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NMR studies of p7 protein from hepatitis C virus

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Abstract The p7 protein of hepatitis C virus (HCV) plays an important role in the viral lifecycle. Like other members of the viroporin family of small membrane proteins, the amino acid sequence of p7 is largely conserved over the entire range of genotypes, and it forms ion channels that can be blocked by a number of established channel-blocking compounds. Its characteristics as a membrane protein make it difficult to study by most structural techniques, since it requires the presence of lipids to fold and function properly. Purified p7 can be incorporated into phospholipid bilayers and micelles. Initial solid-state nuclear magnetic resonance (NMR) studies of p7 in 14-O-PC/6-O-PC bicelles indicate that the protein contains helical segments that are tilted approximately 10° and 25° relative to the bilayer normal. A truncated construct corresponding to the second transmembrane domain of p7 is shown to have properties similar to those of the full-length protein, and was used to determine that the helix segment tilted at 10° is in the C-terminal portion of the protein. The addition of the channel blocker amantadine to the full-length protein resulted in selective chemical shift changes, demonstrating that NMR has a potential role in the development of drugs targeted to p7.

Keywords Hepatitis C virus · p7 · Solid-state NMR · Bicelles

Introduction

Hepatitis C virus (HCV), a member of the *Hepacivirus* genus of the family *Flaviviridae*, primarily infects hepatocytes in humans. It causes persistent infection in a majority of cases, and can lead to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Choo et al. 1991). Current treatments for HCV infection have limited effectiveness (Davis et al. 1998; Di Bisceglie et al. 1995; Hadziyannis et al. 2004; McHutchison et al. 1998). Thus, a major motivation for studying the gene products of HCV is to identify and characterize potential targets for drugs.

The genome of HCV, a single-stranded positive-sense RNA of ~9.6 kilobases, is translated into a single 3,000-amino-acid polypeptide that is subsequently proteolytically cleaved into ten mature proteins. These include three structural proteins (E1, E2, core), six nonstructural proteins (NS2–NS5), and a small membrane protein (p7) whose sequence is located between those of the structural and nonstructural proteins (Lin et al. 1994; Selby et al. 1994).

p7 has been classified as a viroporin, since it is a small channel-forming viral membrane protein that affects the infectivity of the virus (Gonzalez and Carrasco 2003). Viroporins have been shown to participate in a number of viral functions, including the release of mature virus particles from the cell. Viroporins have also been shown to influence the cellular functions of the host (e.g., the cell vesicle system, glycoprotein trafficking, and membrane permeability). p7 has been demonstrated to be essential for infectivity of HCV, since viruses with mutated and deleted versions of p7 are not viable (Sakai et al. 2003). Like other viroporins, p7's amino acid sequence is largely conserved throughout the range of genotypes.

In addition to its functions, p7 has structural features in common with other viroporins. The full-length protein

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contains two hydrophobic segments that are likely to be transmembrane helices, and two conserved basic residues in the interhelical loop. p7 homo-oligomers function as ion channels in liposomes (StGelais et al. 2007). Significantly, the channel activity is affected by the channel-blocking compounds amantadine (Griffin et al. 2003), hexamethylene amiloride (HMA) (Pavlovic et al. 2003), and long-alkyl-chain imino sugar derivatives (Premkumar et al. 2004). The ion channels formed by p7 may play an important role in the lifecycle of the virus, and drugs that block them may affect virus replication, as seen with other viral p7 proteins (Harada et al. 2000).

Nuclear magnetic resonance (NMR) spectroscopy is an important tool for studying membrane proteins, and solid-state NMR is unique in its ability to characterize membrane proteins in their native phospholipids bilayer environment. Other viroporins, including M2 of influenza A and Vpu from HIV-1, have been studied extensively by NMR in membrane environments (Luo et al. 2007; Park et al. 2003; Wang et al. 2001). Recent advances in sample preparation, instrumentation, and experimental methods are used in the studies of p7 described here.

