Expression and Initial Structural Insights from Solid-State NMR of the M2 Proton Channel from Influenza A Virus†

Changlin Tian,‡ Kurt Tobler,§ Robert A. Lamb,|| Lawrence H. Pinto,⊥ and T. A. Cross*,#

National High Magnetic Field Lab, Institute of Molecular Biophysics, and Department of Chemistry and Biochemistry, Florida State University, Tallahassee, Florida 32310, and Department of Biochemistry, Molecular Biology, and Cell Biology, Department of Neurobiology and Physiology, and Howard Hughes Medical Institute, Northwestern University, 2153 North Campus Drive, Evanston, Illinois 60208-3500

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ABSTRACT: The M2 protein from influenza A virus has been expressed, purified, and reconstituted into DMPC/DMPG liposomes. SDS−PAGE analysis of reconstituted M2 protein in DMPC/DMPG liposomes demonstrates a stable tetrameric preparation. Circular dichroism spectra of the intact M2 protein in detergent indicate 67% α-helix. The uniformly ^{15}N-labeled M2 protein and both ^{15}N-Val- and ^{15}N-Leu-labeled M2 protein have been expressed from defined M9 media. The ^{1}H-^{15}N HSQC (heteronuclear single quantum correlation) solution NMR experiments have been performed on the amino acid specific labeled protein in 300 mM SDS-d_{25} micelles, and the data indicate a homogeneous preparation. The reconstituted M2/DMPC/DMPG proteoliposomes were used for preparing uniformly aligned solid-state NMR samples for ^{15}N-^{1}H dipolar/^{15}N chemical shift correlation experiments. The spectra support a transmembrane helix in M2 protein having a tilt angle of approximate 25°, quantitatively similar to results obtained on the isolated M2 transmembrane peptide reconstituted in DMPC bilayers (38°). In addition, the spectra suggest that the tetrameric protein forms a symmetric or at least pseudosymmetric bundle consistent with data obtained by other research groups based on electrophysiological measurements and substituted cysteine scanning mutagenesis experiments that characterize a tetrameric structure.

Solid-state NMR[1] spectra of isotopically labeled M2 protein from influenza A virus are shown along with preliminary structural characterizations of the protein backbone. Membrane proteins represent the majority of drug targets, and this proton-selective channel is already the target for the antiviral drugs amantadine and rimantadine (*1*, *2*). Yet, there is little beyond the primary sequence and partial topology that is known about the structure of M2 protein, which plays a critical role in the viral life cycle (*1*).

The M2 proton channel facilitates the uncoating of virions in endosomes and modulates the pH of the trans-Golgi network (*1*, *2*). Direct electrophysiological evidence that the M2 protein has ion channel activity has been obtained by expressing the M2 protein in oocytes of *Xenopus laevis* (*1*, *3*) or in mammalian cells (*4*, *5*). The M2 protein has 97 residues expressed at the plasma membrane of virus-infected cells, and the protein is oriented such that it has 24 N-terminal extracellular residues, a 19-residue transmembrane domain, and a 54-residue cytoplasmic tail (*1*, *2*). Analysis of ion channel properties of mixed oligomers, cysteine scanning mutagenesis, and chemical cross-linking experiments have shown that the active oligomeric state of M2 is a tetramer (*6*−*9*). The native state consists of either a pair of disulfide-linked dimers or a disulfide-linked tetramer. Disulfide bonds form through the two cysteine residues (Cys17, Cys19) located in the N-terminal extracellular domain (*8*).

The biologically active oligomeric state of many cellular ion channels spans the membrane many times [e.g., Ca^{2+} and Na^{+} channels have 24 transmembrane domains, K^{+} channels have 4 subunits each of 6 transmembrane domains, even the KcsA ion channel has 4 subunits each of 2 transmembrane domains (*10*, *11*)]. Compared to these channels, the homotetrameric M2 ion channel is smaller, representing an excellent opportunity for the development of structure−function correlations.

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* Corresponding author. Tel: (850)-644-0917. Fax: (850)-644-1366. E-mail: cross@magnet.fsu.edu.

‡ National High Magnetic Field Lab, Institute of Molecular Biophysics, Florida State University.

§ Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University.

|| Department of Biochemistry, Molecular Biology, and Cell Biology and Howard Hughes Medical Institute, Northwestern University.

⊥ Department of Neurobiology and Physiology, Northwestern University.

National High Magnetic Field Laboratory, Institute of Molecular Biophysics, and Department of Chemistry and Biochemistry, Florida State University.

