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Structural interactions between lipids, water and S1-S4 voltage-sensing domains

Dmitriy Krepiy¹, Klaus Gawrisch², and Kenton J. Swartz¹

¹Molecular Physiology and Biophysics Section, Porter Neuroscience Research Center, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892 USA

²Laboratory of Membrane Biochemistry and Biophysics, National Institute of Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, MD 20892 USA

Abstract

Membrane proteins serve crucial signaling and transport functions, yet relatively little is known about their structures in membrane environments or how lipids interact with these proteins. For voltage-activated ion channels, X-ray structures suggest that the mobile voltage-sensing S4 helix would be exposed to the membrane, and functional studies reveal that lipid modification can profoundly alter channel activity. Here we use solid-state NMR to investigate structural interactions of lipids and water with S1-S4 voltage-sensing domains, and to explore whether lipids influence the structure of the protein. Our results demonstrate that S1-S4 domains exhibit extensive interactions with lipids, and that these domains are heavily hydrated when embedded in a membrane. We also find evidence for preferential interactions of anionic lipids with S1-S4 domains, and that these interactions have lifetimes on the timescale of 10^{-3} s. Arg residues within S1-S4 domains are well-hydrated and are positioned in close proximity to lipids, exhibiting local interactions with both lipid head groups and acyl chains. Comparative studies with a positively charged lipid lacking a phosphodiester group reveal that this lipid modification has only modest effects on the structure and hydration of S1-S4 domains. Taken together, our results demonstrate that Arg residues in S1-S4 voltage-sensing domains reside in close proximity to the hydrophobic interior of the membrane, yet are well-hydrated, a requirement for carrying charge and driving protein motions in response to changes in membrane voltage.

Keywords

NMR; S4 helix; voltage-activated ion channel; anionic lipids; DOTAP

Introduction

Membrane proteins play many important roles, from salt secretion in the kidney to generating electrical signals in the nervous system. The X-ray structures of an increasing number of detergent solubilized membrane proteins have been solved¹, revealing their molecular design and providing valuable information on their mechanisms. Although X-ray structures of detergent solubilized membrane proteins are widely considered to be valid

Correspondence should be addressed to: Klaus Gawrisch (klausg@mail.nih.gov) or Kenton J. Swartz (swartzk@ninds.nih.gov).

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representations of protein structure in a lipid membrane environment, relatively little direct structural information is available for these proteins in their natural membrane environment. A rare example is aquaporin, for which a high resolution structure within a membrane has been solved using electron crystallography². In addition, there have been relatively few investigations on the link between lipid-protein interactions, protein structure and function or the dynamics of these interactions.

These questions are particularly germane in the case of voltage-activated ion channels, where interactions between the voltage-sensing domains and the membrane are thought to be important for the mechanisms of voltage sensing¹¹. X-ray structures of voltage-activated potassium (Kv) channels show the S1-S4 voltage-sensing domains only loosely associated with the central pore domain, and that all of the helices within the S1-S4 domain would be predicted to have extensive lipid exposure if embedded in a lipid membrane (Fig. 1a)¹². Even the Arg-rich S4 helix that moves in response to changes in membrane voltage appears to be highly exposed to lipids (Fig. 1b,c), with the outer two Arg residues projecting out towards the membrane, inferences that are supported by other studies. Functional studies on related eukaryotic Kv channels show that modification of the lipid sphingomyelin can dramatically alter voltage-dependent gating properties and pharmacology of the channel, and it has been proposed that the lipid interacts intimately with S1-S4 voltage sensors. In previous functional studies on the archaeobacterial KvAP channel reconstituted into membranes, lipids with phosphate head groups are required for channel activation. In particular, KvAP is non-functional when reconstituted in DOTAP (1,2-dioleoyl-3-trimethylammonium-propane), a positively charged lipid lacking a phosphodiester moiety, and the voltage-activation relation is progressively shifted to more negative voltages as the molar ratio of DOTAP to phospholipid is decreased²³. Based on accessibility studies, Zheng and colleagues proposed that lipids like DOTAP prevent the S1-S4 voltage-sensing domain of KvAP from adopting the activated conformation required for channel opening²⁴. The intriguing hypothesis emerging from these studies is that interactions between Arg residues in S1-S4 domains with phosphodiester lipid moieties (Fig. 1a) are essential for voltage-sensor activation and that lipids can influence the structure of S1-S4 voltage sensors.

Results

Our goal in the present studies was to investigate physical interactions of lipids with S1-S4 voltage-sensing domains and to explore the extent to which lipids influence the structure of these important domains. We focused our efforts on the S1-S4 voltage-sensing domain of KvAP, an archaeobacterial channel that can be expressed at high levels in *E. coli*, purified to homogeneity and reconstituted into membranes of varying lipid composition. For all of the experiments described here, we expressed and purified the S1-S4 domain or full-length KvAP channel in *E. coli* as previously described²⁸, and developed protocols for isotope labeling the protein with ¹³C and/or ¹⁵N and reconstitution into lipids for studies using magic angle spinning, solid-state NMR spectroscopy. The standard condition for all of our studies was to reconstitute the protein using dialysis into a 1:1 mixture of the zwitterionic phospholipid POPC and the anionic phospholipid POPG, a lipid mixture that allows us to obtain homogeneous samples of proteoliposomes suitable for structural studies²⁸.

Lipid interactions with S1-S4 domains

Before focusing our studies on isolated S1-S4 domains, we compared lipid interactions for the isolated domain and the full-length channel using Saturation Transfer Difference (STD) NMR experiments designed to detect magnetization transfer from protons on the protein to protons on the lipids. If low power (824 Hz) radiofrequency pulses are applied to the amide region of the protein (8.5 ppm), we observed detectable attenuation of peak intensities for lipid methylene $-(CH_2)_n-$ segments compared to when pulses are applied at a control