Protein samples that are uniformly or selectively isotopically labeled with ^{15}N and/or ^{13}C are essential for NMR studies. Since milligram quantities of the protein constructs are required, it is necessary to express the protein constructs in bacteria. Proteins can be uniformly labeled by growing the bacteria in a minimal media where the sole source of nitrogen and/or carbon is labeled with the appropriate isotope (e.g., ^{15}N ammonium sulfate or ^{13}C -labeled glucose). For the expression of selectively labeled proteins, the media is supplemented with all 20 amino acids, one of which is isotopically labeled. Membrane proteins, especially those that form channels, tend to disrupt cell membranes and cause cell death when overexpressed in bacteria. Therefore, we prepared the p7 constructs as a fusion with another protein that forms inclusion bodies, keeping the disruptive ion channels away from the cell membranes. The protein sequences are subsequently cleaved from the fusion protein, and purified using metal affinity, size exclusion or reverse-phase chromatography.

Protein-containing micelles can be used in solution NMR studies, since for small proteins under favorable conditions, they have relatively short correlation times that result in well-resolved spectra. Although micelles, and related isotropic bicelles or mixed micelle preparations, mimic some aspects of the membrane environment, it is difficult to know if the structure determined under these conditions represents the functional protein, especially if the protein forms oligomers as is the case for p7. The use of magnetically aligned bicelles allows the structure to be determined in a fully hydrated planar-lipid bilayer

environment similar to those used to characterize channel activity (Sanders 1994). Bicelles are made from a mixture of lipids with different chain lengths that self-assemble into bilayers and align with their normal perpendicular to the applied magnetic field (Sanders 1994; Sanders and Prosser 1998; Sanders et al. 1993). For NMR studies the protein is incorporated into and immobilized by the long-chain phospholipids that form the planar bilayer portion of the bicelles.

NMR instrumentation is constantly being improved in order to address the challenges presented by membrane proteins in bilayer environments. In addition to increased magnetic field strengths, probes are being developed to perform experiments on these high-dielectric lossy samples. Improved radiofrequency homogeneity of the coil and reduction of sample heating are two key components being addressed for the purpose of studying protein-containing bilayer samples (Wu et al. 2009).

A range of two-dimensional solid-state experiments has been developed for studying aligned samples of membrane proteins. High-resolution separated local field experiments such as polarization inversion spin exchange at the magic angle (PISEMA) (Wu et al. 1994) and magic sandwich based separated local field spectroscopy (SAMMY) (Nevzorov and Opella 2003a) correlate the orientation-dependent frequencies of the ^{15}N chemical shift and the ^1H - ^{15}N dipolar coupling interactions for each ^{15}N -labeled amide site in the protein. Since the orientations that affect the frequencies are relative to the fixed frame of the magnetic field, the wheel-like patterns of resonances, known as polarity index slant angle (PISA) wheels, that are apparent in the spectra can be used to determine the orientation of the protein backbone relative to the bilayer (Marassi and Opella 2000; Wang et al. 2000). Complete analysis of NMR data leads to the determination of the three-dimensional structure of the protein in the bilayer.

Nuclear magnetic resonance can be used to identify the residues in the protein that interact with drugs (Hajduk et al. 1997; Huth et al. 2005; Yu et al. 2005). This is straightforward for solution NMR, and has been demonstrated for the viroporin Vpu in micelles (Park and Opella 2007); it is demonstrated here for p7 in micelles. If a drug compound binds to the protein it will cause changes in the local chemical environment, which can be observed by changes in the amide chemical shifts in the two-dimensional heteronuclear single quantum correlation (HSQC) spectrum of the protein. By comparing spectra of the protein in the presence and absence of the drug, specific binding sites can be identified. In complementary solid-state NMR experiments, it is feasible to determine not only specific residues that interact with drugs, but also global changes in the protein structure by observing orientational changes in the aligned protein.