[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; IPTG, isopropyl β-D-thiogalactopyranoside; OD, optical density; CD, circular dichroism; HSQC, heteronuclear single quantum correlation; NMR, nuclear magnetic resonance; CP, cross-polarization; PISEMA, polarization inversion spin exchange at the magic angle; OG, β-octyl-glucoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; DPC, dodecylphosphoatidylcholine; PISA wheel, polar index slant angle wheel.

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Membrane protein structural characterization is complicated by many features and consequences of the membrane environment. Here we have initiated this challenging task with solid-state NMR spectroscopy of the M2 protein reconstituted in aligned lipid bilayers of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG). The protein expressed in *E. coli* was uniformly ^{15}N-labeled and with ^{15}N-amino acid specifically labeled. ^{15}N-^{1}H dipolar/^{15}N chemical shift correlated spectra from these aligned samples using the PISEMA (polarization inversion spin exchange at the magic angle) experiment (*12*) have resulted in numerous well-resolved resonances and preliminary structural conclusions. Several efforts have demonstrated the application of PISEMA for structural characterization of other membrane proteins in hydrated lipid bilayers (*13*, *14*).

The analysis of orientational restraints derived from solid-state NMR spectra has been shown to define a unique high-resolution 3D structure (*15*−*19*). The complete structure of gramicidin A was described for the first time in a lipid bilayer environment by solid-state NMR (*15*, *16*). Recently, the backbone structure of the M2 transmembrane peptide, M2-TMP, derived from the M2 ion channel has been characterized and refined (*19*). In achieving such results, the anisotropic chemical shift and dipolar interactions were used as independent restraints. Recently, it was noticed by Cross and co-workers (*20*) as well as by Marassi and Opella (*21*) that the ^{15}N-^{1}H dipolar and ^{15}N chemical shift correlated restraints are much more informative. In fact, in the PISEMA spectra, correlated resonances give rise to images of α helical wheels with 3.6 resonances per turn. These images can be used without resonance assignments to define the tilt of the helix with respect to the bilayer normal.

EXPERIMENTAL PROCEDURES

Protein Expression. The expression plasmid pET37 was constructed based on plasmids pET22 and pET15, which were purchased from Novagen Inc. (Madison, WI). The cDNA for the M2 ion channel has been modified by attaching -(CAC) (CAC) (CAC) (CAC) (CAC) (CAC) UAG for a hex-His tag at the 3′ terminus. The Cys19 and Cys50 residues were mutated to serine, leaving Cys17 available for forming a disulfide bond, which has been shown to play an important role in stabilizing the M2 tetramer in detergent solution (*6*−*8*). This expression plasmid was transformed into *E. coli* (BL21DE3 pLysS). Cells from a single colony were used to inoculate 5 mL of LB media. All media contained 34 μg/mL chloramphenicol and 50 μg/mL carbenicillin. The cells were grown overnight at 37 °C, and each 0.5 mL culture was used to inoculate 50 mL of defined M9 media. The cells were grown at 37 °C until $OD_{600} = 0.8$, at which time IPTG (isopropyl β-D-thiogalactopyranoside) was added to a final concentration of 1 mM to induce expression of M2 protein. After 4 h, the cells were pelleted by centrifugation, and the cell pellets were frozen at −20 °C.

Analysis of Expression and Protein Purification from Inclusion Bodies. Frozen cells were thawed, and the cell pellets from a 1000 mL culture were resuspended in 80 mL of suspension buffer (20 mM Tris-HCl, 50 mM NaCl, pH 8.0), together with 500 μL of 1 mg/mL lysozyme. The aliquot suspension was sonicated using a probe sonicator for 1 min to shear the DNA. The lysed cells were centrifuged at 4 °C and 10000*g* for 20 min to pellet inclusion bodies and cell debris. The pellets were suspended in the suspension buffer without lysozyme, sonicated, and centrifuged again to wash the pellets. After 3 cycles of pelleting and resuspending, the final pellets were dissolved in 36 mL of suspension buffer containing 6 M urea and held on ice for 2 h. The suspension was centrifuged at 4 °C and 20000*g* for 20 min. The supernatant was passed through a 0.4 μm PTFE filter. Then 12 mL aliquots of the supernatant were applied to a NTA-Ni affinity column precharged using Ni_2SO_4. The 10 mL bed volume of this column was washed with 80 mL of the binding buffer (20 mM imidazole, 6 M urea, 50 mM Tris-HCl, pH 8.0, and 50 mM NaCl). The purified protein was collected during elution of the column with 30 mL of the elution buffer (300 mM imidazole, 6 M urea, 50 mM Tris-HCl, pH 8.0, and 50 mM NaCl).

