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Review

Comparative NMR studies demonstrate profound differences between two viroporins: p7 of HCV and Vpu of HIV-1

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ABSTRACT

The p7 protein from hepatitis C virus and the Vpu protein from HIV-1 are members of the viroporin family of small viral membrane proteins. It is essential to determine their structures in order to obtain an understanding of their molecular mechanisms and to develop new classes of anti-viral drugs. Because they are membrane proteins, it is challenging to study them in their native phospholipid bilayer environments by most experimental methods. Here we describe applications of NMR spectroscopy to both p7 and Vpu. Isotopically labeled p7 and Vpu samples were prepared by heterologous expression in bacteria, initial isolation as fusion proteins, and final purification by chromatography. The purified proteins were studied in the model membrane environments of micelles by solution NMR spectroscopy and in aligned phospholipid bilayers by solid-state NMR spectroscopy. The resulting structural findings enable comparisons to be made between the two proteins, demonstrating that they have quite different architectures. Most notably, Vpu has one trans-membrane helix and p7 has two trans-membrane helices; in addition, there are significant differences in the structures and dynamics of their internal loop and terminal regions.

Contents

1. Introduction

1.1. Viroporins

Viroporins are relatively small viral membrane proteins whose functions are crucial to the lifecycle of viruses [1,2]. Proteins classified as viroporins include Vpu from the Human immunodeficiency virus 1

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(HIV-1), p7 from Hepatitis C virus (HCV), M2 from the influenza virus A, poliovirus 2B, and alphavirus 6K. Although these proteins are not involved in replication, they appear to have multiple biological activities, and are essential for viral infectivity by promoting virus release. Structural investigations of viroporins are at an early stage, with M2 from influenza being the most advanced [3–5]. However our NMR studies of Vpu [6–14] and of p7 [15–17] have revealed many of their principal structural features in membrane environments, and the properties of these two proteins are compared here. In general, all of the viroporins have several common features, including at least one hydrophobic trans-membrane helix and conserved basic and/or aromatic residues. There is evidence that at least some of these proteins oligomerize to form ion channels [18–21], several of which are inhibited by the addition of established channel blocking compounds [21–27]. Also, mutations in these proteins can lead to non-viable viruses [28–31]. These proteins are highly conserved across genotypes, and these experimental results suggest that they are potential targets for anti-viral drugs.

1.2. Viroporin proteins p7 of HCV and Vpu of HIV-1

Hepatitis C virus, a member of the Flaviviridae family, infects over 170 million people worldwide. Infections are often chronic, resulting in cirrhosis, end-stage liver disease and hepatocellular carcinoma. HCV's viral RNA, which is 2.6 kb, encodes a single polyprotein that is proteolytically cleaved into ten proteins that are responsible for the functions of the virus life cycle [32,33]. One of these proteins, p7, a small hydrophobic protein with 63 residues, has been shown to play a role in the release of mature virus particles from infected cells [28]. Topology predictions suggest that p7 has two hydrophobic trans-membrane helices connected by a short inter-helical loop that contains the two highly conserved basic residues, K33 and R35 [34–36]. p7 has been shown to form hexamers in phospholipid bilayers, and several groups have demonstrated that the resulting ion channels can be blocked by compounds such as amantadine [21], hexamethylene amiloride [23] and long acyl-chain iminosugar derivatives [24].

Human Immunodeficiency Virus (HIV) is a lentivirus in the retrovirus family. HIV can lead to the acquired immune deficiency syndrome (AIDS) by destroying crucial immune functions. In 2008, an estimated 33.4 million people were infected with HIV worldwide. HIV-1 and HIV-2 are the two principal types of HIV; HIV-1 is more virulent, has higher infectivity, and contains the gene for Vpu. Vpu is an 81-residue membrane protein that is responsible for two biological activities that contribute to the pathogenicity of HIV-1 infections in humans; it accelerates the degradation of CD4 receptors and enhances the release of newly formed virus particles from infected cells [37–40]. These activities are associated with separate domains of the protein. The C-terminal cytoplasmic domain of the protein modulates CD4 degradation via the phosphorylation of two conserved serine residues (S52 and S56). The N-terminal transmembrane domain is responsible for enhancing the release of virus particles. The mechanism by which Vpu enhances virus release is under active investigation in a number of laboratories [18,41–44]. The results of molecular dynamics simulations and channel activity studies have shown that the hydrophobic transmembrane domain of Vpu can form pentamers or tetramers [45]. Notably, a single mutation of alanine at position 18 of Vpu by a histidine (A18H) has been shown to make the virus infection sensitive to rimantadine, a M2 channel blocker [14].

