Structural Influences: Cholesterol, Drug, and Proton Binding to Full-Length Influenza A M2 Protein

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ABSTRACT The structure and functions of the M2 protein from Influenza A are sensitive to pH, cholesterol, and the antiinfluenza drug Amantadine. This is a tetrameric membrane protein of 97 amino-acid residues that has multiple functions, among them as a proton-selective channel and facilitator of viral budding, replacing the need for the ESCRT proteins that other viruses utilize. Here, various amino-acid-specific-labeled samples of the full-length protein were prepared and mixed, so that only interresidue $^{13}$C-$^{13}$C cross peaks between two differently labeled proteins representing interhelical interactions are observed. This channel is activated at slightly acidic pH values in the endosome when the His$^{37}$ residues in the middle of the transmembrane domain take on a $+2$ or $+3$ charged state. Changes observed here in interhelical distances in the N-terminus can be accounted for by modest structural changes, and no significant changes in structure were detected in the C-terminal portion of the channel upon activation of the channel. Amantadine, which blocks proton conductance by binding in the aqueous pore near the N-terminus, however, significantly modifies the tetrameric structure on the opposite side of the membrane. The interactions between the juxtamembrane amphipathic helix of one monomer and its neighboring monomer observed in the absence of drug are disrupted in its presence. However, the addition of cholesterol prevents this structural disruption. In fact, strong interactions are observed between cholesterol and residues in the amphipathic helix, accounting for cholesterol binding adjacent to a native palmitoylation site and near to an interhelix crevice that is typical of cholesterol binding sites. The resultant stabilization of the amphipathic helix deep in the bilayer interface facilitates the bilayer curvature that is essential for viral budding.

INTRODUCTION

Since the first membrane protein and peptide structures obtained in the presence of lipids (1,2), the influence of the membrane environment on protein structure has been the subject of considerable interest (3,4). Well before membrane protein structures were characterized, Anfinsen (5) made it clear that protein structure was the result of the totality of interactions including those within the protein and between the protein and its environment. Structures of M2 protein constructs from Influenza A that have been characterized in detergent crystal lattices, detergent micelles, proteoliposomes composed of liquid crystalline lipid bilayers, and raftlike lipid compositions have extended our understanding of the environmental influences on membrane protein structure (6–14). Here, we present structural restraints obtained from the full-length M2 (M2FL) proton channel in lipid bilayers describing the structural influence of cholesterol, protons, and drug binding on the helices of the M2 conductance domain (M2CD) using solid-state nuclear magnetic resonance (NMR) spectroscopy.

While details of the M2 structure are still debated, especially those associated with the conductance mechanism (15–18), the oligomeric structure is well documented as a left-handed tetramer, with some dimer-of-dimer character from four monomers, each with a single transmembrane (TM) helix followed by an amphipathic helix in a juxtamembrane position in the viral interior. The structure of the N- and C-termini of the protein are largely unknown, except for strong evidence for $\beta$-sheet content in the first 20 residues (19) and recent evidence that a few residues, and maybe many more in the C-terminus, are disordered (20).

Solid-state NMR (ssNMR) is the primary technique for characterizing membrane protein structure in natively bilayer environments at atomic resolution. Recent ssNMR structures include the structure of phospholamban (21) and its phosphorylated pentameric form (22); a mercury transporter, MerF (23); a chemokine receptor, CXCRI (24); the Mycobacterium cell division protein, CrgA (25); sensory rhodopsin (26); and the trimeric autotransporter,
YadA (27). Many more publications provide detailed structural results on other membrane proteins. Such studies are still compromised by the simplified models of the very complex native membrane environment. In important efforts to address this challenge with the M2 protein, Hu et al. (28) and Cady et al. (29) have modeled the viral lipid environment using sphingomyelin, DPPC, DPPE, and cholesterol in a molar ratio of 28:21:1:30 (known as VM) or sphingomyelin, POPC, POPE, and cholesterol in a molar ratio 25.6:25.6:25.6:23 (known as VM+). However, M2CD does not bind amantadine (AMT, an antiviral drug) in either of these environments, suggesting that these environments do not represent native-like M2 environments (29).