^{15}N Uniform Labeling and ^{15}N-Val or -Leu Labeling. E. coli was cultured in defined M9 media with $^{15}NH_4Cl$ as the sole nitrogen source (*22*). Protein expression was induced with IPTG at an $OD_{600} = 0.8$ and purified as described above. For ^{15}N-amino acid specific labeling, *E. coli* was cultured in defined M9 media together with all amino acids except Leu or Val (*22*, *23*). For a 1 L culture, 40 mg of Ala, 40 mg of Glu, 40 mg of Gln, 40 mg of Arg, 25 mg of Asp, 10 mg of Asn, 5 mg of Cys, 10 mg of Gly, 10 mg of His, 10 mg of Ile, 10 mg of Lys, 25 mg of Met, 10 mg of Pro, 160 mg of Ser, 10 mg of Thr, 10 mg of Tyr, 5 mg of Phe, 10 mg of Val, 10 mg of Leu, and 5 mg of Trp were used. Upon induction, a 3-fold quantity of ^{15}N-labeled Val or Leu was added to the culture together with IPTG. The protein was purified as described above.

Detergent Solubilization. The eluted and filtered protein aliquot was dialyzed against Tris-HCl, pH 8.0, 1 mM SDS, and 1 mM DTT using dialysis bags with a 5 kDa molecular mass cutoff. The buffer was exchanged 6 times to remove urea and imidazole. The protein solution was concentrated by centrifugation using a 5 kDa cutoff filter (Fisher Scientific, Pittsburgh, PA) while keeping the SDS concentration at 1 mM. This concentrated protein aliquot was used to prepare solution NMR samples. Alternatively, a concentrated protein aliquot was diluted 40-fold with Tris-HCl, pH 8.0, buffer containing 5 mM octyl-glucoside (OG). This sample was used for CD analysis and for solid-state NMR sample preparation.

Micellar Solution NMR Sample Preparation. The concentrated protein aliquot was diluted 10-fold with 50 mM Na_2HPO_4/NaH_2PO_4, pH 6.6, and 1 mM SDS-d_{25}. The protein/SDS aliquot was run through a 0.40 μm filter to remove precipitates. The solution was put in a cryo-vacuum system to obtain a dry powder. Then 540 μL of H_2O and 60 μL of D_2O were added to dissolve the dry powder and to prepare a micellar sample with 300 mM SDS-d_{25} and 0.2 mM protein concentration for solution NMR experiments. A ^{1}H-^{15}N HSQC experiment (*24*, *25*) was run on the 720 MHz Unity Plus Varian spectrometer equipped with a triple resonance probe, and data were processed using the Varian 6.1 software package.

CD Analysis of M2 Protein. After dissolving the protein in 5 mM OG, 200 mM OG solution was added to achieve a final concentration of 30 mM, and the solution was passed through a 0.40 μm filter to remove any precipitates. CD spectra were collected from purified M2 protein in 10 mM

Tris-HCl, pH 8.0, buffer and in 30 mM OG, 10 mM Tris-HCl, pH 8.0, buffer. A quartz cuvette with a 1 mm path length was used in an AVIV CD instrument model 202. Three scans were collected from 260 to 195 nm with a step size of 0.2 nm, at a rate of 20 nm/min, and a bandwidth of 1.0 nm. The mean residue molar ellipticity was calculated from the averaged CD spectra using $[\Theta]_{mrm} = \Theta \times MW/10 \times c \times l$, where l is the path length in centimeters, c is the protein concentration in milligrams per milliliter, and MW is the molecular weight of the protein calculated from the primary sequence: 11 976.4. The CD data were deconvoluted to analyze the secondary structure information based on the protein concentration (*26*−*28*) determined using a BSA assay according to the manufacturer's protocol (Pierce Chemical Co., Rockford, IL).

Detergent-Mediated Reconstitution of M2 Protein into DMPC/DMPG Liposomes. A 200 mM OG stock solution was added to the M2 protein solution to achieve a 30 mM OG final concentration analogous to protocols developed for other membrane proteins (*29*−*32*). Brief bath sonication generated a homogeneous preparation. DMPC/DMPG liposomes were prepared with a molar ratio of 4:1. The preformed liposomes were added to the M2 protein solution in 30 mM OG, achieving a lipid:protein molar ratio of 200:1. The solution was transferred into a dialysis bag with a 5 kDa cutoff and dialyzed against 50 mM Na_2HPO_4/NaH_2PO_4, pH 8.0, buffer with three cycles of buffer changes, and then dialyzed against 10 mM Na_2HPO_4/NaH_2PO_4, pH 6.6, with two more buffer changes. Proteoliposomes were pelleted by ultracentrifugation from the dialyzed solution (196000*g*, 90 min) in a 70 Ti rotor (Beckman Instrument, Fullerton, CA). The pellet was used for aligned sample preparation by suspending the 50 mg pellet in 1 mL of HPLC-grade H_2O.