Methods

Protein preparation

HCV p7 with the sequence from genotype 1b strain J4 was expressed in *E. coli*. The two protein constructs used in the present study correspond to the full-length protein and to a truncated protein containing only the last 39 amino acids of the sequence ALENLVVLNAASVAGAHGILSFLVFFS (AAWYIKGR LAPGAAYAFYGVWPLLLLLLALPPRAYA). We refer to the truncated protein as p7TM2, since it was designed to represent the second transmembrane helix of p7. The full-length protein has a serine at position 27 in place of the native cysteine to facilitate the purification of p7 by eliminating the possibility of forming disulfide bonds at high protein concentrations. The genes for the p7 protein constructs were ligated into the pET vector (Novagen, www.novagen.com) downstream of the fusion protein TrpΔLE that directs the expressed protein to inclusion bodies. For uniformly ^{15}N -labeled samples, the bacteria were grown in M9 minimal media with ^{15}N ammonium sulfate as the sole nitrogen source. For expression of selectively ^{15}N -leucine labeled p7TM2, M9 media was supplemented with ^{15}N -leucine and each of the other 19 unlabeled amino acids. Expression was induced by the addition of isopropyl β -D-1-thiogalactopyranoside (IPTG). After harvesting the cells by centrifugation, the fusion protein was isolated from the cell lysate and dissolved in Tris buffer containing 6 M guanidine. The solubilized fusion protein was purified by nickel affinity chromatography by relying on its N-terminal His₆ tag. Bound protein was washed and eluted with imidazole. After extensive dialysis and lyophilization, the protein was cleaved from the fusion protein with cyanogen bromide in formic acid at the methionine residue between p7 and the TrpΔLE protein. After further dialysis and lyophilization, p7 was purified from the other cleavage products using size exclusion fast protein liquid chromatography (FPLC) in a sodium phosphate buffer containing sodium dodecyl sulfate (SDS). The elution fractions containing the isolated p7 protein were exhaustively dialyzed and dried on a lyophilizer overnight. The protein was stored at 4°C until it was incorporated into lipids for the NMR experiments.

Samples for solution NMR were prepared by dissolving pure protein powder in a solution containing 400 mM 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC). The sample was vortexed until clear, then diluted to a final concentration of 125 mM DHPC with 90% H₂O and 10% D₂O. The pH was adjusted to 4.0 by the addition of a small amount of sodium hydroxide. For the drug studies, 10 μl of a stock solution of amantadine was added to the sample to yield a final concentration of 10 mM (20 times the protein concentration).

Samples for solid-state NMR were prepared using previously described methods for making magnetically aligned bicelles (De Angelis and Opella 2007). Purified p7 protein was dissolved in solution containing 9.5 mg of the short-chain lipid, 1,2-di-*O*-hexyl-*sn*-glycero-3-phosphocholine 6-*O*-PC (6-*O*-PC), and 200 μl of water. The pH was adjusted to 4.0 and the sample was vortexed. The clear solution was added to 45.6 mg 1,2-di-*O*-tetradecyl-*sn*-glycero-3-phosphocholine (14-*O*-PC). The sample was subjected to several temperature cycles (42°C/4°C) with vortexing until the solution was clear and exhibited the appropriate phase transitions. The protein-containing bicelles were transferred to a flat-bottomed 5 mm NMR tube and capped with a rubber stopper.

Protein samples with their amide hydrogens replaced by deuterons were prepared by freezing with liquid nitrogen, and placing them on a lyophilizer overnight. The dried samples were dissolved in the appropriate concentrations of D₂O and H₂O.

NMR studies

Solution NMR studies were performed on a Bruker Avance 800 MHz spectrometer equipped with a standard $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ triple-resonance probe. All experiments were performed at 50°C. The fast ^1H - ^{15}N HSQC (Mori et al. 1995) experiment was performed with 1024 t1 points and 256 t2 points.

Solid-state NMR studies were performed on a Bruker Avance 700 MHz spectrometer equipped with a home-built $^1\text{H}/^{15}\text{N}$ double-resonance probe with a solenoid coil of 5 mm inner diameter and a strip shield (Wu et al. 2009). The experiments were carried out at 42°C where the protein-containing bicelles are aligned in the field. One-dimensional ^{15}N NMR spectra were obtained using mismatch optimized I-S transfer (MOIST) (Levitt et al. 1986) cross-polarization and SPINAL16 modulated decoupling (Fung et al. 2000; Sinha et al. 2005) with 512 t1 points and 2 k scans. Two-dimensional separated local field spectra were obtained using the SAMMY experiment (Nevzorov and Opella 2003a) with 48 t1 points and 512 t2 points. The acquisition time was 5 ms, and the recycle delay 7 s. The B1 fields were 50 kHz. The chemical shifts are referenced to external ^{15}N -labeled solid ammonium sulfate set to 26.8 ppm and H₂O set to 4.7 ppm.

