75 (Teller et al. 1979). To investigate the oligomerization state of gA solubilized by RMs, we compared the translational diffusion coefficients of a gA-RM sample with an analogous RM sample sans gA. Our diffusion results are consistent with RM dimerization, since the average diffusion coefficient dimer to monomer ratio is 0.75 ± 0.02. The most straightforward interpretation of the data is that the gA dimer bridges two RM particles.

The NOESY data can also be used to determine whether the AOT-solubilized, folded gA peptide monomers form the canonical N–N terminal helical dimer, and in fact we detect interchain NOEs that are consistent with homodimerization (see Fig. 1 of the Electronic Supplementary Materials). The interchain NOEs that we observe correspond to the through space correlations of 1-Val HN to 6-D-Val HN and 3-Ala HN to 4-D-Leu HC_{α}. Based on the gramicidin A coordinate file 1JNO present in the Protein Data Bank, we expect the interproton distances of these interchain correlations to be $\sim$4.7 Å and $\sim$3.1 Å respectively, whereas the expected intrachain distances would be much longer, at $\sim$7.8 Å and 5.1 Å. We also detect correlations corresponding to the 1-Val HN to the 5-Ala HN and HC_{α}, however there is insufficient resolution to distinguish these crosspeaks from other nearby crosspeaks with high confidence. Based on the diffusion measurements and the gA interchain NOE data we conclude that gA bridges two reverse micelles.

Reverse micelles constantly interact: combining, reorganizing and then reforming individual particles. In addition, the thermodynamic penalties for reverse micelle bridging appear to be small. Application of reverse micelles as a membrane mimetic for integral membrane proteins thus appears to be promising, and the current results are an important initial step towards more complex reverse micelle based investigations of membrane proteins.

Spectroscopic benefits of NMR studies utilizing reverse micelles

A notable aspect of the comparison of spectra that appears in Fig. 2 is the difference in the resolution of the 1D ^{1}H spectra. The narrower linewidths in resonances in the gA/AOT-RM spectrum result from the difference in the bulk viscosities of n-pentane and water at 20°C. The Stokes-Einstein expression suggests that the rotational correlation time is directly proportional to viscosity and inversely proportional to temperature. Thus, the SDS detergent micelle data originally recorded by Hinton and coworkers which was recorded at 55°C would exhibit higher resolution than that shown in Fig. 2 due to a decrease in the correlation time. Specifically, since the viscosities of water at 20°C and 55°C are 1.002 cp and 0.5040 cp, the correlation time would decrease by a factor of 2. The influence of the temperature change (independent of its influence on viscosity) would further reduce the correlation time by a factor of 328/293 or $\sim$1.1. Taking the influence of temperature both directly and indirectly (viscosity) into account then, the cumulative effect of the increased temperature (coupled with the lower viscosity) should decrease the correlation time by a factor of $\sim$2.2 when compared with the correlation time of the aqueous sample at 20°C. The overall effect of reconstituting gA in the reverse micelle system in n-pentane thus provides a decrease in viscosity equivalent to a 35°C increase in the temperature for the gA-micelle sample in water. Furthermore, lower viscosity solvents compatible with reverse micelle samples could further decrease the correlation time far below what could be achieved using any type of aqueous system.

Three additional related points on the subject of temperature versus viscosity based changes in correlation time are worth mentioning. First, since it is generally the case that studies of detergent solubilized peptide/proteins must be conducted at elevated temperatures, only thermostable proteins can be studied by this approach, and thus alternative methods that allow access to investigations at more moderate temperatures are important. Secondly, we would like to point out that 55°C is outside the operational temperature range of the current generation of cold probes, and since these devices figure prominently in studies of membrane proteins, the ability to work at lower temperature is again, a potentially important advantage. On a related third point, reverse-micelle based studies employ very low bulk electrolyte concentrations, e.g., even though the concentration of salt in the encapsulated water can be quite high, the overall concentration with respect to the total volume remains low. In the current generation of both room-temperature and cold-probes this feature of RM-based studies is a notable advantage (Flynn et al. 2000).

Finally, as pointed out above, although the line width of AOT-solubilized gA is significantly smaller than that observed in SDS-solubilized gA, the chemical shifts of the resonances are virtually identical. This confirms that RM solubilized gA has the same native-like fold found for SDS-solubilized gA. The inherent spectroscopic benefits of the RM/alkane system will be generally applicable to all membrane polypeptiders that are solubilized in RMs, and the effect becomes more significant as the protein size increases (Flynn 2004). This signal enhancement will simplify and improve experimental aspects of membrane protein structural biology.

The gA used in the current study was a natural abundance sample, which naturally precludes measurement of ^{15}N relaxation which can be used to accurately determine the global rotational correlation time. Bax and coworkers have determined that a good estimate of the rotational correlation time can be obtained based on the average selective T_2 value of amide protons (Sklenar and Bax 1987; Anglister et al. 1993; Cavanagh et al. 2007), e.g., $\tau_c \cong 1/5T_2$ ns. Application of this analysis to data recorded for AOT-solubilized gA yields an average T_2 value of 16.0 ms ± 0.5 ms and therefore an approximate value for the correlation time of ~12.5 ns. The average T_2 value for amide protons in the AOT-solubilized gA sample in n-pentane is roughly double that found for the SDS-solubilized gA sample at 20°C, which suggests a significant decrease (~2×) in the global rotational correlation time for that sample relative to the SDS-solubilized form in aqueous solution. Thus, reverse micelle solubilized gA shows spectroscopic benefits specific to this membrane mimetic system relative to the commonly used aqueous micelles. This benefit could be enhanced even further with even lower viscosity apolar solvents such as propane or ethane (Flynn et al. 2002; Peterson et al. 2005).