Electrophoretic Analysis of Reconstituted M2 Protein in DMPC/DMPG. A three-layer Tricine-based polyacrylamide gel has been used for electrophoretic analysis of the reconstituted M2 protein in DMPC/DMPG liposomes (*33*). Long slab gels with dimensions of 27 cm × 22 cm have been prepared with 16.5% *T* and 6% *C* for the separating gel, 10% *T* and 3% *C* for the spacer gel, and 4% *T* and 3% *C* for the stacking gel, in which *T* denotes the total percent concentration of monomeric acrylamide and bisacrylamide and *C* denotes the percent concentration of the bisacrylamide relative to the total concentration *T* (*33*). The reconstituted M2 protein in liposomes has been dissolved in the loading buffer [1% SDS, 20% glycerol (w/v), 50 mM Tris-HCl, pH 6.8] in the presence or absence of 1 mM DTT, the reductive reagent. The samples have been boiled for 0, 5, and 10 min, respectively, before loading on the gel (*34*). Protein bands have been stained using Coomassie Brilliant Blue R-250.

Aligned Sample Preparation. The suspended proteoliposomes of M2 protein were briefly sonicated in a water bath, fast-frozen with liquid nitrogen, and then thawed at room temperature. After 3 cycles of bath sonication, freezing, and thawing at room temperature, the proteoliposome samples were dispersed onto clean glass slides (approximately 2.0 mg of sample dry weight per slide with dimensions of 5.7 × 12 mm). The hydrated sample on 50 glass slides was placed in a clean hood and air-dried for 2 h, followed by vaccum-drying for at least 24 h. HPLC-grade H_2O was added to each slide at a weight ratio of 1:1 (2 μL of H_2O/slide). The slides were immediately stacked into a square glass tube (6 × 6 × 15 mm inner dimensions), and the tube was sealed. Following incubation at 43 °C for 24 h, a sample with approximately 50% hydration was achieved. The samples were ready for ^{31}P and ^{15}N solid-state NMR experiments.

^{31}P Solid-State NMR Experiments. A home-built ^1H-^{31}P double resonance static probe with an 8 mm cylindrical coil was used for ^{31}P experiments. Proton decoupled ^{31}P single pulse experiments were run on a 300 MHz wide bore Bruker DMX spectrometer.

^{15}N PISEMA Solid-State NMR Experiments. A home-built ^1H-^{15}N double resonance static probe with an 8 × 15 mm cylindrical coil was used for ^{15}N experiments. All of these experiments were run on a 400 MHz wide bore spectrometer with a home-built console around a Chemagnetics data acquisition system. A cross-polarization (CP) Hahn spin−echo pulse sequence was used for ^{15}N chemical shift spectra and also used for power match condition calibration. ^{15}N chemical shifts are referenced to $^{15}NH_4NO_3$ at 0 ppm. PISEMA (polarization inversion spin exchange at the magic angle) pulse sequence was used for ^{15}N-^1H dipolar coupling/^{15}N chemical shift 2D correlation experiments (*12*). The PISEMA experiments were set up with a 5.5 μs ^1H 90° pulse width and 60 kHz ^1H decoupling power, and the 2D data were acquired with 4000 accumulated FIDs using 256 complex data points in the t2 time domain and 18 real points in the t1 time domain. Data were processed with 100 Hz line broadening in the first dimension and a 90° sinebell window function in the second dimension using the spinsight software package and lab-developed MATLAB scripts.

PISA Wheel Simulation and Tilt Angle Analysis The magnetic field axis B_0 direction was set parallel to the lipid bilayer normal. Averaged values from experimental data for the chemical shift tensors ($\sigma_{11} = 31.3$ ppm, $\sigma_{22} = 55.2$ ppm, $\sigma_{33} = 201.8$ ppm) were used, as well as a value for the dipolar coupling of 10.375 kHz. A typical angle between the σ_{33} tensor element and $\nu_{||}$ of the dipolar tensor of 17° was used as confirmed (*20*). PISA (polar index slant angles) wheels of a protein helix were calculated by rotating the helix about its axis while calculating the anisotropic dipolar and chemical shift observables (*20*). A series of simulated PISA wheels for different tilt angles of the helix were overlaid with the resonances in the PISEMA spectrum of ^{15}N uniformly labeled M2 protein to determine the tilt angle of the transmembrane helix of the M2 protein with respect to the lipid bilayer normal.