1.3. Applications of NMR spectroscopy to p7 and Vpu

Information about the structure and dynamics of these proteins has the potential to provide insights into their mechanisms of action and contribute to the rational design of drugs. NMR spectroscopy can be used to characterize the structure of these proteins at atomic resolution in micelle and bilayer environments [14,46–48], something that is not possible using other experimental methods of protein structure determination. Another advantage of NMR spectroscopy is that experiments are available that can be used to monitor molecular motions on multiple timescales to provide a detailed description of the dynamics of the proteins. Recent developments in instrumentation, specifically, the design and construction of solid-state NMR probes that reduce the sample heating due to high frequency radiofrequency irradiations and provide higher sensitivity facilitate the study of samples with high dielectric constants like viroporins in their native environments of fully hydrated phospholipid bilayers [49].

2. Expression, isotopic labeling, and purification of p7 and Vpu

2.1. Fusion protein expression

The over expression of polypeptides with sequences corresponding to those of p7 and Vpu in bacteria is challenging because in high concentrations they can disrupt the integrity of cell membranes, resulting in the early death of rapidly growing bacteria. The use of fusion proteins overcomes this obstacle, since the over expressed fusion proteins are generally not toxic to bacteria because they are sequestered in inclusion bodies, which keeps them away from the vulnerable cell membranes. Fusion partner proteins such as Trp-leader (TrpΔLE) and ketosteroid isomerase (KSI) are commonly used, and have been successfully employed in the bacterial expression of p7 and Vpu.

The methods used for the expression and purification for p7 and Vpu have evolved from those previously described by Park et al. [10] and by Cook et al. [15]. Coding sequences corresponding to p7 from HCV genotype J4 and to Vpu from HIV-1 genotype NL43 were designed with codon optimization to facilitate heterologous expression in *Escherichia coli.* Amplified sequences were inserted into plasmid vectors containing N-terminal fusion proteins. The p7 sequence was fused to that of TrpΔLE and that of Vpu to KSI. Both constructs include histidine tags to enable facile purification by nickel affinity chromatography. The plasmids were transformed into BL21 (DE3) cells. Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) when the growth media reached an optical density of 0.5–0.6. Cells were allowed to grow an additional 4–6 h and then harvested by centrifugation. The expression of the fusion proteins is visualized in the SDS-PAGE shown in Fig. 1A and D, where the total cell proteins before and after 4 hours of induction by the addition of IPTG are compared.

2.2. Isotopic labeling

NMR experiments require milligram quantities of highly purified isotopically labeled proteins. The expression of membrane proteins as fusion proteins in *E. coli* can be carried out in a variety of media for this purpose. M9 minimal media with ^{15}N–ammonium sulfate, ^{13}C-glucose and/or deuterium oxide can be used to produce uniformly ^{15}N, ^{13}C and ^{2}H-labeled proteins as required. M9 media can also be prepared with specific isotopically labeled amino acids along with other unlabeled amino acids to produce selectively labeled (by residue type) proteins.

2.3. Purification

Following expression in *E. coli*, the purification of the polypeptides is accomplished in several discrete steps. Following the disruption of the cells, the fusion proteins are separated from soluble proteins and membrane fractions using a detergent-containing resuspension buffer. A denaturing buffer containing guanidine hydrochloride is then used to solubilize the fusion proteins. Metal affinity column with nickel is used to separate the fusion protein from contaminants in the inclusion bodies. To separate the polypeptide sequence of interest from its fusion partner, site-specific cleavage is accomplished either as