frequency of 11 ppm (Fig. 2a). When the frequency-dependence of presaturation was investigated, we observed the anticipated intensity distribution of protein amide resonances (Fig. 2b). Magnetization transfer through the protein is fast (diffusion constant of $0.3 \text{ (nm)}^2/\text{msec}$), whereas transfer from protein to lipid is rate-limiting (diffusion coefficient of $0.0025 \text{ (nm)}^2/\text{msec}$), suggesting that the attenuation factor of lipid resonances from presaturation will be a reflection of exposure of the particular lipid species to the surface of the protein. The low power saturating ^1H radiofrequency pulses cause attenuation of lipid resonances only in the presence of the protein (Fig. 2c; $<1300 \text{ Hz}$), supporting this interpretation. Very strong saturating radiofrequency pulses can cause attenuation of lipid resonances in protein-free liposomes (Fig. 2c; $>1300 \text{ Hz}$), likely due to direct saturation of nearby lipid and water resonances (e.g. double bonds at 5.3 ppm and water at 4.8 ppm; see Fig. 2b). Spin diffusion from head group to hydrocarbon chain resonances along the fluid lipid molecules has low efficiency and is unlikely to make a significant contribution to saturation of chain resonances³¹. Collectively, these results suggest that the protein was successfully reconstituted into the lipid matrix and assumes a transmembrane orientation. The attenuation factors observed for 0.25 mol% of the monomeric S1-S4 domain are similar to those for 0.32 mol% of the full-length KvAP channel (Fig. 2c; where mol% of the full-length channel is calculated as monomeric), suggesting that lipid interactions detected in these experiments with the full-length channel are dominated by those occurring with S1-S4 domains.

The superposition of ^1H NMR lipid resonances of POPC and POPG prevents distinguishing between interactions of POPC or POPG with the protein in the experiments described thus far. To explore whether there are preferential interactions of these two lipids with S1-S4 domains, we implemented ^{13}C -detected Insensitive Nuclei Enhancement by Polarization Transfer (INEPT) experiments with pre-saturation, where the magnetization from protein protons is transferred to lipid protons and detected via natural abundance of ^{13}C lipid resonances through J couplings. Although this experiment does not require the use of ^{13}C labeled protein, we used uniformly $^{15}\text{N}/^{13}\text{C}$ labeled S1-S4 domain because it allowed us to perform important control experiments (see below). We assigned the lipid resonances in POPC and POPG (Fig. 3b), and studied attenuation of these resonances when magnetization was transferred from protein to a 1:1 mixture of POPC and POPG using low power (740 Hz) presaturating pulses (Fig. 3c). We observed more pronounced attenuation for the resolvable carbons of POPG when compared to POPC, and this distinction could be observed over all power settings, suggesting that magnetization transfer from protein to POPG is more efficient (Fig. 3d). In control experiments, we investigated how quickly magnetization equilibrates within the protein when saturating ^1H -radiofrequency pulses are applied at 8.5 ppm. When the ^{13}C spectrum of the protein was detected via ^1H - ^{13}C cross-polarization, increasing duration and power of presaturation caused decreases in the intensity of all ^{13}C resonance bands of the protein (Supplementary Fig. 1), indicating that during presaturation ^1H magnetization spreads rapidly throughout the entire protein and from there to the surrounding lipids.

To investigate the influence of the protein on the structure and dynamics of the lipid hydrocarbon chains, we prepared samples containing the full-length KvAP channel reconstituted into mixtures of either $^2\text{H}_{31}$ -POPG:POPC or $^2\text{H}_{31}$ -POPC:POPG with perdeuterated palmitoyl chains, and obtained powder pattern ^2H NMR spectra with quadrupolar splittings, from which order parameters of C-H bonds were obtained (Supplementary Fig. 2). Protein-induced changes in the lipid order parameters are smaller than the experimental uncertainty at a concentration of 0.32 mol % of the full length KvAP in membranes. The absence of significant changes of lipid order with and without added protein indicates that the thickness of the hydrophobic core of the lipid matrix matches the length of hydrophobic transmembrane helices of the protein very well, such that hydrocarbon chains of lipids are not stretched or compressed near the protein. We used the

order parameters for the C-H bonds of the palmitoyl chains to calculate the hydrophobic thickness of the bilayer³⁴ and obtained values of 27 Å, consistent with neutron diffractions studies for the S1-S4 domain at comparable protein concentrations²⁸. In these ²H NMR spectra with quadrupolar splittings we could not see evidence of substantial demixing of lipid species due to the presence of the protein as we observed only one set of order parameters for all lipids in the mixture.

Short-range lipid and water interactions with S1-S4 domains

To more directly explore the nature of interactions between lipids, S1-S4 domains and water, we set out to measure short-range (<5 Å) NOE-based interactions with ¹⁵N or ¹³C detected ¹H spin diffusion experiments. In these experiments, magnetization is transferred from protons of the lipids and water through spin-diffusion to protons of the protein, and then to ¹⁵N or ¹³C of the protein via cross-polarization. ¹⁵N spectra have the advantage of having negligible contributions from ¹⁵N natural abundance lipid signals, but have somewhat lower sensitivity, whereas ¹³C spectra have higher sensitivity, but contain many ¹³C natural abundance lipid signals. In these experiments, the dipolar coupling depends on the distance, and therefore the rate of the transfer is dependent on the proximity of the protein to the lipid moieties or water. A 2-D ¹⁵N-detected ¹H spin-diffusion spectrum of the uniformly ¹⁵N/¹³C labeled S1-S4 in POPC:POPG, with a mixing time of 100 msec is shown in Fig. 4a. The horizontal axis shows the ¹⁵N protein spectrum, whereas the vertical axis shows the ¹H spectra containing resolved peaks for water and many lipid protons. The well-defined cross-peaks between amide ¹⁵NH(CO) and lipid ¹H resonances arising from the terminal methyl (0.9 ppm), methylene segment (1.3 ppm), and both interfacial and head-group regions (3.37–4.45 ppm), confirms that the S1-S4 domain assumes a transmembrane orientation, consistent with the above STD results, and that the protein reaches as far as the choline group of POPC (3.2 ppm cross-peak) (Fig. 4a). A strong cross-peak between water at 4.8 ppm and NH(CO) amide groups is also observed, indicating heavy hydration of the protein in a lipid bilayer. We also obtained 2-D ¹³C-detected ¹H spin-diffusion spectra (Supplementary Fig. 3a), and in these we observed interactions of lipid and water with C_α and C_β regions of the protein, demonstrating interactions of the protein backbone and side-chains with both lipid and water. Examination of the relationship between mixing time and intensity of the cross-peaks between the protein and either lipid or water (Fig. 4b) suggest that the lifetimes of interactions detected in these experiments is on the timescale of 10⁻³s. These results are consistent with STD experiments where the bulk lipid must be populated with ¹H-saturated lipid species, requiring that the lifetimes of lipid-protein interactions are in the range of 10⁻³s.