RESULTS AND DISCUSSION

The M2 protein was expressed well in *E. coli* BL21(DE3)-pLysS in the form of inclusion bodies, and the yield of the purified membrane protein was up to 10 mg/L from the M9 culture. After the M2 protein was expressed and purified from *E. coli*, the SDS−PAGE gel of the reconstituted M2 protein in DMPC/DMPG liposomes has been obtained (Figure 1). In the absence of the reductive reagent DTT, M2 protein in liposomes forms tetramers at room temperature, while most of the M2 protein dissociates to dimers after 5 or 10 min of heating in boiling water before being loaded onto the SDS−PAGE gel. In the presence of the reductive reagent DTT, most of the M2 proteins in liposomes form monomers, which is consistent with the importance of the disulfide bond in stabilizing the functional tetrameric M2

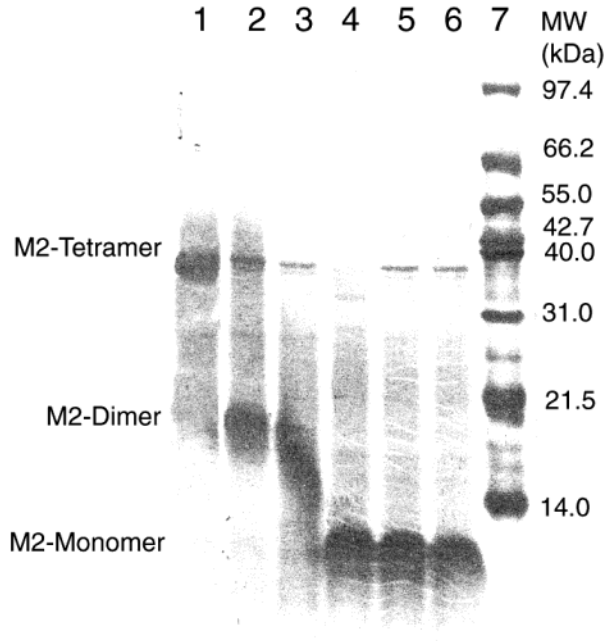

FIGURE 1: SDS−PAGE of reconstituted M2 protein in DMPC/DMPG liposomes. Lane 1, in the absence of reductive reagent, DTT, at room temperature; lane 2, in the absence of DTT, 5 min boiling before loading sample onto the gel; lane 3, in the absence of DTT, 10 min boiling; lane 4, in the presence of DTT, at room temperature; lane 5, in the presence of DTT, 5 min boiling; lane 6, in the presence of DTT, 10 min boiling; lane 7, molecular mass markers.

proton channels (*6*, *8*, *35*). Since the monomeric M2 protein migrates as expected for the 12 kDa polypeptide, these observations are most naturally explained if the bands represent denatured, monomeric protein. The dimeric M2 protein and tetrameric M2 proteins in liposomes migrate as 20 and 39 kDa, respectively, according to the molecular mass markers running in the same SDS−PAGE gel. The oligomeric complexes run somewhat below the expected molecular mass, as is sometimes observed with oligomeric membrane proteins on glycine-based gels (*34*, *36*). The sharp band of tetrameric M2 protein suggests that the complex has a well-defined stoichiometry since nonspecific aggregation of polypeptides often results in a ladder or smear of bands on SDS−PAGE. Extensive boiling irreversibly disrupts the tetrameric association as shown in lanes 2 and 3 of Figure 1, resulting in a smeared band of unstable dimeric M2 protein as shown in lane 3 of Figure 1.

The protein is soluble in Tris-HCl, pH 8.0, buffer at approximately 1 mg/mL, but between pH 7.0 and 4.5, the solubility of the intact M2 protein in aqueous buffer is negligible. The solubility was monitored using scanning UV spectra between 340 and 240 nm. Considerable light scattering was observed when the buffer was below pH 7.0.

In Figure 2 the CD spectra of intact M2 protein in 10 mM Tris-HCl, pH 8.0, with and without 30 mM OG are shown. The analysis of the CD spectra shows that the M2 protein in aqueous solution at pH 8.0 (used in sample preparation protocol) is approximately 40% α-helix and 28% random coil. When the protein is dissolved in OG solution, there is approximately 66% α-helix, and the random coil percentage is reduced to 15%. Hence, the protein has substantially more α-helix in the presence of detergent. Also, in the presence of 100 mM SDS, a much less mild detergent, the M2 protein