From functional assays conducted by Schroeder et al. (30), AMT inhibits proton flux through M2FL in liquid crystalline lipid bilayers containing 30% cholesterol, so it does not appear to be the high concentration of cholesterol that is responsible for the lack of AMT binding in Hong’s studies (30). Recently, it has become clear that M2 prefers an environment on the edge of the raftlike domains where the pyramidal-shaped M2CD conformation induced by its amphiphilic helix interacting with the lipid bilayer facilitates viral budding (30–32). Indeed, a major strength of the solid-state NMR technology for membrane protein structures is the flexibility for preparing samples in a wide variety of pH and ionic strength conditions and lipid compositions.

Potentially facilitating the location of the M2 protein on the periphery of raftlike domains are the relatively short and significantly tilted TM helices that will be better accommodated in the lipid bilayers surrounding the raftlike domains (30). While it has been suggested that M2 binds cholesterol, many of these claims are based on cholesterol-recognition amino acid-consensus sequence (CRAC) motifs occurring in the amphiphilic helix of numerous influenza strains (33). However, it is unlikely that these sequences are predictive of cholesterol binding, because these motifs were originally characterized for TM helices. Furthermore, in the influenza A/Udorn 307/72 H3N2 strain (the M2 sequence studied here), the amino-acid sequence does not meet all of the requirements for a CRAC domain. However, the M2 amphiphilic helix has a Cys residue (mutated in our constructs to Ser) that is natively palmitoylated, a feature that is often associated with cholesterol binding (34).

In this article, we demonstrate the application of mixed asymmetrically isotopically labeled samples (18,35), in which two different 13C labeled preparations of M2FL are mixed in a 1:1 molar ratio to selectively observe cross peaks between monomers in this tetrameric structure. For this, 13C-13C dipolar-assisted rotational resonance (DARR) experiments (36) are performed to observe local interactions between helices. Consequently, no such cross peaks will arise from within a monomer, and the cross peaks will only arise when the different labeled monomers are adjacent to each other in the tetrameric structure. The 1:30 molar ratio of protein monomer/lipid represents a 1:120 tetramer/lipid, which is more than adequate to suppress any tetramer-to-tetramer cross peaks, because there is no propensity for the tetramers to form a higher-order oligomeric structure. However, the dilute protein and dilute pairs of labels leads to limited sensitivity for these experiments.

**MATERIALS AND METHODS**

Isotopically labeled M2FL protein was expressed with N-terminal histidine-6 tag from the BL21 RP codon plus *Escherichia coli*. Initial cultures were grown in rich media. Two liters of cells were pelleted and washed with 50 mL of M9 minimal media before growing 1 L of M9 culture supplemented with a 100 mg of an isotopically labeled amino acid (Cambridge Isotope Labs, Tewksbury, MA; Sigma-Aldrich, Miamisburg, OH). Purification was carried out using Empigen-BB solubilized M2FL after cell disruption with a French press. After high-g centrifugation the soluble fraction was loaded onto a nickel affinity column and purified M2FL was eluted from the column at pH 8.0 with 0.5% wt/vol octylglucoside detergent in Tris buffer. This purified protein was then treated with 5 mM diithiothreitol for obtaining the protein in monomeric form. Additional details are provided in the Supporting Material.

A 4:1 molar ratio of DMPC and DMPG lipids was used for reconstitution of the protein in lipid bilayers, and when cholesterol was added a molar ratio of 1:4 (cholesterol/other lipids or ~20% cholesterol lipid fraction) was used. For reconstitution of M2FL into these lipid environments, the lipid mixture was made 3% (wt/vol) with the octylglucoside followed by the addition of a 1:1 molar ratio of two different amino acid-labeled M2FL preparations to yield a protein/lipid of 1:30. The detergent was removed by dialysis and confirmed by HPLC using evaporative light scattering detection (37,38). The final buffer was either 20 mM HEPES buffer for pH 7.5 or 20 mM citrate buffer for pH 5.0. For AMT-binding experiments, the drug was added after the removal of detergents to the resulting proteoliposome suspension at a 1:2 molar ratio of channel/drug. Labeled cholesterol was obtained from Sigma-Aldrich (Miamisburg, OH).