We were particularly interested in investigating interactions of Arg residues in the S1-S4 domain with lipids and water, as these residues drive conformational rearrangement in response to changes in membrane voltage, and their hydration and interactions with lipids are thought to be critical for the mechanism of voltage-sensing. The S1-S4 domain of KvAP contains 11 Arg residues, 5 of which are located within the S4 helix that drives conformational changes in response to membrane voltage (Fig. 1b,c). In the ¹⁵N dimension of the 2-D ¹⁵N-detected ¹H spin-diffusion spectrum for the uniformly ¹⁵N-labeled S1-S4 domain in phospholipid (Fig. 4a), well-resolved peaks are observed for both N_η and N_ε of Arg guanidinium groups (at ~75 and 85 ppm), and cross-peaks are observed with water, demonstrating that Arg residues in the S1-S4 domain are well-hydrated. These cross-peaks are observed for mixing times as short as 10 msec (Fig. 4c), but are not observed for the C_α of Arg residues in ¹³C-Arg labeled S1-S4 domain (Supplementary Fig. 3a), suggesting that the cross-peaks in the 2-D ¹⁵N-detected ¹H spin-diffusion spectrum have a significant contribution from direct chemical exchange with water protons. Together, these results

indicate that guanidinium groups of Arg residues in the S1-S4 domain are in direct contact with water.

We also observed well-defined cross-peaks between the guanidinium groups and lipid methylenes (Fig. 4a), demonstrating that Arg residues are positioned in close proximity to the surrounding lipids. In contrast, the terminal amines (N_ϵ) of Lys residues exhibit a barely detectable cross-peak with lipid methylenes even though a well-defined cross-peak with water is observed (Fig. 4a), suggesting that Lys and Arg residues are both hydrated but exhibit distinct interactions with acyl chains of lipids. This observed difference in environment for Arg and Lys residues is consistent with the X-ray and solution structures of the S1-S4 domain of KvAP, where most of the Lys residues are clustered at the intracellular end of the domain, whereas many of the Arg residues are located within the membrane spanning regions of the domain (Fig. 1b,c).

To explore whether we could detect interactions between Arg residues and the head group region of lipids we obtained 2-D ^{13}C detected ^1H spin diffusion spectra for uniformly ^{13}C -labeled and Arg- ^{13}C labeled S1-S4 domains (Supplementary Fig. 3a,b). Although the spectrum for the uniformly labeled protein provides information on interaction of protein side chains with lipids and water, signals from Arg residues cannot be specifically resolved. However, in the spectrum for the Arg- ^{13}C labeled S1-S4 domain, we can detect interactions of lipid moieties with C_α , C_δ and $C_{\beta\gamma}$ of Arg from cross-peaks of these carbons with lipid methylenes (at 1.3 ppm), and lipid glycerol and head-groups (3.37–4.45 ppm), demonstrating that Arg residues interact with the head-group region of the bilayer. These resolved interactions of Arg residues with lipid head groups provide experimental support for the notion that some of the Arg residues within S1-S4 domains interact with lipid phosphodiester.

Reconstitution of S1-S4 domains in DOTAP

To explore the importance of the lipid phosphodiester moiety and whether lipids can influence the structure of voltage sensors, we reconstituted the S1-S4 domain of KvAP in DOTAP, a lipid lacking a phosphodiester group. Since relatively little biochemical or structural characterization has been performed for membrane proteins reconstituted into DOTAP, we first examined the topology, hydration and structural integrity of S1-S4 domains following reconstitution in this cationic lipid. We reconstituted the $^{15}\text{N}/^{13}\text{C}$ labeled S1-S4 domain of KvAP in DOTAP, obtained a 2-D ^{15}N -detected ^1H spin-diffusion spectrum, and quantitatively compared interactions of the protein with lipids and water by measuring intensity build-up curves (Fig. 4b,c). Spin diffusion build-up curves from H_2O and lipid methylenes to the S1-S4 domain amide NH and Arg N_η 1,2 in DOTAP and POPC:POPG are similar, demonstrating that in both lipid matrices the protein assumes a transmembrane topology and is heavily hydrated. We were also unable to detect differences between phospholipid and DOTAP in saturation transfer difference experiments where magnetization is transferred from either lipid methylenes or water to uniformly ^{13}C -labeled S1-S4 domain (Supplementary Fig. 4a-c), supporting these conclusions.

Next we characterized S1-S4 domains reconstituted into DOTAP using circular dichroism and fluorescence spectroscopy (Fig. 5). The S1-S4 domain of KvAP contains a single Trp residue (Trp70 in the S2 helix; Fig. 1b,c) that exhibits a fluorescence emission spectra indicative of a hydrophobic environment when reconstituted into phospholipid²⁸, a feature that is unaffected when the protein is reconstituted into DOTAP (Fig. 5a). In addition, circular dichroism spectra of S1-S4 domains reconstituted into phospholipid or DOTAP are indistinguishable (Fig. 5b), and both are consistent with the high helical content (~85%) expected from X-ray and solution NMR structures in detergents. We also labeled the S1-S4 domain of KvAP with 6-bromoacetyl-2-dimethylaminonaphthalene (BADAN) after

introducing a Cys at A111 at the outer end of the S3 helix (Fig. 1b). Comparison of BADAN-labeled S1-S4 domains reconstituted into phospholipid or DOTAP reveals that fluorescence emission is quenched in DOTAP when excited at 385 nm, and that the emission λ_{max} shifts from 475 nm to 500 nm (Fig. 5c), suggesting that the dielectric constant of the environment at the outer S3 helix exhibits a pronounced change in DOTAP. Addition of empty DOTAP liposomes to POPC:POPG liposomes containing the S1-S4 domain of KvAP also altered the BADAN fluorescence similar to what was observed with DOTAP proteoliposomes (Fig. 5d). Taken together, these results suggest that S1-S4 domains can be reconstituted in a transmembrane orientation in DOTAP without disrupting the structural integrity of the protein.