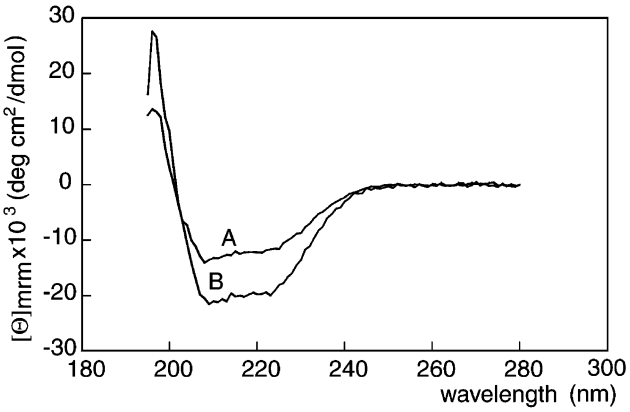

FIGURE 2: Three CD spectra were collected from solutions with or without M2 protein. The spectra were averaged, and the spectrum without M2 protein present was subtracted from the spectrum with M2 protein. Spectra in 10 mM Tris-HCl, pH 8.0, aqueous buffer exhibit approximately 40% α-helix secondary structure while in 30 mM OG/Tris-HCl, pH 8.0, the spectra exhibit around 67% α-helix secondary structure. Minima in the spectra at 208 and 221 nm are indicative of a high α-helical content.

adopts a similar CD spectrum to that in OG and similar secondary structure distribution (data not shown). Duff and co-workers have investigated the secondary structure of the transmembrane region of M2 protein in DMPC vesicles showing that the region is predominantly α-helix (*37*). Here, M2 protein in DMPC/DMPG vesicles also shows a high percentage of α-helix based on the CD spectrum, but considerable light scattering compromises the quantitative analysis of the secondary structure distribution. The molar ratio of M2 protein to lipid (DMPC:DMPG 4:1 molar ratio) has been optimized at 1:200, equivalent to a 1:11 ratio by weight. The optimization was achieved by maximizing the 221 nm CD observation indicative of α-helix.

Solution NMR HSQC (^{1}H-^{15}N correlation) spectra were used to characterize the homogeneity of the expressed M2 protein structure. Figure 3 shows the spectrum of ^{15}N-Leu-labeled M2 protein/SDS micelles using 300 mM SDS-d_{25} in buffer of 50 mM Na_2HPO_4/NaH_2PO_4, pH 6.6. Resonances from 8 of the 10 Leu residues can be clearly observed. Variable intensity in the spectral peaks appears to be correlated with the putative number of buried residues in the micelles. Six leucine residues are in the transmembrane domain, two in each of the terminal segments. Surface-exposed residues in non-α-helical domains or at the aqueous membrane interface may display unfavorable dynamics and exchange kinetics, leading to reduced intensity (*38*). Results for valine (data not shown) are similar. Both preparations illustrate that a homogeneous preparation of protein has been obtained, since multiple resonances are not observed for each residue. Therefore, it can be concluded that the sample is pure and uniformly folded, at least in the transmembrane domain. Furthermore, it is clear that there has been no scrambling of the amino acid labels.

The ^{31}P NMR spectrum of an oriented sample of M2 protein in DMPC/DMPG (4:1 molar ratio) with a 1:200 protein:lipid molar ratio is shown in Figure 4. The ^{31}P resonance at 27 ppm (relative to $\delta_{iso} = 0$ ppm) represents alignment of the lipid bilayers with the bilayer normal parallel to the B_0 axis of the magnet, as required for static solid-state NMR experiments. The lack of a substantial signal at −13 ppm demonstrates minimal unoriented material

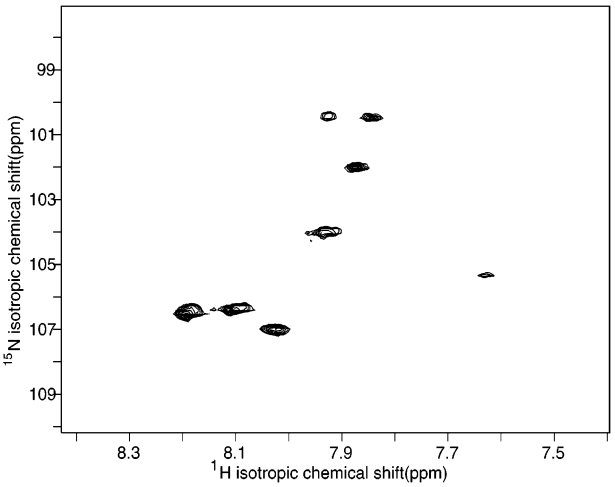

FIGURE 3: ^{15}N-^{1}H HSQC solution NMR spectrum of approximately 0.2 mM ^{15}N-Leu-labeled M2 protein in 300 mM SDS, 50 mM Na_2HPO_4/NaH_2PO_4, pH 6.6, buffer. The spectrum was acquired in a 720 MHz spectrometer equipped with a standard triple resonance probe using 2048 × 512 sample points at 15 °C. Data were processed using 2 Hz line broadening in both the t2 and t1 dimensions with zero-filling to 4096 × 512 data points. The ^{15}N-^{1}H correlation spectrum shows 8 high-intensity resonances indicating a homogeneous structure for M2 protein in an SDS micellar environment.