Proteoliposomes containing M2 were packed into 3.2 mm-thin walled magic-angled spinning rotors. Spectra were acquired on a Bruker Avance 600.1 MHz (14.1 T) NMR spectrometer (Bruker, Billerica, MA) at 262 K using an NHMFL Low-E triple resonance probe for minimal sample heating and optimized sensitivity (39,40). The observation of intermonomer structural restraints were obtained with DARR experiments (36) using mixing times of 300 or 500 ms. Different spinning rates (9–12.2 kHz) were used to avoid spinning side-band overlap with the potential cross peaks. All 13C chemical shifts were referenced externally, using 13C-labeled glycine sample, by setting the carbonyl carbon resonance to 178.4 ppm relative to TMS.

**RESULTS AND DISCUSSION**

The labeling strategy was based on the M2CD structure (PDB: 2L0U (10)) and the amino-acid sequence from the influenza A/Udorn 307/72 H3N2 strain of M2 (Fig. 1 A). Five different amino acid-specific 13C-labeled protein preparations were expressed and purified using uniformly 13C labeled Ala, Ile, Leu, Phe, and Val. The distributions of the labels are shown in Figs. 1 A and in S1. The two-dimensional magic-angle spinning ssNMR spectra are shown for each of these single amino acid-labeled samples illustrating that there is no observable scrambling of the labels. Four different mixed labeled samples were prepared by...
combining pairs of amino acid-labeled samples in a 50:50 mixture: Ala-Ile, Ile-Leu, Val-Leu, and Phe-Leu. The first three samples were prepared to search for unambiguous interhelical distance restraints between amino acids of different types in the TM helices and the last sample was prepared for a similar search between the amphipathic helices and the adjacent monomer.

In considering the cross-peak possibilities, we and others have found that the TM domain resonances are stronger than resonances from the terminal regions of the protein. Consequently, in general it can be anticipated that our observed cross peaks would be dominated by magnetization transfer within the conductance domain. In addition, the only ambiguities for Ala29 and Ala30 residues in the TM helix are two Ala residues in the C-terminus where there is a single Ile residue (Ile94). This Ile residue is directly involved in the virus’s mechanism to evade autophagy by binding the essential autophagy protein, LC3, to the F91xxI94 sequence—an interaction recognized as monomer-monomer protein interaction (41). Consequently, it is very unlikely that this site would be in close proximity to an adjacent monomer for an Ala-Ile interaction, and therefore any observed interactions between Ala and Ile residues are likely to be associated with packing of the TM helices. The Ile-Leu residues in the N-terminus are far apart in the monomer, and therefore they are likely to be far apart in the tetramer, where there is suspected β-structure (19). There is a pair of these residues in the C-terminus (Ile94; Leu96), but again it is likely that this sequence acts as a monomer to bind LC3 and therefore it is unlikely to form an Ile-Leu cross peak in the 13C correlation spectra. The Val-Leu pair presents a possible interaction in the N-terminus if intermonomer β-strand hydrogen bonding occurs, but even here the distance between Ca sites would be >>10 Å for a parallel sheet structure. In the C-terminus, the Val residue (Val28) is also in the LC3 interacting region and unlikely to have intermonomer interactions with Leu26 as described above. The fourth sample was Phe-Leu labeled, and the only possible intermonomer interactions between these residues are those involving the Phe residues of the amphipathic helix, because the only terminal region Phe residue is Phe91 in the LC3 binding site. Below we will only discuss the ambiguities that might arise within the conductance domain (residues 23–62) of the full-length protein.

**Proton binding**

In studies of the M2FL protein in lipid bilayer preparations the structural influences of proton, AMT, and cholesterol binding are assessed through the use 13C-13C distance restraints between monomers. At high pH (≥7.5) the four histidines (His37) responsible for proton transport are in their neutral state, and at low pH (5.0) the proton channel is activated by having the histidine tetrad charged with a mix of +2 and +3 states (42–44). The structural influence of pH activation has been assessed by observing each of these mixed labeled samples at pH 7.5 and 5.0 (Figs. 2 and S2) with a spectral slice through a well-resolved resonance of one of the two amino acid-labeled monomers. Based on the structure of the M2CD in lipid bilayers (PDB: 2L0J) at pH 7.5, the minimal Ca-Ca distance between Val and Leu residues is the Val28 and Leu26 distance of 8.1 Å (Fig. 3 A; Table 1). In the DARR spectrum at a 500 ms mixing time, a cross peak is not observed (Fig. 2 A, pH 7.5), while at pH 5.0 this cross peak is observed. For Ala Ca and Ile Cγ1, the minimal distance in PDB: 2L0J is 3.6 Å between residues Ala30 and Ile35, respectively. This cross peak is observed at pH 7.5, but not at pH 5.0 (Fig. 2 B). Based on the PDB: 2L0J structure, two Ile and Leu Ca interactions could contribute to observed cross peaks: an 8.1 Å distance between residues Ile32 and Leu26, and a 7.7 Å distance between residues Ile34 and Leu38. An Ile-Leu cross peak is observed from both pH 7.5 and 5.0 samples.