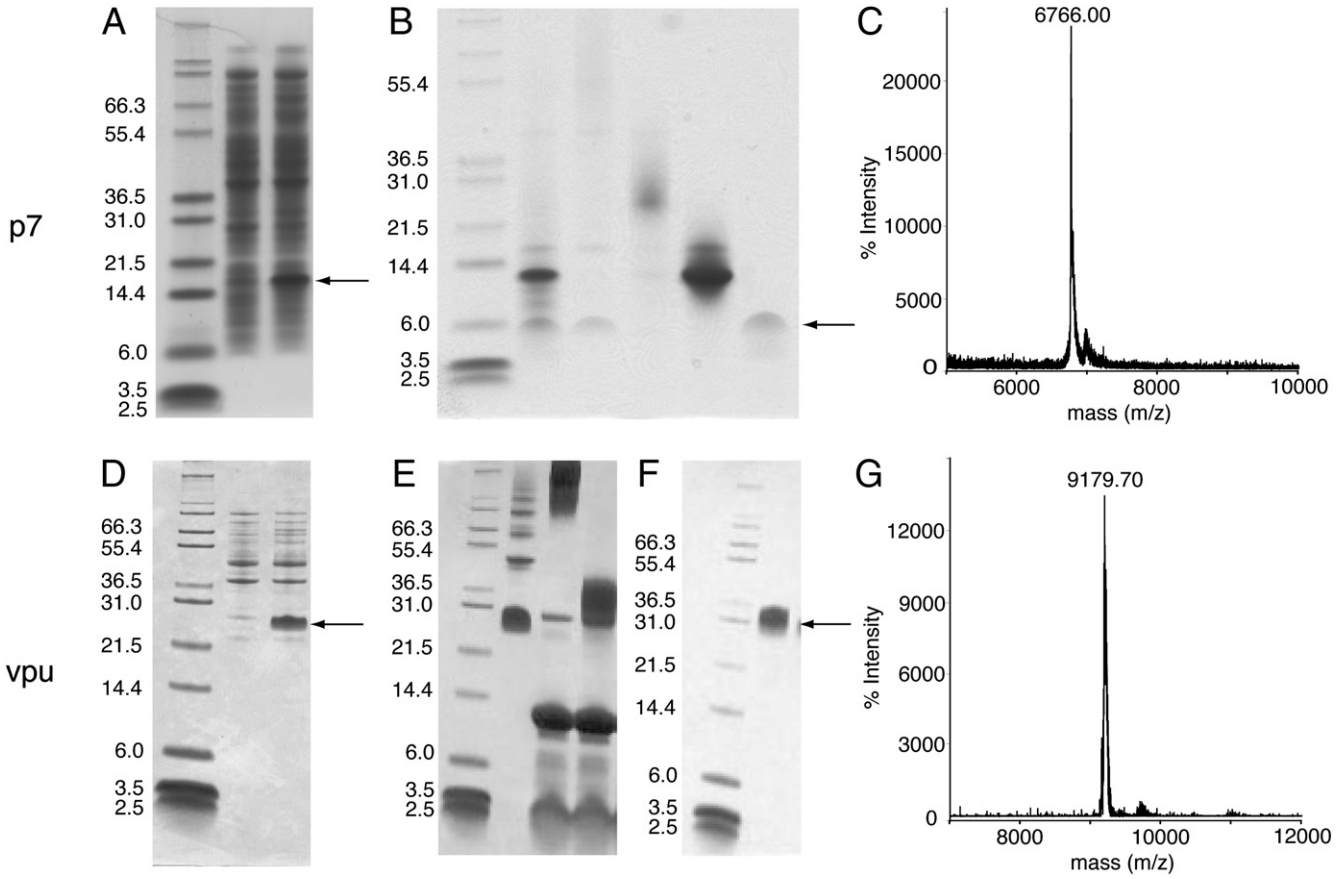

Fig. 1. The expression and purification of p7 and Vpu as fusion proteins in *E. coli* strain BL21 (DE3). (A–F) SDS-PAGE analysis. (A–B) p7. (D–F) Vpu. (A) The expression of His_6-TrpLE-p7. Left lane: protein size markers. Middle lane: total cell protein before induction. Right lane: total cell protein after induction with IPTG. The over expressed fusion protein is marked with an arrow. (B) The purification of p7 by size exclusion FPLC. Pure p7 is marked with an arrow in the rightmost lane. (D) The expression of KSI-Vpu. Left lane: protein size markers. Middle lane: total cell protein before induction. Right lane: total cell protein after induction. Pure Vpu is marked with an arrow. (E) For the purification of Vpu samples from the HPLC injection solvent test are shown in an SDS-PAGE gel (lane 2 shows pre-cleaved protein while lanes 3 and 4 show that the protein aggregates in a typical HPLC solvent, TFE/H_2O/TFA, and in a more hydrophobic solvent, HFIP/H_2O/TFA. (F) HPLC purified Vpu is marked with an arrow. The mass spectra of both proteins show that the purified proteins have the correct mass. (C) p7 at 6766.00 Daltons. (G) Vpu at 9179.70 Daltons.

an enzymatic cleavage using a sequence selective protease such as thrombin or enterokinase, or chemical cleavage using a chemical reagent such as cyanogen bromide. Final purification of the protein is generally accomplished with reverse-phase high performance liquid chromatography (HPLC), as well as ion-exchange chromatography and size exclusion chromatography as necessary.