Data processing

All NMR data were processed using NMRPipe (Delaglio et al. 1995) and analyzed using NMRView (Johnson and Blevins 1994) or SPARKY (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco). PISA wheel analysis was performed as previously

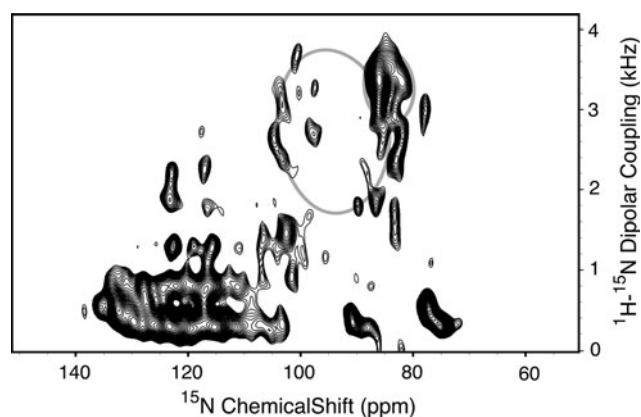


Fig. 1 Two-dimensional solid-state NMR $^1\text{H}/^{15}\text{N}$ separated local field spectrum of uniform ^{15}N -labeled p7 in magnetically aligned 14-O-PC/6-O-PC bicelles ($q = 3.2$, $w/v = 15\%$). The SAMMY experiment was performed at 42°C at 700 MHz. For comparison, PISA wheel simulations for helices with tilt angles of 10 and 25° are overlaid on the spectrum

described (Nevzorov and Opella 2003b) using MATLAB (Mathworks).

Results

The two-dimensional solid-state NMR spectrum of uniformly ^{15}N -labeled full-length p7 in 14-O-PC/6-O-PC bicelles in Fig. 1 is characteristic of a membrane protein aligned in a bilayer with its normal perpendicular to the direction of the applied magnetic field. The spectral intensity between 70 and 120 ppm indicates the presence of many residues in transmembrane helices. The experimental data were compared with simulations of ideal α -helices, and shown to be consistent with the tilt of two helical segments of approximately 10° and 25° relative to

the bilayer normal. The overall spectral quality is quite good with a number of resonances clearly resolved.

In order to interpret the spectrum in Fig. 1 with its high degree of spectral overlap, a truncated construct of p7 was designed, expressed, and purified for NMR studies. The sequence p7TM2 corresponds mainly to the second transmembrane helix of p7. The sequence includes the loop region and C-terminal portion of the full-length protein. The two-dimensional $^1\text{H}/^{15}\text{N}$ HSQC solution NMR spectra of the full-length and truncated constructs of p7 are well resolved and indicate that the truncated construct behaves very similarly to the full-length protein in DHPC micelles. The uniformly ^{15}N -labeled full-length protein in 90% H_2O (Fig. 2a) contains ~ 60 resonances, as expected. To determine which residues are in the helical transmembrane region the method of deuterium exchange was used (Veglia et al. 2002). By adding deuterium oxide to the sample the more readily exchanged amide sites lose signal intensities. For membrane proteins, residues in stable transmembrane helices do not exchange even at high percentages of D_2O . Only seven signals are observed for the uniform ^{15}N -labeled full-length protein in the 98% D_2O sample (Fig. 2b). Five of the seven residues in the deuterium-exchanged spectrum are also present in the spectrum of selectively ^{15}N -Leu-labeled p7TM2 in H_2O and there are negligible chemical shift differences for the resonances from residues Leu 51 through Leu 55 in the two constructs (Fig. 2c). This suggests that the chemical and structural environments of the leucine-rich region of the protein are similar in the two constructs.