The specific labels and relatively long mixing times permit spin diffusion over relatively long distances (45) and therefore, with the possible exception of the ambiguous Ile-Leu restraints (Fig. 2 C), the pH 7.5 data is consistent with the PDB: 2L0J structure of M2CD obtained at the same pH. Despite the long distances in the PDB: 2L0J structure for the Ile32-Leu36 and Ile34-Leu38 Ca-Ca pairs (Fig. 3 A), the combination of a modest change in the full-length protein structure at the helix-helix interface and/or a slightly different tilt or rotation angle for the helix...
the distances by a couple of Ångstroms, accounting for the pH 7.5 results in Fig. 2 tabulated in Table 1. Recently, conformational heterogeneity in the neutral His37 tetrad has been observed, and it was suggested that it could have been induced by heterogeneity in helix packing (44). In other words, it is not expected that a single set of rotameric states represents the native structure at even the low temperatures used here.

Although no significant pH dependence in the N-terminal helical structure has been observed previously from truncated constructs of the M2 protein (46), here it is clear that a structural change does occur in the full-length protein based on specific interhelical distance differences upon channel activation. At pH 5.0, Val28-Leu26 are closer together, while Ala30-Ile35 are further apart and the ambiguous Leu-Ile results remain constant; however, compensating differences between these ambiguous pairs could occur. This structural perturbation could be the result of a small decrease in the helical tilt or small change in the rotational orientation of the helices.

For the amphipathic helix, cross peaks are observed at pH 7.5 between the Phe ring carbons and Leu C\text{g} sites (Fig. 2 D). The M2CD PDB: 2L0J structure is considered an excellent model for this portion of the protein, because the amphipathic helix has the tilt and rotation angle as previously characterized for the full-length protein at pH 8.0 (47). From the PDB: 2L0J structure, two possible interactions (Phe48-Leu59 and Leu46-Phe54) could contribute to these cross peaks (Fig. 3 B). Both of these are potential sources for the observed cross-peak intensity that represents interactions between the C-terminus of the amphipathic helix (Leu59 and Phe54) and the start of the amphipathic helix (Phe48) or the C-terminus of the TM helix (Leu46). In the PDB: 2L0J structure, Leu46 C\gamma-Phe54 C\delta distances range from 4.6 to 7.0 Å for three of the four residue pairs in this
tetrameric structure; and for the Leu46 Cγ-Phe54, Cγ distances range from 6.4 to 7.4 Å (Table 1). In part, these increased distances account for the weaker Phe Cγ cross-peak intensity in Fig. 2, D and E. For three of the Phe48 Cδe-Leu59 Cγ pairs, distances range from 5.2 to 6.7 Å and for the Phe48 Cγ-Leu59 Cγ distances range from 5.0 to 5.7 Å. In Fig. 2 D, cross peaks from Leu Cγ to Phe Cδe and Phe Cγ are observed at both pH 7.5 and 5.0. When the Leu-labeled L59V M2 mutant was mixed with Phe-labeled M2WT, a cross peak was still observed to Phe Cδe (Phe54) from Leu46 Cγ (Fig. 2 E), but at substantially reduced intensity, suggesting that both pairs (Phe48-Leu59 and Leu46-Phe54) contribute to the strong Phe-Leu wildtype (WT) cross peaks at pH 7.5 in Fig. 2 D. However, the Leu46 Cγ-Phe54 Cγ cross peak is not observed, in part due to the weaker transfer to the nonprotonated Phe Cγ and the longer Leu46 Cγ-Phe54 Cγ distance (Table 1). Hence, even this distance subtlety seems to be consistent with the PDB: 2L0J structure. Interestingly, at pH 5.0, intensity values are observed for the WT sequences that are similar to those seen at pH 7.5. This appears to be at odds with electron spin resonance (ESR) results that noted different water accessibility at low pH for spin labels on the amphipathic helix and hence a different structural arrangement at low pH (48).