Specifically, we utilize a four-step protocol for the purification of p7 and Vpu. Cell pellets are resuspended in resuspension buffer and disrupted by sonication. Inclusion bodies are isolated by centrifugation and denatured in binding buffer containing Tris and 6M guanidine prior to purification by nickel affinity chromatography. The fusion protein is bound to the column, and after several wash steps is eluted with guanidine-containing buffer containing a higher concentration of imidazole; it is dialyzed to remove the denaturing guanidine, and lyophilized to yield a dry powder. The protein is then dissolved in 70% formic acid for cleavage by cyanogen bromide at a methionine residue located between the sequence of the target protein and its fusion partner. After neutralization and complete removal of cyanogen bromide by dialysis, the protein is lyophilized. The final purification step is performed by size exclusion fast protein liquid chromatography (FPLC) for p7 and by reverse phase high performance liquid chromatography (HPLC) on a C4 column for Vpu. Samples of cleaved p7 and the FPLC elutions were subjected to SDS-PAGE; the last lane in Fig. 1B contains pure p7 protein. For Vpu the SDS-PAGE showing the solubilization of the protein in an organic solvent prior to purification by HPLC is in Fig. 1E. The sample tends to aggregate in the typical HPLC solvent of trifluoroethanol (TFE), water and trifluoroacetic acid (TFA) (50:49.9:0.1%) in lane 3, but was completely dissolved when the loading solvent contained hexafluoroisopropanol (HFIP)/water/TFA (50:49.9:0.1%) as shown in lane 4. Mass spectrometry was used to confirm the identity of the purified proteins. The spectra show single peaks corresponding to the correct masses for p7 (Fig. 1C) and Vpu (Fig. 1G). p7 appears as a monomer of apparent molecular weight of ~7 kDa in the SDS-PAGE, in contrast, Vpu appears as an oligomer of ~35 kDa, suggesting that Vpu is oligomeric in the SDS micellar environment.

3. Lipid environments for membrane proteins

3.1. Micelles, isotropic bicelles, and aligned bilayers

Micelles and isotropic bicelles can be used to study small membrane proteins by solution NMR spectroscopy. They are shown in cartoon form in Fig. 2A and B, respectively. Although not ideal as a membrane environment because of the absence of a bilayer, the small size of protein-containing micelles provide a hydrophobic environment similar to that of a membrane that is capable of undergoing overall reorientation fast enough to yield solution NMR spectra with relatively narrow linewidths. The most commonly used micelle-forming detergents and lipids are SDS (sodium dodecyl sulfate), DPC (dodecylphosphocholine) and DHPC (1,2-dihexanoyl-sn-glycero-3-phosphocholine). Many other micelle-forming lipids and detergents are typically surveyed in the

Fig. 2. Cartoon representations of the phospholipid assemblies in the various NMR samples. (A) Micelles that consist of detergents or short chain lipids. (B) Isotropic bicelles that consist of a mixture of long and short chain lipids with a $q<1.0$. (C) Magnetically aligned bilayers with $q=3.2$. Defects are shown in the bilayers.

course of optimizing the samples for solution NMR studies. Isotropic bicelles are prepared by the addition of small amounts of long chain lipids like DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) to the micelle samples, typically with DHPC as the short chain lipid. Bicelles are characterized by the molar ratio of long chain to short chain lipids (q). For $q<1$, the rotational correlation times of small membrane proteins are typically short enough to enable solution NMR experiments.

Solid-state NMR spectroscopy provides the opportunity to study membrane proteins in their native environment of fully hydrated phospholipid bilayers under physiological conditions. This is a significant advantage over other experimental methods, but it requires greater technical sophistication in the instrumentation and experimental methods. In high-resolution solid-state NMR experiments, intense radiofrequency irradiations replace rapid molecular motions as the principal line-narrowing mechanism. Two approaches are most commonly employed, either oriented sample (OS) solid-state NMR of membrane proteins in aligned bilayers or magic angle spinning (MAS) solid-state NMR of unoriented samples. In general, magnetic alignment of bilayers consisting of DMPC and DHPC with a $q=3.2$ provides convenient samples for high-resolution solid-state NMR experiments. A schematic drawing of the aligned bilayers is shown in Fig. 2C. The bilayers self-assemble and align with their normals perpendicular to the direction of the applied magnetic field, which induces alignment of the incorporated membrane protein [50–52]. The bilayers can be "flipped" with the addition of lanthanides or the use of biphenyl lipids to provide an alignment of the bilayers with their normals parallel to the magnetic field [53]. As part of the sample optimization process, a variety of additional lipids are incorporated in order to improve the resolution of the spectra or stabilize the samples; these include lipids with charged head groups, different acyl-chain lengths, ester or ether linkages, and various sites of unsaturation.