Gramicidin A bridging models

A model structure consistent with both the observed interchain NOEs as well as the results of translational diffusion measurements is shown in Fig. 4. The N-term to C-term distance of gA folded into the $\beta^{6.3}$ conformation is a very good match to the AOT acyl chain length. In our model we suggest that the C-term portion of gA points inward toward the aqueous core as it would in a natural biological membrane, while the N-term is directed outward into either the solvent or to form an N-N gA-dimer, thus bringing two RM particles together. This model has previously been suggested by Braco and coworkers who conducted an earlier investigation of AOT-solubilized gA based on the combination of chromatographic (HPLC) and spectroscopic (CD and fluorescence) methods (Salom et al. 1992).

Kruijff and coworkers and Killian have proposed that hexagonal H_{II} phases can be stabilized by the presence of gA in a compatible lipid (DOPC), which has features that are similar to the gA-mediated (AOT) RM dimer model (Van Echteld et al. 1982; Killian and De Kruijff 1986; Tournois et al. 1987a; Tournois et al. 1987b; Killian 1992). Hexagonal lipid phases (H) consist of close-packed tubes or columns of amphiphilic molecules in which the length of the individual structures are long compared with their diameters. Thus, if we imagine a cross section of a conventional micelle extended normal to the plane of the section, we obtain the H_I phase. The cross section of a reverse micelle extended in the same way generates the (inverted) H_{II} phase. Reverse micelles can be thought of as a limiting example of the H_{II} phase, e.g., in the limit as the length of the columns decreases to that of the cross-sectional diameter. This again indicates that significant structural plasticity exists in inverted micellar forms, which in turn suggests that these systems an excellent general model system for studies of membrane proteins.

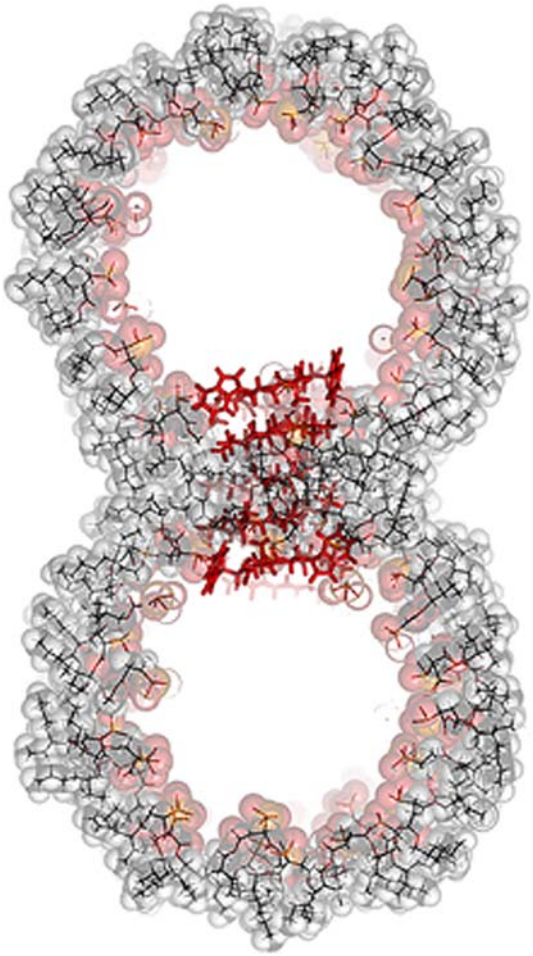

Fig. 4 Structural model of RM solubilized gramicidin A indicating bridging interactions. The model was prepared by docking the gramicidin A structure (PDB accession number 1JNO.pdb) to two energy minimized atomically explicit RM models, with the water removed and clipping planes adjusted to indicate how gA might span the two reverse micelles

Conclusions

Hydrodynamic measurements as well as intra- and inter-residue NOE connectivities both support the model in which single-stranded N-N gA-dimers bridge two RM particles. In turn, the observation of gA–mediated RM

dimerization suggests the potential of RMs as hosts for membrane proteins, and it is proposed that with adjustment, the encapsulation based method may be generally applicable to structural studies of a wide range of membrane proteins.

Membrane proteins are challenging structural targets due to inherent difficulties with solubilization, aggregation, and inherent particle size. Results presented here demonstrate that RM-based solubilization can be an effective alternative method for investigating structures of membrane proteins. The techniques interface directly with commonly used membrane protein purification and reconstitution protocols. The method is directly compatible with the standard suite of multinuclear multidimensional NMR experiments that are commonly used in protein resonance assignment and structure determination and requires no special adjustments or modifications to established NMR procedures. The approach also produces relatively high concentrations of the peptide, which should lead to improved sensitivity in all of the key NMR experiments.

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