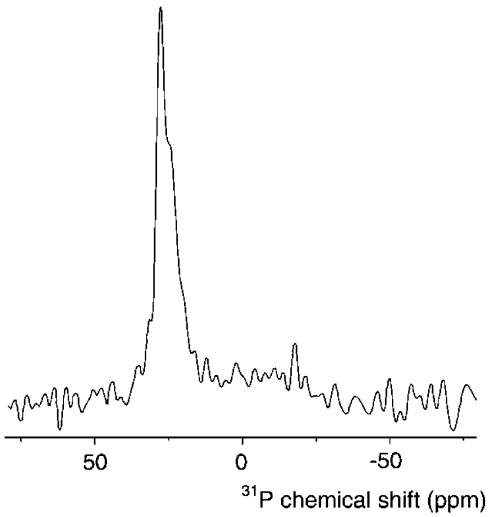

FIGURE 4: Proton-decoupled ^{31}P chemical shift spectrum of M2 protein in aligned DMPC/DMPG lipid bilayers. 32 transients were averaged to obtain the spectrum at room temperature in a wide bore 300 MHz spectrometer with 50 kHz proton decoupling. Data were processed with 10 Hz of line broadening.

in the samples. Second, the ^{31}P resonance is only slightly asymmetric. Asymmetry would suggest defect structures in the sample leading to a mosaic spread of orientations (*39*). Alternatively, the mixture of the lipid may contribute to the asymmetry, but here the small asymmetry is most likely due to a small mosaicity.

Shown in Figure 5 are ^{15}N solid-state NMR spectra of M2 protein incorporated into these aligned DMPC/DMPG membranes. The spectrum of uniformly ^{15}N-labeled M2 protein is shown in Figure 5A, while the ^{15}N-Val-labeled sample is shown in Figure 5B. The 170 ppm range of chemical shifts in the spectrum of uniformly ^{15}N-labeled protein indicates that large-amplitude motions, if they exist, are restricted to motions about the bilayer normal, for much of the protein.

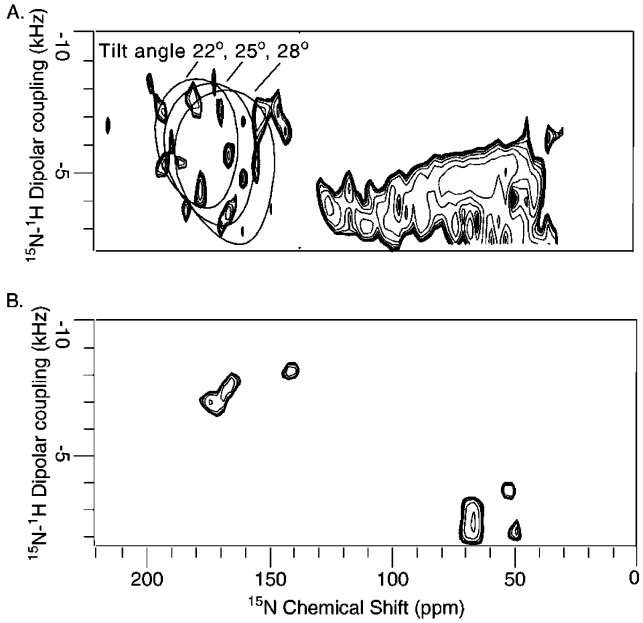

FIGURE 5: PISEMA spectra of uniformly aligned intact M2 protein in DMPC/DMPG lipid bilayers at 30 °C. (A) Uniformly ^{15}N-labeled M2 protein. Approximately two-thirds of the ^{15}N sites are from backbone amides. (B) ^{15}N-Val specifically labeled M2 protein. Valines at positions 7, 27, 28, 68, 84, and 92 have been labeled. Regions between ±2 kHz were deleted because of spectral artifacts.