3.2. Sample preparations

It is crucial that the proteins used in the NMR experiments are highly purified in order to have homogeneous samples and avoid spurious signals. This requires the complete removal of denaturants, organic solvents, and any unwanted detergents or lipids. This also requires careful optimization of all sample conditions (pH, temperature, and buffer and salt concentrations, etc.). Careful attention must be paid during the solubilization of the protein with detergents or lipids in order to ensure that the sample is completely clear and homogeneous, and that the protein is fully solubilized and properly folded. This is often done by adding a concentrated amount of the detergent or lipid prior to diluting it for the final sample. For solid-state NMR the sample is put through a number of heat/cold/vortexing cycles until the sample is clear, and displays the properties of a magnetically alignable bilayer phase. Preliminary one-dimensional NMR spectra are obtained to determine whether the sample is ready for more detailed investigations.

4. Solution NMR spectroscopy

4.1. Structure and dynamics

Following sample optimization, well-resolved, two-dimensional ^{1}H-^{15}N heteronuclear single quantum correlation (HSQC) solution NMR spectra of uniformly ^{15}N-labeled p7 (Fig. 3A) and Vpu (Fig. 3E) were obtained in DHPC micelles. The complete backbone resonance assignments of p7 and Vpu were obtained using standard methods that included triple resonance experiments of uniformly ^{15}N and ^{13}C labeled samples, two- and three-dimensional homonuclear $^{1}H/^{1}H$ NOE experiments, and comparisons among spectra of selectively labeled (by residue type) samples [54,55]. The sequential resonance assignments are an essential prerequisite to the spectroscopic measurements used to characterize the secondary structure and internal dynamics of the proteins. The chemical shift frequencies of several sites in the amino acid residues (^{13}CO, $^{13}C\alpha$, $^{1}H\alpha$ or $^{13}C\beta$) are valuable data. Deviations from the random coil values of the chemical shift index (CSI) are suggestive of the secondary structure and can be used to estimate the bond angles in the protein backbone [56].

Deuterium/hydrogen fractionation is an effective method for identifying the basic topology of membrane proteins in micelles by monitoring the relative facility for hydrogen exchange at backbone amide sites [57]. With an increasing percentage of D_2O in the solution, the intensities of resonances in solvent accessible loop and terminal regions are reduced compared to those in hydrophobic transmembrane helices. Heteronuclear ^{1}H-^{15}N NOE measurements provide a reliable guide to differences in the local dynamics of membrane proteins [58], since it is generally possible to distinguish qualitatively between structured helical regions, and mobile loops, and terminal regions. Bicelle q titration experiments can also be used to probe the dynamics of a protein. By increasing the q of the protein-containing bicelles, the overall reorientation rate of the protein is decreased, causing the selective broadening of signals of those residues that are structured within the bicelle environment. Fig. 3 demonstrates the effect of increasing q on the spectra of uniformly ^{15}N labeled p7 and Vpu. Notably, essentially all of the signals are broadened beyond detection in $q=3.2$ samples where both proteins are immobilized in the bilayers and aligned in the magnetic field. These samples are suitable only for solid-state NMR experiments.

Paramagnetic relaxation enhancement (PRE) measurements are increasingly being used to measure relatively long-range distances between residues in proteins [59,60]. Site-directed mutagenesis enables the attachment of electron spin labels such as a nitroxide groups to specific sites where a cysteine is located. The paramagnetic form of the label causes selective broadening of the resonances from proximate residues that can be measured and converted into quantitative distance constraints for structure calculations. The reduced form of the label serves as the control. The results of these experiments complement the angular restraints associated with heteronuclear residual dipolar couplings (RDCs) to calculate the three-dimensional structures of the proteins.