Lipid-induced changes in secondary structure of S1-S4 domains

Our next objective was to explore the extent to which DOTAP influences the secondary structure of S1-S4 voltage-sensing domains. The changes in fluorescence observed for BADAN fluorescence would be consistent with a possible influence of the lipid on S1-S4 domain structure. In addition, previous solution NMR experiments on detergent micelles indicate that the structure and dynamics of the S1-S4 domain of KvAP are sensitive to the surrounding detergents. We initially focused our efforts on resolving the Arg spin systems in uniformly ^{13}C -labeled samples because these can provide valuable information on the local environment of the different carbons of Arg residues. In a 2-D ^{13}C - ^{13}C cross-polarization dipolar assisted rotational resonance (CP-DARR) correlation experiment we observe well-resolved cross-peaks for $\text{C}_\zeta\text{-C}_{\beta,\gamma}$, $\text{C}_\zeta\text{-C}_\delta$ and $\text{C}_\zeta\text{-C}_\alpha$ (Fig. 6a). Definitive assignment of the Arg cross-peaks is supported by a CP-DARR spectrum obtained for a sample specifically ^{13}C -labeled for Arg residues (Supplementary Fig. 5). Comparison of these Arg cross-peaks for uniformly labeled samples reconstituted in phospholipid and DOTAP (Fig. 6a) reveal that the lipids do not cause dramatic changes in these Arg cross-peaks. Closer examination of the $\text{C}_\zeta\text{-C}_\alpha$ and $\text{C}_\zeta\text{-C}_{\beta,\gamma}$ cross-peaks (Fig. 6b,c, respectively) is particularly informative because the chemical shifts in the C_α and $\text{C}_{\beta,\gamma}$ dimensions are sensitive to dihedral angles and therefore indicative of secondary structure⁵⁹. The envelope of chemical shifts for the Arg C_α band has a maximal intensity at 60 ppm in either phospholipid or DOTAP, which indicates that a majority of the Arg residues in the S1-S4 domain are positioned within α -helices⁶⁰. The resolution of the spectra is not high enough to distinguish between Arg residues in α or 3_{10} helices, the latter of which would exhibit C_α shifts in the upfield direction⁶¹. In the case of the Arg $\text{C}_{\beta,\gamma}$, the maximal intensity is at 30 ppm, in support of α -helical secondary structure⁶⁰. Comparison of the C_α region for the entire protein from ^{13}C spectra obtained in phospholipid and DOTAP suggest that the overall secondary structure of the protein is similar (Supplementary Fig. 4d), and in both cases are consistent with predictions using assigned chemical shifts for C_α atoms from the solutions NMR structure of the S1-S4 domain in detergent⁴⁸.

Lipid-induced changes in tertiary structure of S1-S4 domains

Although the results thus far suggest that DOTAP does not influence the secondary structure of S1-S4 domains, we sought more direct constraints on whether lipids can influence the tertiary structure of the protein. We initially took advantage of the fact that the S1-S4 domain of KvAP contains only one Trp residue (Trp70 in the S2 helix; Fig. 1b,c), and that a similarly positioned Phe residue in the Shaker Kv channel has been proposed to exhibit dynamic interactions with Arg residues during the process of voltage sensing. To explore whether Trp70 in the S1-S4 domain of KvAP interacts with Arg residues, we labeled the indole of Trp70 with either ^{13}C or ^{15}N and obtained 1-D ^{13}C or ^{15}N spectra of the protein in either phospholipid or DOTAP (Fig. 7a,b). The unique chemical shifts of the eight indole carbons in Trp (100–120 ppm) allowed unambiguous assignment of two peaks in the ^{13}C spectra as arising from carbon atoms in Trp70 (Fig. 7a). In ^{15}N spectra we observed a

resolved peak corresponding to the N_ϵ of indole (Fig. 7b). Both ^{13}C and ^{15}N spectra obtained following reconstitution into DOTAP reveal that the lipid does not cause any detectable change in the indole chemical shifts, as would be expected if the chemical environment of Trp70 is influenced by lipid composition.

Electrophysiological and computational studies suggest that many S4 Arg residues have electrostatic interactions with acidic residues in the S2 and S3 helices (Fig. 1a), and that these interactions are essential for proper helix packing and for changing conformation between resting and activated conformations. The X-ray structures of the S1-S4 domain of KvAP (Fig. 1b,c) and the full-length Kv1.2 channel reveal that many S4 Arg residues interact with acidic residues from S1-S3, and it has been proposed that these electrostatic interactions reorganize in an ion exchange like mechanism as S4 moves between resting and activated states (Fig. 1a). Although the S1-S4 domain of KvAP used in our studies contains 13 acidic residues, only E45 in S1, D62 in S2 and E107 in S3 are positioned in the X-ray structure of the activated state where they could in principle form ionic bonds with S4 Arg residues (E107 with R123, E45 with R126 and D62 with Arg 133)²⁰. Only one Arg outside the S4 helix (Arg76 in S2) is positioned in the X-ray structure where it could form interactions with acidic residues (D72 in S2 and E93 in S3), meaning that any interactions between Arg and acidic residues we might detect in our experiments would be dominated by S4 Arg residues. The structure also reveals that two additional S4 Arg residues are positioned external to those that interact with acidic residues, and thus as S4 moves inward during the conformational change from the activated to the resting state, we would anticipate a substantial rearrangement of electrostatic interactions (Fig. 1). To investigate whether Arg residues in the S1-S4 domain form ionic bonds with acidic residues, we looked for cross-peaks between Arg C_ζ and carboxyl groups of Asp/Glu in 2-D ^{13}C - ^{13}C correlation CP-DARR spectra of the uniformly ^{13}C -labeled S1-S4 domain reconstituted in phospholipid. A well-defined cross-peak is observed at the appropriate chemical shifts for C_ζ and CO of either carboxyl or carbonyl groups in the uniformly labeled sample (Fig. 8a), but not in samples where only Arg was specifically labeled (Fig. 8b), indicating that the cross-peak does not arise from an C_o - C_ζ correlation within the Arg residue spin system. Although the cross-peak we observe could arise from local interactions between Arg C_ζ and either backbone carbonyls or carboxy groups of Asp/Glu, it is likely that the latter make a contribution as these are prominently observed in X-ray structures of S1-S4 domains^{12; 20}. We obtained similar spectra for samples reconstituted into DOTAP, and observed little change in the C_ζ -CO cross-peak compared to phospholipid. To further explore whether the network of ionic bonds is sensitive to the lipid environment, we obtained 2-D CP ^{13}C - ^{15}N spectra and observed a well-defined cross-peak corresponding to guanidinium $^{13}\text{C}_\zeta$ and $^{15}\text{N}_\eta$ correlation (Fig. 8c). Although the chemical shifts of this cross-peak should be particularly sensitive to the local environment of the Arg side chain, we observed no chemical shift perturbation when comparing phospholipid and DOTAP.