Solid-state NMR was used to examine the properties of the truncated construct of p7 in phospholipid bilayers. The NMR spectra of a uniformly ^{15}N -labeled sample of p7TM2 (Fig. 3a, b) show improved resolution compared with the full-length protein; however, the small tilt angle of a helical

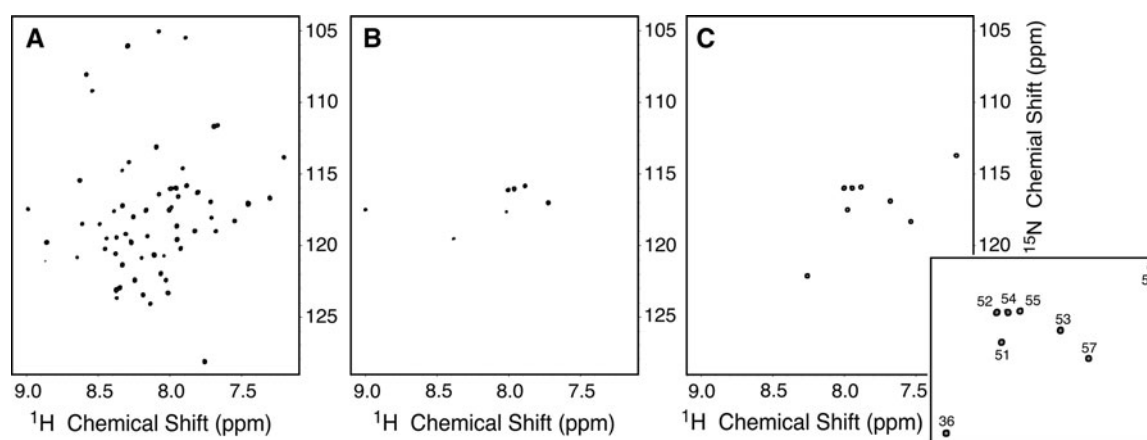


Fig. 2 Two-dimensional solution NMR $^1\text{H}/^{15}\text{N}$ HSQC spectra of p7 constructs in DHPC micelles. **A** Uniformly ^{15}N -labeled full-length p7 in 90% H_2O . **B** Uniformly ^{15}N -labeled full-length p7 in 98% D_2O .