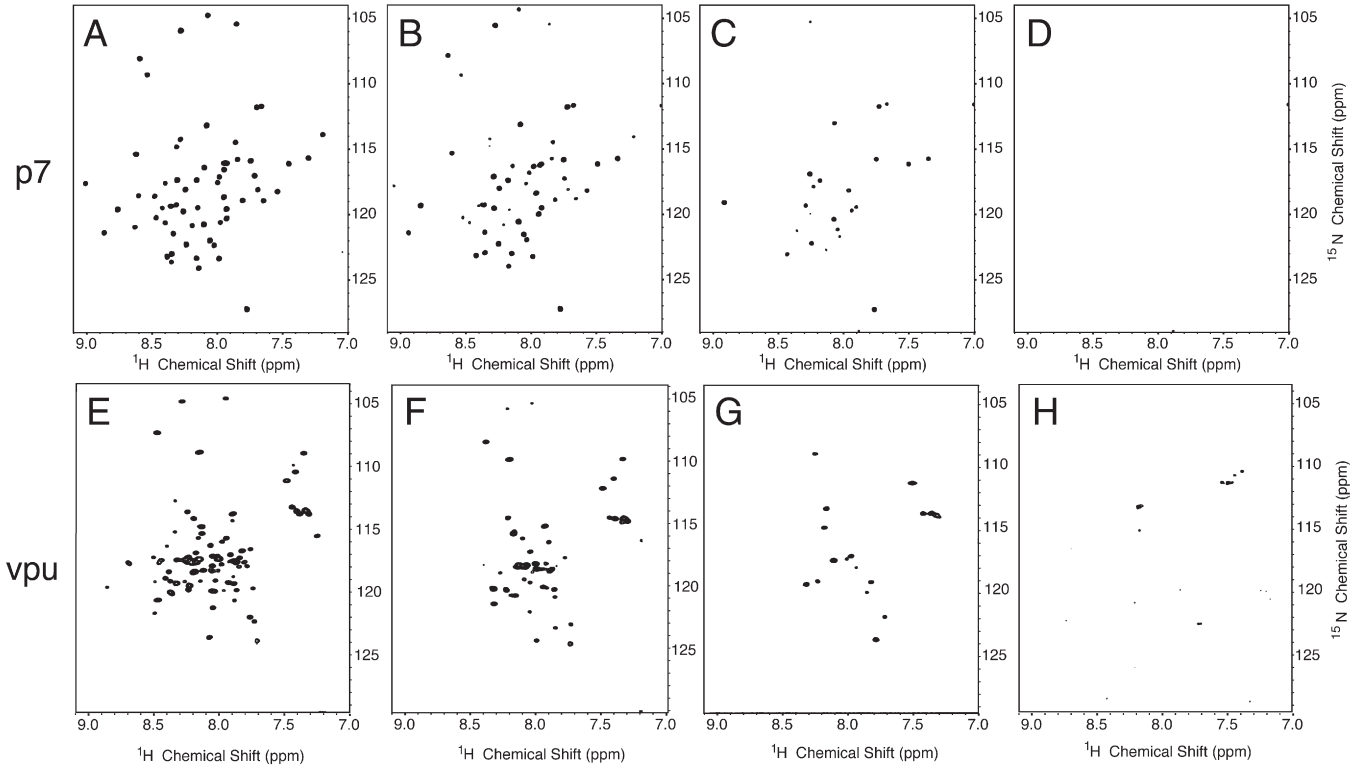

Fig. 3. Two-dimensional ^{1}H/^{15}N HSQC solution NMR spectra of uniformly ^{15}N labeled p7 and Vpu in micelles, isotropic bicelles, and magnetically aligned bilayers obtained at 50 °C. Top row: spectra of p7. (A) $q=0$ DHPC micelles. (B) $q=0.1$ bicelles. (C) $q=0.5$ bicelles. (D) $q=3.2$ bilayers. Bottom row: spectra of Vpu. (E) $q=0$ DHPC micelles. (F) $q=0.25$ bicelles. (G) $q=1.0$ bicelles. (H) $q=3.2$ bilayers.