Discussion

The objective of the present study was to investigate structural interactions of lipids and water with S1-S4 voltage-sensing domains and to explore the extent to which lipids can influence the structure of the protein. Relatively little is known about how lipids interact with membrane proteins, or whether they are capable of influencing protein structure and dynamics⁷⁹. Our focus on S1-S4 voltage-sensing domains was motivated by X-ray structures of these proteins, which predict interactions between key mobile motifs in the protein and the membrane, and by functional studies showing that lipids can have profound effects on the activity of voltage-activated ion channels.

Our NMR experiments on the S1-S4 domain of KvAP reconstituted into fully hydrated phospholipids show that S1-S4 domains interact with both the hydrophobic acyl chains as well as head-group region of phospholipids (Figs. 2–4, Supplementary Figs. 1, 3, 4), demonstrating that these domains are embedded in the membrane and assume a transmembrane orientation, consistent with previous neutron diffraction experiments²⁸. We compared interactions of the S1-S4 domain with POPC and POPG (Fig. 3), and found that the anionic POPG interacts preferentially with the protein domain, similar to what has been observed in studies on the KcsA potassium channel. Although the strength and lifetime of anionic lipid interactions with KcsA are unknown, our collective experiments indicate that the interactions of both POPC and POPG with the S1-S4 domain have lifetimes on the order of 10^{-3} s, which is comparable to the ‘semi-specific’ interactions that have been described for lipids interacting with rhodopsin. Using ^1H spin diffusion experiments designed to identify short-range NOE-based interactions, we detected interactions between Arg residues in the S1-S4 domain and both acyl chains and head-group regions of phospholipids (Fig. 4, Supplementary Fig. 3), providing direct support for the view that crucial elements of S1-S4 voltage-sensing domains interact with the lipid membrane.

S1-S4 domains are unique membrane proteins that sense changes in membrane voltage using a mobile S4 helix that is rich in charged Arg residues¹¹. If these domains are fully embedded in the membrane, as our results demonstrate, the Arg residues must reside within a local environment that has a high dielectric in order to maintain their charge. The X-ray structures of S1-S4 domains contain crevices that could provide a pathway for water to hydrate Arg residues, and their hydration has been inferred from spin label studies. Previous NMR results suggest that S1-S4 domains are hydrated, but they do not specifically address hydration of the critical Arg residues. Our ^1H spin diffusion experiments unambiguously demonstrate that the guanidinium groups of Arg residues in the S1-S4 domain are hydrated in the membrane (Fig. 4, Supplementary Fig. 3). We were also able to detect interactions of the guanidinium groups of S4 Arg residues with CO groups (Fig. 8), consistent with the notion that Arg residues are stabilized within the membrane by ionic bonds with acidic residues.

To investigate how lipids can influence the structure of S1-S4 domains, we performed a series of comparative experiments in phospholipid and DOTAP, a lipid lacking a phosphodiester moiety. S1-S4 domains retain structural integrity following reconstitution into DOTAP, and the protein maintains its strong hydration and interaction with acyl chains of the lipid (Fig. 4; Supplementary Fig. 3). NMR experiments with the Arg spin system and Trp70 indicate that the lipid modification does not alter the secondary structure of Arg residues or the local environment of Arg and Trp side chains (Fig. 5–8). The S1-S4 domains in the available X-ray structures of Kv channels are thought to represent an activated state, and although the structure of the resting state is unknown, a range of constraints and models of voltage-sensor activation implicate structural rearrangements that are substantial enough to have been detected in our experiments. We did observe DOTAP-induced changes in BADAN fluorescence when labeling the outer end of the S4 helix, however, in light of the NMR results we think that these likely reflect alterations in the local dielectric constant in the head-group region produced by the cationic lipid rather than a change in the conformation of the protein. This would imply that DOTAP does not inhibit the activity of the KvAP channel by interfering with voltage-sensor activation²⁴. Studies on the related Shaker Kv channel suggest that voltage-sensor activation is followed by a final opening transition that involves a modest motion of the S4 helix⁸⁷, a conformational change that may be sensitive to replacement of phospholipids with DOTAP. It will be interesting to further explore the mechanism of DOTAP inhibition of the KvAP channel to examine whether the final opening transition is sensitive to lipid modification.

Taken together, our experiments with the S1-S4 domain of KvAP suggest that lipids interact intimately with the protein, but that they do not detectably alter the structure of the domain. Indeed, all of our data obtained on S1-S4 domains embedded in lipid membranes are remarkably consistent with both X-ray and solution NMR structures of the domain obtained in detergent micelles. It will be interesting to use the approaches developed here to explore interactions between lipids and other membrane proteins. It will be particularly interesting to investigate S1-S4 domains in eukaryotic voltage-activated ion channels, which reside in membranes with heterogeneous lipid compositions, and where lipids such as sphingomyelin have been proposed to interact specifically with voltage sensors.

Materials and Methods

S1-S4 protein expression, purification and characterization

The recombinant S1-S4 voltage-sensing domain or the full-length KvAP channel was expressed in XL1-Blue strain of *E.coli* as previously described²⁰. Briefly, the *E.coli* culture was grown in LB broth supplemented with Ampicillin (100 μ g/ml) and the protein expression was induced with 1 mM isopropyl- β -D-thiogalactopyranoside (Calbiochem). For expression of full length KvAP, BaCl₂ was added to the final concentration of 10 mM. After induction, cells were collected, cell walls were disrupted by using a homogenizer (Avestin), the membrane fraction isolated by centrifugation at 40,000xg, solubilized in 2.5 % (w/v) decylmaltoside (Anatrace) in 20 mM Tris/HCl, 100 mM KCl, pH 7.8. The lysate was then centrifuged at 100,000xg for 1 hour at 4°C, and the supernatant was mixed with Co-Talon resin (Clontech) and equilibrated for 3 hours. The resin with bound protein was washed with 0.25% (w/v) decylmaltoside, 10 mM imidazole in 20 mM Tris/HCl, 100 mM KCl, pH 7.8. The buffer was then exchanged to 3 % (w/v) n-octyl- β -D-glucopyranoside (OG) in 20 mM Tris/HCl, 100 mM KCl, pH 7.8 and the protein eluted with 400 mM imidazole in the same buffer. One unit of thrombin (Sigma) was added per mg of protein and the mixture was dialyzed against 3 % (w/v) OG in 20 mM Tris/HCl, 100 mM KCl, pH 7.8 overnight at 4°C in a dialysis cassette with MW cutoff of 10 kDa (Pierce). The protein fraction was further purified using size exclusion chromatography with a Superdex 10/300 GL FPLC column (GE Healthcare) in 3 % (w/v) n-octyl- β -D-glucopyranoside (OG) in 20 mM Tris/HCl, 100 mM KCl.