Based on the 97 amino acid residues of M2 protein together with 6 histidine residues in the tag, the uniformly ^{15}N-labeled solid-state NMR spectrum was expected to show 98 backbone amides, 4 prolyl imides (nonprotonated, therefore, 0 kHz dipolar splitting), and a mobile amino terminus. In addition, 48 ^{15}N sites occur in the side chains including 7 Arg, 2 Asn, 2 Gln, 3 Lys, 2 Trp, and 9 His. The three Lys and amino terminus primary amines will cross-polarize poorly. From the CD spectrum of the M2 protein in detergent OG solution, approximately 66% of the M2 protein sequence is α-helical while 15% is indicated to be random coil. This estimate may be an underestimate for the α-helical content in planar lipid bilayers, but much more structural data are needed before final conclusions can be made. Some highly dynamic regions (e.g., loops and terminal regions) may polarize weakly in the standard cross-polarization procedure and therefore may not be observed. Residues in structured random coil regions (e.g., bends between two α-helical regions) will give rise to resonances dispersed throughout the spectrum rather than being in a localized region of the spectrum. Considerable resolution is observed in the region around 170 ppm where the resonances from the transmembrane region are anticipated. Indeed, this region is simulated well with PISA wheels, suggesting a helix tilt with respect to the bilayer normal of 25° ± 3° (Figure 5A). This result strongly suggests the existence of a transmembrane helix, and similar to the study of the transmembrane peptide of M2 protein (M2-TMP), the helix has a very substantial tilt with respect to the bilayer normal. However, quantitatively, the tilt value is different from the 38° value of the refined M2-TMP structure in DMPC bilayers at a peptide to lipid molar ratio of 1:8 (*40*). Both M2-TMP and the intact M2 protein have been shown to conduct protons (*41*, *42*), but the structural data presented here and previously for M2-TMP are at pH 7.0 or above where these structures are in the closed state. Consequently, no conclusions about the

conducting state can, at this time, be made. Furthermore, the influence of different lipid environments is not clear. Previously, we have published that the helix tilt of M2-TMP was an intrinsic property of the peptide based on studies using different fatty acid chain lengths (*43*). However, we did not assess the influence of the lipid headgroups in these studies. Therefore, it may be that the different tilt angles are the result of different lipid headgroup mixtures for M2-TMP and the intact protein.

The large intensity in the spectral region around 80 ppm is likely to reflect considerable interfacial α-helix, based on the rationale above. However, the lack of resolution in this region demands other approaches to resolve the resonances. The amino acid ^{15}N specifically labeled spectrum shows excellent resolution (Figure 5B). The M2 protein has six valines at sites 7, 27, 28, 68, 84, and 92. Two of them, 27 and 28, are located in the transmembrane region, one in the N-terminus of the protein and three in the C-terminus of the protein. While six resonances can be identified in the PISEMA spectrum as anticipated, variable intensity and line width in the resonances may again result from differences in the dynamics along the peptide backbone. The resolution of the single amides in the two-dimensional PISEMA spectrum confirms the ^{31}P spectral results that the protein not just the lipid (i.e., ^{31}P results) is uniformly aligned with respect to the magnetic field axis. The resonance counting also confirms that there has been little, if any, scrambling of the ^{15}N labels. The overlap of the resonances in the ^{15}N-Val and ^{15}N uniformly labeled samples is well within a line width in these spectra with modest signal-to-noise.

The native M2 protein is known to be a tetramer, and ultracentrifuge experiments of the chemically synthesized full-length M2 protein reconstituted into dodecylphosphatidylcholine (DPC) micelles (*44*) and SDS−PAGE analysis of the reconstituted M2 protein in DMPC/DMPG liposomes (Figure 1) were also found to prove the existence of tetrameric M2 protein. Additional PISEMA experiments using proton/deuterium exchange of the M2 protein indicate the presence of an aqueous pore in our samples of M2 reconstituted into DMPC/DMPG liposomes (Tian and Cross, unpublished results). These results are all suggestive of a tetramer being present in our samples. However, from resonance counting in Figure 5B, it is clear that the tetrameric structure present in the solid-state NMR sample is symmetric with each monomer having the same orientation with respect to the bilayer normal.

In conclusion, M2 protein has been overexpressed in defined media for both uniform and amino acid specific labeling. High purity of the protein has been achieved, and it has been reconstituted into both detergent and lipid bilayer environments. SDS−PAGE analysis of the reconstituted M2 protein in DMPC/DMPG liposomes demonstrates the stable tetrameric stoichiometry of the preparation at room temperature. Solid-state NMR spectra demonstrate that homogeneous and well-aligned samples are prepared. Analysis of these first spectra of M2 protein is consistent with a transmembrane helix tilted by 25° with respect to the bilayer normal and with helices on the bilayer surface. The oligomeric structure is also shown to be symmetric or at least pseudo-symmetric. These data clearly show the potential for biosynthetic labeling and solid-state NMR of uniformly aligned samples to resolve the resonances from a 100 amino acid residue protein in a hydrated liquid-crystalline lipid bilayer environment. As with smaller peptides, prospects for resonance assignments and 3D structural characterization are very high.

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