4.2. Residual dipolar couplings

It is difficult to observe a sufficient number of "long-range" homonuclear ^{1}H-^{1}H NOEs for structure determination of membrane proteins in micelles or isotropic bicelles. Instead, the measurement of residue dipolar couplings (RDCs) of weakly aligned samples using an in-phase/anti-phase (IPAP) experiment [61] provide the principal orientational restraints [62–64] that are used for structure determination of membrane proteins. The protein-containing micelle or isotropic bicelle samples can be weakly aligned by incorporating them into polyacrylamide gels that are stretched or compressed [65–67], or by adding intact bacteriophage particles [68], DNA [69] or lanthanide ions [70,71] to the solutions. Fig. 4 shows plots of experimental ^{1}H-^{15}N RDCs as a function of residue number for the backbone amide sites of p7 and Vpu. Dipolar Waves are highly effective at identifying and characterizing the principal secondary structure features of membrane proteins [62]. Fitting of sine waves with periodicities of 3.6 residues to the RDCs plotted in Fig. 4 demonstrates the presence of helical regions that encompass residues 9 to 26, 33 to 49 and 57 to 70 in Vpu, and residues 6 to 16, 17 to 27, 41 to 49 and 50 to 57 in p7. The presence of a break in the dipolar wave is an indicator of a kink in the helices. Also mobile loops and terminal regions have negligible RDC values. In addition to describing the secondary structure of the proteins, these measurements provide input for the calculation of the three-dimensional structure of a protein, although for this purpose it is necessary to obtain data from multiple alignment media to eliminate angular ambiguities.

5. Solid-state NMR

5.1. Solid-state NMR experiments of aligned samples

The protein-containing bilayer samples are carefully checked by ^{31}P NMR to ensure that the lipid bilayers are uniform and well aligned in the magnetic field before the initiation of further experiments. Samples containing p7 or Vpu yield high quality ^{31}P spectra in magnetically

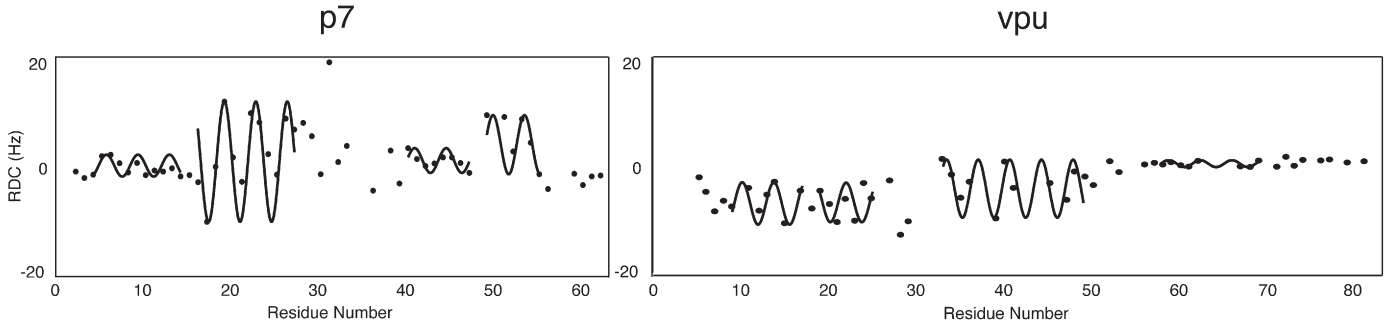

Fig. 4. Plots of ^{1}H-^{15}N RDCs as a function of residue number. Left panel: p7. Right panel: Vpu. The RDCs were measured from weakly aligned protein samples in DHPC micelles at 50 °C. The alignment was induced by stressed polyacrylamide gels. Dipolar waves were quantitatively fit to the data. The regions with best fits to a sinusoid determine the helical segments of the proteins.

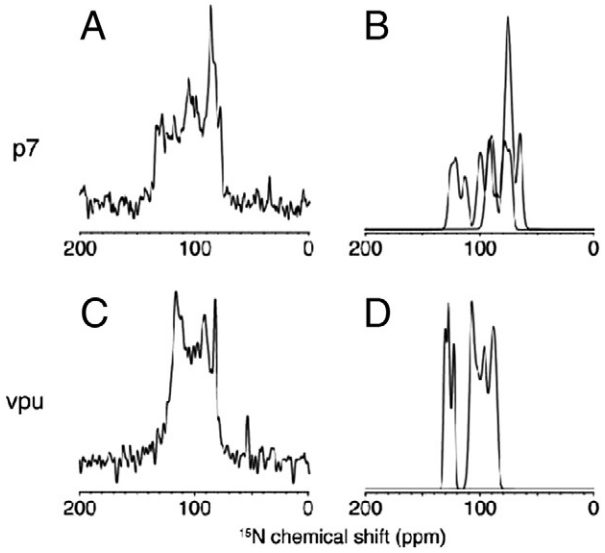

Fig. 5. ^{15}N solid-state NMR spectra of uniformly ^{15}N labeled proteins in bilayers aligned with their normals perpendicular to the magnetic field. (A and B) p7. (C and D) Vpu. (A and C) Experimental spectra obtained at 700 MHz and 42 °C. (B) Simulated spectra of p7 based on helices tilted 10° and 25° from the bilayer normal. Bottom: (D) Simulated spectra of Vpu based on helices with tilt angles of 30° (trans-membrane) and 70° (cytoplasmic).