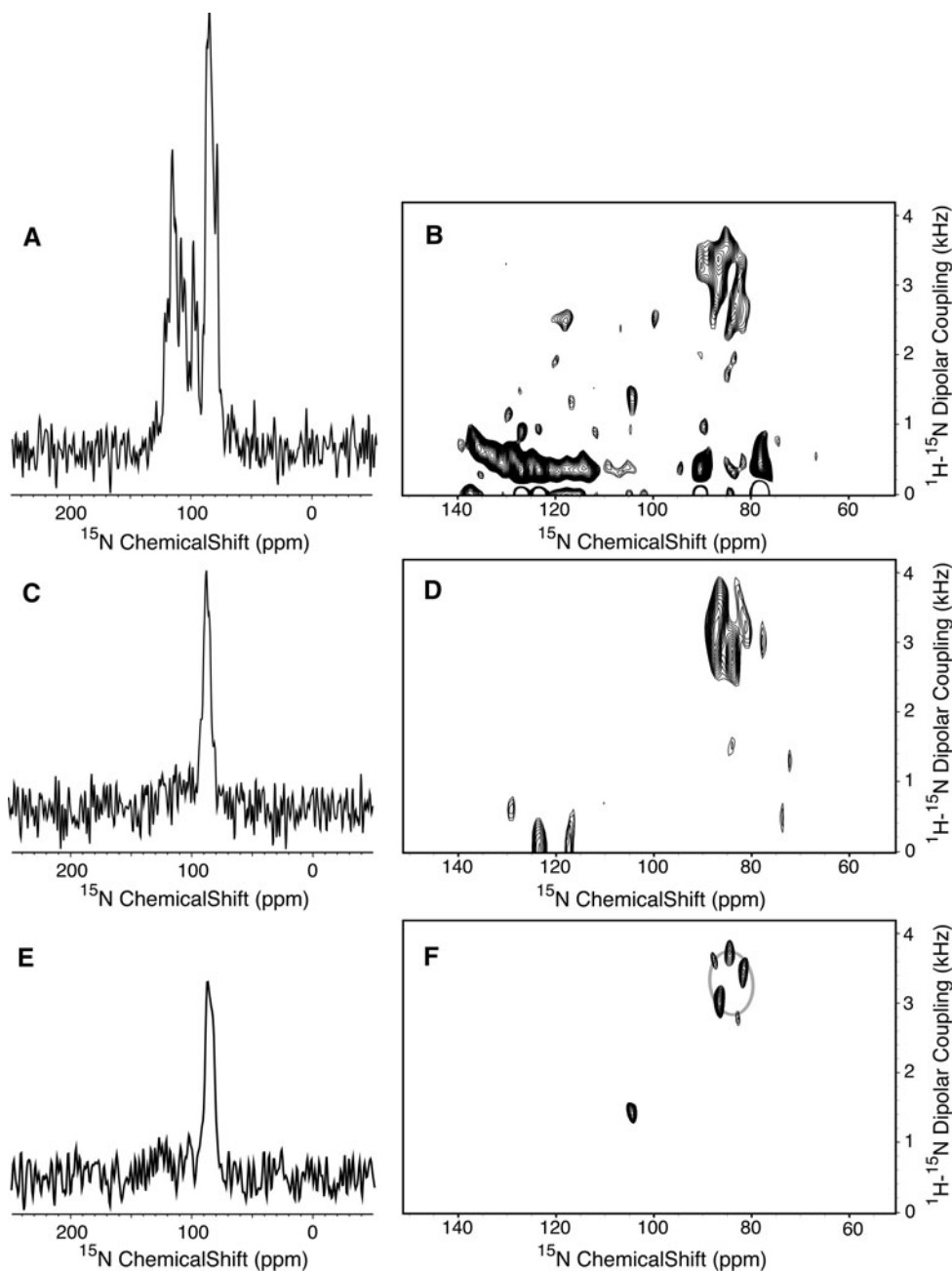
C Selectively ^{15}N -Leu labeled p7TM2. The inset shows the assignment of the eight leucine resonances in the p7TM2 protein construct. The experiments were performed at 50°C at 800 MHz

segment results in limited dispersion of the signals. There is also a large band (110–140 ppm) of resonance intensity near zero in the dipolar coupling frequency, which may be associated with dynamic or surface residues. Following hydrogen/deuteron exchange, the remaining resonances (Fig. 3b, d) were limited to the helical region, demonstrating that the residues in this segment of the protein were resistant to exchange. The one-dimensional ^{15}N NMR spectrum of the selectively ^{15}N -leucine labeled sample (Fig. 3e) indicates that the stretch of five leucines contributes to the intensity between 80 and 90 ppm in the spectrum obtained following deuterium exchange. This was confirmed in the two-dimensional NMR spectrum

(Fig. 3f), which showed at least five leucine signals in the same region. A simulated PISA wheel of an ideal α -helix with a 10° tilt is superimposed on the spectrum, and appears to fit well to the experimental data. Notably, the resonance intensity in this region is also present in the spectrum of the full-length protein, suggesting that the helical segment has a similar tilt angle in both the full-length and truncated constructs.

Initial drug binding studies were performed with full-length p7 in DHPC micelles by comparing solution NMR spectra. Upon the addition of a 20:1 molar excess of amantadine several of the protein resonances were selectively shifted in the two-dimensional $^1\text{H}/^{15}\text{N}$ HSQC

Fig. 3 One- and two-dimensional solid-state NMR spectra of p7TM2 in magnetically aligned 14-O-PC/6-O-PC bicelles. **A** and **B** Uniformly ^{15}N labeled in H_2O . **C** and **D** Uniformly ^{15}N labeled in 99% D_2O . **E** and **F** Selectively ^{15}N -Leu labeled in H_2O . The experiments were performed at 42°C at 700 MHz