### Cholesterol binding

When cholesterol is added to the lipid composition of the membrane at pH 7.5, the binding of AMT has no influence on the cross peaks between Leu and Phe (Figs. 5 and S4). This result suggests that cholesterol is stabilizing the structure of the amphipathic helix in the lipid interface, as it is represented in the PDB structure, 2L0J. When cholesterol is labeled with 13C2,3,4 and mixed with 13C Phe-labeled M2FL, strong cross peaks are observed in slices through the cholesterol C2 resonance frequency to the ring carbons of Phe, as shown in Fig. 6, A and B. No cross peaks were observed in spectral slices observed through the C25 resonance of cholesterol labeled with 13C25,26,27 mixed with 13C Phe-labeled M2FL. These results suggest a standard orientation for cholesterol in the lipid bilayer, including for DMPC lipids used in these experiments (51), with the

### AMT binding

The binding of AMT results in no significant differences in the cross-peak intensities compared to protein samples at the same pH without AMT in the N-terminus of the TM helix (Figs. 4, A–C, and S3). This is consistent with the solid-state NMR studies of the truncated samples of the M2 protein (7,11). The ESR studies using spin labels tethered to the N-terminus of the TM domain showed changes upon AMT binding that were interpreted as pore diameter restrictions, but a change in structure of the N-terminal residue could account for the spectral changes (49).

<table>
<thead>
<tr>
<th>Residues Involved</th>
<th>Distance Range in PDB: 2L0J (Å)</th>
<th>pH 7.5 Cross Peaks Observed</th>
<th>pH 7.5 Agreement with PDB: 2L0J Distances</th>
<th>pH 7.5 Cross Peaks Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu46-Cα-Val56-Cα</td>
<td>8.1–8.2</td>
<td>—</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Ala46-Cα-Ile52-Cγ2</td>
<td>3.6–3.9</td>
<td>+</td>
<td>—</td>
<td>+b</td>
</tr>
<tr>
<td>Leu46-Cα-Ile52-Cα</td>
<td>7.5–8.1</td>
<td>+b</td>
<td>+b</td>
<td>+b</td>
</tr>
<tr>
<td>Leu46-Cγ-Phe54-Cδe</td>
<td>4.6–7.0</td>
<td>+c</td>
<td>+c</td>
<td>+c</td>
</tr>
<tr>
<td>Leu46-Cγ-Phe54-Cδe</td>
<td>4.6–7.0</td>
<td>+c</td>
<td>+c</td>
<td>+c</td>
</tr>
<tr>
<td>Leu46-Cγ-Phe54-Cγ</td>
<td>5.0–5.7</td>
<td>+d</td>
<td>+d</td>
<td>+e</td>
</tr>
<tr>
<td>L59V-Leu46-Cγ-Phe54-Cδe</td>
<td>4.6–7.0</td>
<td>+c</td>
<td>+e</td>
<td>+e</td>
</tr>
<tr>
<td>L59V Leu46-Cγ-Phe54-Cγ</td>
<td>6.4–7.4</td>
<td>+c</td>
<td>+e</td>
<td>+e</td>
</tr>
</tbody>
</table>

*aMeasured range for three out of four residue pairs in the PDB: 2L0J WT protein.
*bThe observed interaction could be the result of a short distance for either of these two Leu/Ile pairs.
*cThe observed interaction could be the result of a short distance for either of these two Leu/Phe Cδ pairs.
*dThe observed interaction could be the result of a short distance for either of these two Leu/Phe Cγ pairs.

TABLE 1 pH Dependence of Cross Peaks and Correlation with Conductance Domain Structure PDB: 2L0J
hydroxyl in the bilayer interface and the hydrocarbon tail buried in the interstices of the lipid bilayer.