aligned bilayers of DMPC/DHPC with $q = 3.2$. ^{15}N solid-state NMR spectra demonstrate that the proteins are well aligned along with the bilayers. The lack of underlying powder pattern intensity or a distinguishable isotropic intensity, and observing the bulk of the signal intensity between 70 ppm and 140 ppm (Fig. 5A and B) indicates that the proteins assume folded conformations in bilayers aligned with their normals perpendicular to the direction of the magnetic field.

The chemical shifts of the backbone amides are orientation dependent because the proteins are not undergoing isotropic reorientation, and the frequencies of the resonances can be directly correlated to the tilt angle of the helical segments of the proteins within the bilayers by comparisons with simulated spectra. An overlay of simulated spectra for helices that are tilted 10° and 25° from the bilayer normal (Fig. 5C) is consistent with the experimental data for p7. Vpu has one transmembrane helix that is tilted 27° or 30° with respect to the bilayer normal in DMPC/DMPG bilayers [11] or DMPC/DHPC bicelles, respectively [12]. The comparison of an experimental spectrum of full-length Vpu and a simulated spectrum of an ideal transmembrane helix tilted 30° (80 ppm–110 ppm) and in-plane helix titled 70° (120 ppm–135 ppm) demonstrates that the orientation of the cytoplasmic domain in bicelles is similar to that in lipid bilayers aligned macroscopically on glass slides [6].

6. Conclusions

p7 and Vpu are representative examples of the class of viral membrane proteins known as viroporins. Their roles in the infectivity of HCV and HIV-1, respectively, make them potential targets for antiviral drugs. The combination of structural and functional studies will play an important role in the development of new drugs. The relatively small sizes of these proteins makes them well suited for studies by NMR spectroscopy. The successful incorporation of these proteins into micelles, isotropic bicelles, and lipid bilayers and the optimization of the samples and experimental conditions result in well-resolved spectra that enable their dynamics and secondary structures to be characterized.

Although p7 and Vpu are thought to play similar roles in the life cycles of their respective viruses, they have very different structures (Fig. 6). The measurement of ^{1}H-^{15}N residual dipolar couplings of

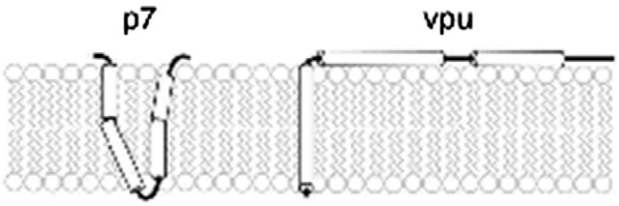

Fig. 6. Cartoon drawings of p7 and Vpu in bilayers based on the analysis of the dipolar waves from the solution NMR data and the comparison between the experimental and simulated spectra from the solid-state NMR data.

weakly aligned samples by solution NMR, and the subsequent fitting of dipolar waves to the plots of RDCs as a function of residue number enabled the secondary structures of the protein to be defined. p7 was found to have what appears to be two transmembrane segments between residues 6–27 and 41–57, each of which consists of two distinct helical segments. In contrast, Vpu has a single transmembrane helix between residues 9 and 26, and a cytoplasmic domain that contains two shorter helices between residues 33 and 49 and between 57 and 70 separated by a short flexible loop that contains the phosphorylation sites essential for one of its biological functions. The ^{15}N solid-state NMR spectra of the proteins in magnetically aligned bilayer samples provide insights into the structures of the proteins as well as their arrangements within the bilayers. Comparison of simulated and experimental NMR spectra for p7 in aligned bilayers indicates that two of the transmembrane helical segments are tilted approximately 10° and 25° from the bilayer normal, while for Vpu, a tilt angle of 30° was observed for the single transmembrane helix. Considering the mobile N- and C-terminal residues, the inter-helical loops, and the helical segments, these small membrane proteins have remarkably complex structures. As the NMR studies proceed toward completion and atomic resolution, the structural basis for the biological activities of p7 and Vpu should begin to be revealed.

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