Uniformly ¹³C, ¹⁵N labeled protein was obtained by protein expression in *E.coli* grown on M9 media supplemented with MEM vitamin solution (Gibco), 100 μ M CaCl₂, 250 mg/L MgCl₂, 5 mg/L thiamine, 0.2 % (w/v) ¹³C-(u)-glucose (Sigma-Aldrich), 0.1% ¹⁵NH₄Cl, 1 g/L ¹³C, ¹⁵N-Isogro (Cambridge Isotope Laboratories) and 100 μ g/ml Ampicillin. For labeling specific amino acids in the protein (e.g. ¹³C or ¹⁵N-Trp, ¹³C or ¹⁵N-Arg), we supplemented the M9 media with a mixture of unlabeled amino acids and the labeled amino acid of choice to a final concentration of 100–200 mg/L (Sigma-Aldrich). The media was supplemented with vitamins and a solution of trace elements⁸⁸.

Purified S1-S4 domains or the full length KvAP channel was analyzed by SDS-PAGE electrophoresis, followed by Coomassie staining (Invitrogen) and by MALDI-MS. The efficiency of the thrombin cleavage and the His-tag removal during the dialysis was confirmed with a His-Probe HRP reagent kit (Pierce) and by MALDI-MS. Isotope labeling efficiency for the S1-S4 domain was confirmed by mass spectrometric (MS) analysis of protein digest fragments. N-terminal sequencing revealed that the first 5 amino acids are removed during protein expression, consistent with the earlier observations⁴⁷. Both uniform and specific amino acid labeling efficiencies were typically ~70%. Protein concentration was determined spectrophotometrically using an extinction coefficient of $\epsilon_{280\text{ nm}} = 17,210\text{ M}^{-1}\text{cm}^{-1}$ for the S1-S4 domain and $\epsilon_{280\text{ nm}} = 34990\text{ M}^{-1}\text{cm}^{-1}$ for the monomeric full length channel, both calculated from the deduced protein composition⁸⁹.

In order to label protein with BADAN, the Ala111Cys mutant was generated by site-directed mutagenesis. Ala111Cys mutant of S1-S4 protein was incubated with 1mM 6-bromoacetyl-2-dimethylaminonaphthalene (BADAN) (Invitrogen) in 3 % (w/v) OG in 20 mM Tris/HCl, 100 mM KCl, pH 7.8 overnight at 4°C, β -mercaptoethanol was added to final 12 mM concentration and labeled protein was purified with size exclusion chromatography using a Superdex 10/300 GL FPLC column (GE Healthcare). The labeling efficiency was analyzed by the relative absorbance of the sample at 280 nm and 387 nm using extinction coefficient of 21 mM⁻¹cm⁻¹ for BADAN⁹⁰ and was found to be ~55%.

Lipid reconstitution of the voltage-sensing domain of KvAP

The S1-S4 voltage-sensing domain or full length KvAP channel were reconstituted into different molar ratios of protein to lipid as previously described using a 1:1 mixture of POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) and POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)]) or DOTAP (1,2-dioleoyl-3-trimethylammonium-propane). All lipids were purchased from Avanti Polar Lipids were mixed in chloroform and the solvent removed under a stream of nitrogen gas followed by application of vacuum overnight. Lipid films were solubilized in 20 mM Tris/HCl, 100 mM KCl buffer, pH 7.8 with 3% (w/v) OG and the protein was added to form mixed detergent-lipid/protein micelles. Proteoliposomes were formed by removal of detergent by dialysis against 20 mM Tris/Cl and 20–100 mM KCl solution, pH 7.8 for 96 hours while changing dialysis buffer daily.

Proteoliposome pellets were collected by ultracentrifugation at 200,000 g at 4°C using an Optima TL 100 TLX ultracentrifuge with a 100.3 TLA rotor (Beckman). Proteoliposome pellets were resuspended in H₂O and sedimented by ultracentrifugation, resuspended and mildly sonicated for 1 minute in a water bath sonicator and passed through an Avestin extruder fitted with a polycarbonate filter of 100 nm pore size. The resultant proteoliposomes were analyzed for lipid and residual detergent content by dissolving aliquots of the sample in deuterated MeOH for analytical ¹H NMR experiments.

Circular Dichroism and Fluorescence Spectroscopy

The 100-nm extruded proteoliposomes with a POPC: POPG (1:1) or DOTAP lipid matrix were dispersed in 20 mM Tris/Cl, 100 mM KCl for analysis. Circular Dichroism spectra were recorded using a JASCO J-815 spectropolarimeter equipped with athermally controlled cuvette holder. Spectra were recorded in 0.1–1 mm quartz cuvettes, from 180 nm to 250 nm with 1 nm step resolution and 4 sec integration time. Fluorescence emission spectra were recorded between 300 and 400 nm (3 nm band pass, 0° polarizer) using an excitation wavelength of 295 nm (3 nm band pass, 90° polarizer) for Trp. The BADAN fluorescence was recorded over the ranges of 400–600nm using an excitation wavelength of 387 nm. Samples were investigated in 1cm × 1cm quartz cuvette at 25°C with continuous stirring using a SPEX FluoroMax 3 spectrofluorometer (Horiba Jobin Yvon), and the data corrected for vesicle scattering⁹⁴ and normalized to zero lipid fluorescence intensity (F₀) read at 320 nm.