A consensus recognition sequence for cholesterol binding has long been recognized (52), originally based on studies of a TM helix in peripheral-type benzodiazepine receptors. This CRAC sequence (–L/V-(X)1-5-Y-(X)1-5-R/K-) was observed as a surface from TM helix 4 of these receptors that interacted with cholesterol, such that the Leu/Val residue was in the hydrophobic region of the bilayer and the Arg/Lys was in the bilayer interface with a Tyr residue midway between these sites. Subsequently, similar sequences have been recognized in G-protein-coupled receptors (GPCRs) (53,54) and generalized to include residues lining a cleft between a pair of TM helices that form a pocket for binding cholesterol. These sequences are referred to as “cholesterol consensus motifs”. In the β-adrenergic receptor, the interfacial positively charged residue was shown to be in an amphipathic helix (53), but the other residues of the CRAC or cholesterol consensus motifs are in TM helices and not in the amphipathic helices, as has been suggested for M2 (30,55–57).

Stewart et al. (55) mutated a couple of the consensus residues of M2, without significantly influencing viral replication in vitro, suggesting that cholesterol binding may not be important for viral budding although it had some effect on morbidity and mortality in a mouse model. However, M2 has affinity for the periphery of the raftlike domains where there is a considerable cholesterol lipid fraction. Indeed, M2 is recognized as facilitating viral budding from raftlike domains by inducing membrane curvature (58) in the absence of ESCRT proteins that are not expressed by influenza viruses (32,56). Specifically, the critical role of amphipathic helix by inducing membrane curvature during viral budding is known (59). Furthermore, it is known that the native Cys50 residue (here C50S) in the middle of the amphipathic helix is palmitoylated, which is often affiliated with cholesterol binding (34). Schroeder et al. (30) showed that the M2FL is copurified with cholesterol. Consequently, there is reason to anticipate that M2 would bind cholesterol as a way to attract the protein to the viral bud zone, although it is also attracted there by its affinity for the water-soluble M1 protein (31,60). Thaa et al. (57) showed that raft-targeting of M2 was not dependent on the CRAC motif, but was dependent on palmitoylation. More recently, Thaa et al. (57) have also shown via FRET studies that neither palmitoylation nor the key cholesterol binding consensus residues are necessary to target M2 to the viral bud zone. Consequently, it is not clear that any of the CRAC motif residues are involved in cholesterol binding to M2.

Four Phe residues (47, 48, 54, and 55) in the amphipathic sequence are distributed on either side of the palmitoylation site. Only two of these four Phe residues are oriented toward the lipid environment, residues Phe47 and Phe54, which would be in an appropriate position to interact with...
cholesterol and form cross peaks with the C₂ carbon of cholesterol. Note that these residues are in an I-to-I+7 sequential arrangement so that they are on the same surface of the helix. Moreover, the PDB: 2L0J structure suggests that Phe⁵⁴ may be in a more appropriate position for this interaction than Phe⁴⁷, for several reasons. First, the carbonyl group of Ser⁵⁰ is the most exposed carbonyl on this surface of the amphipathic helix as a result, in part, of the small Ser (Cys) sidechain (Fig. 6 C). This would be an ideal location for hydrogen bonding of the cholesterol hydroxyl to the protein. Second, this would position cholesterol in a cleft between the C-terminal TM helices and be flanked in the native protein by the palmitoyl group linked to the Cys⁵⁰ residue. In this position, as shown in the structural model in Fig. 6 C, the Phe⁵⁴ aromatic ring would be in van der Waals contact with cholesterol. Indeed, the orientation of the cholesterol and palmitoyl group to the amphipathic helix in the β₂-adrenergic GPCR (61) appears to be similar to what is modeled here and for which we have observed very strong [¹³C]Phe-[¹³C]cholesterol cross peaks consistent with close contact in the structural model with Phe⁵⁴.

Above, it was noted that at pH 5.0 the binding of AMT did not significantly influence the protein structure as assessed by the Phe/Leu cross peak (Fig. 4 E), but at pH 7.5 the binding of AMT resulted in the loss of the Phe/Leu cross peak. The addition of cholesterol to the pH 7.5 preparation with AMT prevented the loss of the Phe/Leu cross peaks as shown in Fig. 5. Consequently, cholesterol appears to stabilize the amphipathic helix structure in the membrane interface. While this stabilizing effect of cholesterol was not required in the absence of drug, its presence can be assumed from the multiple potential interactions between cholesterol and the amphipathic helix (hydrogen bonding directly or indirectly with Ser⁵⁰ (Cys⁵⁰) and van der Waals interactions with Phe⁵⁴) as well as interactions with the C-terminal region of the TM helices and with the palmitoyl group of Cys⁵⁰ (van der Waals interactions). These interactions could result in considerable stabilization of the amphipathic helix position in the lipid interface, and potentially this is important when the protein is inducing substantial curvature strain on the membrane in the budozone upon membrane fusion in the endosome.