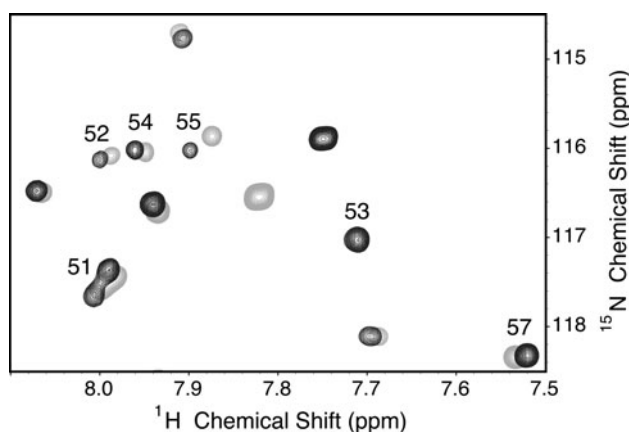


Fig. 4 Chemical shift perturbations in p7 as result of the addition of the channel blocker amantadine. The $^1\text{H}/^{15}\text{N}$ HSQC spectra were obtained without (*grey*) and with (*black*) the addition of 10 mM amantadine

spectrum (Fig. 4). In particular, the resonances assigned to leucines 51 through 57, with the exception of 53, were shifted by addition of the compound, indicating that residues in the second helix are affected. These are the same residues that are resistant to hydrogen/deuterium exchange. The shifts are not limited to the second transmembrane helix, with some observed for resonances associated with the N-terminal portion of the protein.

Discussion

The expression and purification of both full-length p7 and the truncated p7TM2 construct in milligram quantities from bacteria enabled the preparation of isotopically labeled samples suitable for both solution NMR and solid-state NMR experiments. This allowed the protein to be examined in micelle and bilayer environments. The limited dispersion of resonances in the solution NMR spectra is characteristic of a helical membrane protein (Fig. 2). The alignment of the constructs in 14-O-PC/6-O-PC bicelles (Fig. 1) for solid-state NMR indicates that the reconstituted protein, which was overexpressed into the insoluble inclusion bodies, is properly folded and well behaved in these lipid environments. Both types of spectra indicate that the p7TM2 construct, which contains not only the interhelical loop region, but also the complete C terminus of the full-length protein, resembles the native full-length protein in micelles and bilayers. Comparative studies of various combinations of full-length and truncated constructs should be helpful in sorting out the functional and structural properties of the protein.

Solid-state NMR is well suited for studying membrane proteins in phospholipid bilayers (Opella and Marassi

2004). Magnetically aligned bicelle samples provide a unique opportunity to determine the three-dimensional structure of proteins such as viroporins in their native environments. The orientational dependence of the resonance frequencies are especially informative about the structural details of the residues in the transmembrane helices, which are responsible for their ion channel activity and, as shown here, for binding to channel-blocking drugs. The stretch of six leucine residues in the second transmembrane helix has been helpful in determining the tilt angle of this segment of helical residues. By labeling only the leucine residues, the number of peaks in the spectrum was reduced, making it possible to resolve and partially assign their resonances. Their frequencies indicate that the tilt of the helix within the bilayer is approximately 10° . The use of other selective labels will help determine the length of the helix and the orientation of residues prior to the proline 49 at the start of the leucine-rich region of the protein.

A number of compounds have been shown to affect the channel activity of p7. Amantadine, one of the drugs shown to block the ion channels, perturbs the chemical shifts of a number of residues in the full-length protein in micelles. Of the affected residues, five are leucines that the NMR experiments show to be in a transmembrane helix. In a recent report, with these leucine residues replaced by alanine residues, L(50–55)A, p7 exhibited greater resistance to the effects of amantadine (StGelais et al. 2009). Although preliminary, the results of mutagenesis and NMR spectroscopy are consistent in suggesting the involvement of the leucine-rich region of p7 in drug interactions. There is also evidence of involvement of residues in the first transmembrane helix, which includes a histidine residue that Patargias et al. (2006) have predicted on the basis of modeling to interact with amantadine. With more thorough drug binding studies it may be possible to identify the functional groups on both the drug molecules and the protein responsible for binding. Combined with determining the three-dimensional structure of the protein in its native bilayer environment, this should provide a platform for the development of drugs targeted to p7.

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