Nuclear Magnetic Resonance spectroscopy

Proteoliposomes with different protein-lipid ratios (molar 1:100–1:2000) were sedimented by ultracentrifugation and the pellet transferred to 4mm MAS rotors (Bruker). 1-D ¹H saturation transfer difference experiments⁹⁵ were conducted as previously described. ¹H MAS NMR spectra of lipids were recorded at an MAS frequency of 15 kHz and resonance attenuation measured in response to saturating radio frequency (RF) pulses (field strength 0–2 kHz) consisting of twenty Gaussian-shaped 50-msec pulses. The saturation frequency was set to the amide region of the protein (8.5 ppm). The attenuation of the lipid methylene

signal, defined as resonance amplitude recorded without saturation divided by the amplitude with saturation, was followed as an indicator of magnetization transfer to lipid. A total of 16 scans with a recycle delay of 10 s were acquired at 295.1°K. T_1 relaxation times were measured using a standard inversion-recovery sequence. The J-based ^{13}C INEPT spectra optimized for a 130 Hz C-H coupling constant were recorded with ^1H pre-saturation consisting of ten Gaussian shaped 50 msec pulses applied at 8.5 ppm. The two-dimensional ^{13}C - ^{13}C INEPT-TOBSY spectra were recorded at a MAS frequency of 8 kHz as previously described using a refocused ^1H - ^{13}C -INEPT step with a 5 ms ^1H - T_2 filter, with a ^{13}C carrier frequency set at 100 ppm. The ^{15}N -, ^{13}C detected ^1H spin diffusion experiments recorded at a proton mixing time of 100–300 ms were recorded as described in. The ^1H decoupling field strength was $\gamma B_1/2\pi = 70$ kHz.

^1H - ^{13}C - and ^1H - ^{15}N -MAS cross-polarization with and without ^1H presaturation using ten Gaussian-shaped 50-msec pulses at a ^1H frequency at 8.5 ppm were recorded at a MAS frequency of 15 kHz using a ^1H 3.5- μs 90°-pulse a cp-spin lock pulse of 0.5–1 ms length at 50 kHz with a 100%-80% linear gradient on the ^1H channel and 70 kHz of ^1H decoupling using a spinal-64 sequence and a recycle delay of 2s. 2-D cross-polarization dipolar assisted rotational resonance (CP-DARR) experiments were conducted with a 50–300 ms mixing time and the same cross-polarization parameters and ^1H decoupling as above⁹⁷. The ^{15}N - ^{13}C correlation experiments were performed through a double cross-polarization (CP) (^1H - ^{15}N ; ^{15}N - ^{13}C) resulting in selective transfer of the ^{15}N magnetization to directly bonded ^{13}C as previously described⁹⁸. 3.5 μs ^1H pulse length, ^1H - ^{15}N -CP-MAS, 80–100% ramp with 32 kHz CP-power and 1 ms contact time was used in first cross-polarization. Second cross-polarization ^{15}N - ^{13}C -CP was performed at 21 kHz and a contact time of 5 ms with a 90–100% ramp on the ^{15}N channel. A ^1H decoupling power of 51 kHz was used during the ^{15}N - ^{13}C cp as well as during acquisition utilizing a TPPM decoupling sequence. The MAS frequency was set to 8 kHz and temperature to 250°K. All spectra were recorded on a 800 MHz Bruker AV800 spectrometer equipped with a 4 mm $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ CP-MAS probe (Bruker Biospin Corp., Billerica, MA).

^2H static, solid-state NMR spectra were recorded to determine the chain order parameters, $S(n)$, of the perdeuterated palmitoyl chain of $^2\text{H}_{31}$ -POPC or $^2\text{H}_{31}$ -POPG in a 1:1 POPC:POPG mixture in the presence of the S1-S4 voltage-sensing domain or of the full-length KvAP channel. The protein was reconstituted into a mixture of POPC:POPG containing either $^2\text{H}_{31}$ -POPC or $^2\text{H}_{31}$ -POPG, passed through a polycarbonate filter (pore size 100 nm) using an Avestin extruder, the proteoliposome pellet sedimented via ultracentrifugation and transferred to a sample container. The spectra were recorded using a $^1\text{H}/^{13}\text{C}/^2\text{H}$ CP-MAS probe (Bruker) non-spinning operating at a ^2H NMR resonance frequency of 122.8 MHz. The ^2H NMR spectra were acquired with a quadrupolar echo pulse sequence⁹⁹ with a 5.2- μs 90° pulse, a spectral width of 500 kHz and a delay time of 0.2 s. The ^2H NMR spectra were de-Paked and order parameter $S(n)$ of the C-D bonds calculated according to the formula:

$$S(n) = \Delta\nu_Q(n) / (3/4 * e^2 q Q / h),$$

where n is the carbon atom number of the palmitoyl chain, the $\Delta\nu_Q(n)$ is quadrupolar splitting, $e^2 q Q / h$ is the quadrupolar coupling constant (167 kHz for deuterons in the C-H bond).

Smoothed order parameter profiles for the carbons C_2 to C_{11} were calculated as described¹⁰². The hydrophobic thickness of the bilayers was calculated from chain order

parameters as described by³⁴. Spectral processing and analysis of order parameters were performed in Mathcad (PTC, Needham, MA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The nature of lipid-protein interactions for S1-S4 voltage sensors is unknown
Lipids interact with S1-S4 domains and have lifetimes on the order of 10^{-3} s
Arg residues in S1-S4 domains are position close to lipids and are hydrated
The structure of S1-S4 domains is not detectably altered by cationic lipids

Figure 1. Structure and conformational changes in S1-S4 voltage-sensing domains

a) Cartoon representation of an S1-S4 voltage-sensing domain embedded in a lipid bilayer in either activated or resting states. Arg residues are depicted as blue sticks and acidic residues (Glu/Asp) as red sticks. Lipid head groups are depicted as gray ovals. **b)** Solution NMR structure of the S1-S4 domain of KvAP in detergent (pdb accession code 2KYH) viewed from the side. Approximate boundaries of the membrane are indicated with gray lines. Arg side chains are shown in dark blue, Lys in light blue, and Glu/Asp in red. Trp70 is colored orange and position of the Ala111Cys mutant used for Badan labeling is indicated with a yellow sphere. **c)** Structure of the S1-S4 domain viewed from the external side of the membrane.