CONCLUSIONS

Numerous specific structural restraints have been observed here using mixed [¹³C]-labeled M2FL monomers in the tetrameric channel. These restraints have characterized pH-dependent structural perturbations in the N-terminal region of the TM helices that had not been observed in prior studies of truncated protein constructs in various detergent-based or lipid-based environments. Other structural perturbations that had been observed in the C-terminal region of the TM domain were not confirmed in the spectra of M2FL, suggesting increased stability as a result of the presence of the C-terminal domain. NMR interhelical distance restraints between the amphipathic and TM helices had not been observed previously; here the restraints provided by both Leu⁵⁹-Phe⁴⁸ and Leu⁴⁶-Phe⁵⁴ are very significant for characterizing the M2 structure at the inner membrane interface of the viral capsid. These restraints document the proximity of the C-terminal end of the amphipathic helix with the C-terminal end of the TM helix in the adjacent monomer. When AMT was bound at pH 7.5, these Leu-Phe interactions were lost, suggesting a significant structural change.

Cholesterol binding to M2 has been demonstrated, and that it binds in a typical cholesterol binding site and orientation, one that utilizes the amphipathic helix where it interacts with one of the Phe side chains, mostly likely Phe⁵⁴, and it may hydrogen-bond with the Ser(Cys)⁵⁰ carbonyl oxygen. In addition, there is an interhelical crevice in the PDB: 2L0J structure into which cholesterol can optimally form van der Waals interactions. This crevice represents a weak point in the M2 structure with a relatively large separation of the helices. It is important for proteins to avoid fenestrations into the fatty acyl environment that could lead to fatty acyl penetration into the pore, thereby disrupting the functional mechanism. In the native protein, interactions with the Cys⁵⁰ palmitoyl group could further stabilize cholesterol binding. The binding of cholesterol...
to the amphipathic helix and stabilization of this conformation has important consequences for the functionality of the protein. Influenza A does not have the ESCRT proteins that facilitate viral budding for many viruses (60). Instead, M2 facilitates membrane curvature through the position of its amphipathic helix, generating a dramatic pyramidal shape that induces considerable membrane curvature. If the amphipathic helix changes position, such as in the formation of a water-soluble, four-helix bundle (e.g., the solution NMR M2 structure, PDB: 2RLF (8)), it eliminates M2’s ability to induce curvature. The virus buds out from a raftlike domain and the relatively short hydrophobic dimension of the M2 TM helices largely restricts M2 to the thinner surrounding membrane domain rather than the raftlike domain. Consequently, M2 clusters at the periphery of the raftlike domain where cholesterol is present in high concentration, thereby facilitating the binding of cholesterol to M2 and thus stabilizing the amphipathic helix in the lipid interface-inducing curvature of the host membrane needed for viral budding from the host cell. A similar process may occur when the viral interior becomes acidified in the endosome before the fusion of the viral membranes with the endosomal membrane.

**SUPPORTING MATERIAL**

Supporting Materials and Methods, four figures, and one table are available at http://www.biophyjs.org/biophysyjsupplemental/S0006-3495(16)00152-1.

**AUTHOR CONTRIBUTIONS**

E.V.E. and T.A.C. designed the research; E.V.E. expressed the protein and prepared the samples; and E.V.E. and R.F. conducted the NMR experiments and analyzed the data. All authors contributed to the writing of the article.