Figure 2. Saturation transfer difference NMR experiments to detect protein-lipid interactions

a) ^1H MAS spectrum of POPC:POPG lipid mixture in the presence of 1 mol % S1-S4 following a presaturation, proton B_1 -field strength of $\gamma_{\text{H}}B_1/2\pi = 824$ Hz with Gaussian-shaped, 50 ms-long pulses applied for 1 sec at 11 ppm (red) or 8.5 ppm (blue). Spectra were recorded at 14.9 kHz MAS frequency at 295.1°K in a fully hydrated state of the proteoliposome sample. Structure of POPC lipid is depicted above the spectra. **b)** Saturation of the lipid methylene resonance (1.3 ppm) with the radiofrequency pulses applied at different presaturation frequencies. Attenuation factor follows the anticipated intensity distribution of protein amide resonances. **c)** Attenuation factor for lipid methylene segment plotted as a function of radiofrequency pulse applied on the amide region of protein. Attenuation factors were calculated as the resonance amplitude recorded without saturation divided by the amplitude with saturation. Attenuation of lipid alone is shown in black open circles. Attenuation of the resonance for lipid methylene segments of the proteoliposomes containing 0.25 mol % S1-S4 domain and 0.32 mol % full length KvAP channel (calculated as a monomer) are shown as blue filled triangles and squares, respectively.

Figure 3. INEPT experiments comparing interactions of POPC and POPG with S1-S4 domains
a) Head group regions of POPC and POPG lipid structures. **b)** POPC (green) and POPG (red) lipid head-group region of ^{13}C - INEPT natural abundance spectra with 130 Hz $J_{\text{C-H}}$ coupling constant. Spectra were acquired at 14.9 kHz MAS frequency at 295.1°K. **c)** Lipid head-group region of J-based natural abundance ^{13}C - INEPT spectrum with 130 Hz C-H coupling constant for POPC:POPG mixture with 1 mol % S1-S4 domain. Pre-saturation consisting of twenty Gaussian shaped 50 msec radio frequency (RF) pulses of B_1 field strength of 740 Hz with the saturation frequency set to the amide region of the S1-S4 protein (8.5 ppm). **d)** The attenuation of the lipid natural abundance ^{13}C signals for the head-group carbons of POPC and POPG. The signals corresponding to carbons of lipid segments are depicted with numbers. POPG signals are shown in red; POPC signals are shown in green. The composite signals with overlapping POPC and POPG resonances are shown with tan.

Figure 4. ^{15}N -detected ^1H spin-diffusion experiments to detect local interactions of S1-S4 domains with lipids and water

a) 2-D ^{15}N -detected ^1H spin-diffusion spectrum of the S1-S4 in POPC:POPG, with a mixing time of 100 msec. A T_2 filter time of 2 msec, a relaxation delay of 2 s, and a MAS spinning frequency of 7 kHz were used. The 128 t_1 increments were acquired. Above the 2-D spectrum, the ^{15}N projection of the protein is shown. On the left projection is a ^1H spectrum with T2 filter at the same experimental conditions. **b)** Intensity build-up curves from spin diffusion experiments as shown in a. Spin diffusion from H_2O and lipid methylenes to S1-S4 amide NH in POPC:POPG (tan) or DOTAP (blue). **c)** Intensity build-up curves from spin diffusion experiments as shown in a. Spin diffusion from H_2O and lipid methylenes to Arg $\text{N}_{\eta 1,2}$ in POPC:POPG (tan) or DOTAP (blue).

Figure 5. Characterization of S1-S4 domains reconstituted in DOTAP using fluorescence and circular dichroism spectroscopy

a) Fluorescence emission of Trp70 when S1-S4 protein labeled with BADAN reconstituted in POPC:POPG (tan) and in DOTAP (blue). **b)** Circular Dichroism spectra of the S1-S4 in POPC:POPG (tan) or DOTAP (blue) proteoliposomes. Mean residue ellipticity (Θ) ($10^3 \times \text{deg cm}^2 \text{dmol}^{-1}$) is plotted as a function of wavelength. **c)** Fluorescence emission of BADAN label (excitation at 387 nm) when the S1-S4 domain is reconstituted in POPC:POPG (tan) or DOTAP (blue). **d)** Fluorescence emission of BADAN label when the S1-S4 domain is reconstituted in POPC:POPG vesicles and fused with empty DOTAP vesicles.

Figure 6. Cross-polarization dipolar assisted rotational resonance experiments to resolve the Arg spin system in S1-S4 domains

a) 2-D ^{13}C - ^{13}C CP-DARR spectra of uniformly ^{13}C -labeled S1-S4 domain reconstituted in POPC:POPG (tan) or DOTAP (blue) with 300 ms mixing time at 14.9kHz MAS frequency at 275 °K. Cross-peaks indicate ^{13}C - ^{13}C correlations in the protein. Arg residue spin system cross-peaks are labeled. **b)** Enlarged view of the $^{13}\text{C}_\zeta$ - $^{13}\text{C}_\alpha$ cross-peak colored as in a. **c)** Enlarged view of the $^{13}\text{C}_\zeta$ - $^{13}\text{C}_{\beta\gamma}$ cross-peak colored as in a.

Figure 7. Investigating the influence of DOTAP on the environment of Trp70

a) ^{13}C spectra of uniformly ^{13}C -indole Trp70 labeled S1-S4 in POPC:POPG (tan) or DOTAP (blue) recorder at 14.9 kHz MAS frequency at 273 °K. **b)** ^{15}N spectra of ^{15}N -indole labeled Trp70 residue of S1-S4 domain reconstituted in POPC:POPG (tan) or DOTAP (blue).

Figure 8. Exploring the influence of DOTAP on the local environment of Arg residues in S1-S4 domains

a) Enlarged view of CP-DARR spectra showing $^{13}\text{C}_\zeta$ - $^{13}\text{C}_\alpha$ correlations for uniformly ^{13}C -labeled S1-S4 in POPC:POPG (tan) and DOTAP (blue). **b)** Enlarged view showing ^{13}C -Arg S1-S4 in POPC:POPG (1:1) at 300 msec mixing time. **c)** 2-D ^{13}C - ^{15}N CP correlation spectra of uniformly ^{13}C and ^{15}N -labeled S1-S4 domain reconstituted in POPC:POPG (tan) or DOTAP (blue) at 8kHz MAS frequency. A ^{13}C - ^{15}N cross-peak is observed for the guanidinium groups of Arg residues in the protein (C_ζ - N_η).