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**REFERENCES**


Supplemental Information

Structural Influences: Cholesterol, Drug, and Proton Binding to Full-Length Influenza A M2 Protein

E. Vindana Ekanayake, Riqiang Fu, and Timothy A. Cross
Material and Methods

• Expression of M2 full-length

Protein was expressed with N-terminal Histidine-6 tag similar to previously reports\(^1\). Briefly, a single colony of BL21 (DE3) RP codon plus cells from a transformation plate were used to inoculate two 50 ml aliquots of LB media with 100 µg/ml ampicillin antibiotics. This culture was grown at 37 °C overnight (< 14 h) in a shaker incubator (250 rpm) and transferred into 1L of LB media with 100 µg/ml of ampicillin. These cells were allowed to grow at 37 °C till an OD600 value of 0.7 (1 cm pathlength) was reached. The cells were centrifuged at 4000 g for 10 mins at room temperature. Any residual LB was washed away by re-suspending the combined pellet (from 2 L LB) in 50 mL of M9 salt followed by another round of centrifugation. The pelleted cells were re-suspended in 1 L of M9 salts supplemented with 100 mg of the \(^{13}\)C-labeled L-amino acid of interest (Cambridge Isotope Labs, Sigma–Aldrich) and unlabeled amino acids for all remaining amino acids. Then this mixture was incubated at 37 °C for 40 min in the same shaker, before adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM to induce protein overexpression. The culture was incubated for an additional 4 h at 22°C before cells were harvested by centrifugation at 5000 g for 10 mins at room temperature.
Then the pellet was re-suspended in 30 mL of buffer containing 20mM Tris pH 8.0, 500 mM NaCl and 15% glycerol and stored at -80 °C.

- **Purification of M2 full-length**

  The frozen bacterial cell suspension at -80°C was thawed and treated at room temperature. Then Benzonase nuclease (Novagene; 4µL of 250 U/µl) was added to the cell culture to degrade nucleic acids (DNA, RNA). To help disrupt the bacterial cell-walls 0.25mg of lysozyme (Sigma-Aldrich)/L of cell culture was added to the cell suspension at room temperature and all components were mixed using an orbital shaker at room temperature for 1 h prior to cooling the suspension on ice for ~ 1 h. Mechanical cell disruption (French Press; Thermo Scientific) was used to lyse cells. Cells were subjected to French Press three times at 12,000 PSI. Then, Empigen-BB (Sigma-Aldrich- 35% active substance in H2O) was added to the resulting lysate to solubilize the cell membranes until the concentration becomes 3% (wt/vol). The suspension was rotated gently using a tube shaker (Thermo Scientific Labquake) overnight (~14 h) at 4 °C. The resulting membrane solubilized cell lysate was subjected to ultracentrifugation at 228,000 g for 45 min at 8 °C to remove insoluble cell debris as the proteins (membrane and soluble) remained in the supernatant. Nickel affinity column chromatography was used for purification. A 5 ml HisTrap HP Nickel Column (GE Healthcare Life Sciences) was pre-packed with Ni Sepharose High Performance resin. The column was equilibrated with the equilibration buffer (500mM NaCl, 20mM Tris pH 8.0, 1% wt/vol Empigen-BB, 20mM imidazole, 15% glycerol) using an ÄKTAxpress (GE Healthcare Life Sciences) protein purification system and the protein supernatant was loaded onto this column. The column was washed with equilibration buffer (until UV280 absorbance returned to baseline) and subsequently, washed with wash 1 buffer (500mM NaCl, 20mM Tris, pH 8.0, 0.7% wt/vol Empigen-BB, 60
mM imidazole, 15% glycerol). The detergent was exchanged using five column volumes (20 mL) of exchange buffer [500mM NaCl, 20mM Tris, pH 8.0, 0.5% wt/vol octyl-β-D-glucopyranoside (OG), 15% glycerol]. Finally, the protein was eluted using elution buffer (500mM NaCl, 20mM Tris, pH 8.0, 0.5% wt/vol OG, 250 mM imidazole, 15% glycerol) resulting in ~ 35-40 mg of protein for 1 L of M9 media. To break any disulphide bonds that might be present in the N-terminus, the purified protein was treated with 5 mM dithiothreitol (DTT-from Sigma-Aldrich), so that different isotopically labeled monomers from different cultures would blend properly in the next step.

• **Preparation of distinct isotopically labeled mixture, reconstitution into lipid bilayers and MAS sample preparation**

  Different isotopically labeled monomers are mixed 1:1 (mol/mol), in a small round-bottomed flask and bath sonicated (Branson) for ~ 5 mins to make sure the mixture was optimally blended. A 4:1 molar ratio of dimyristolphosphatidylcholine (DMPC) and dimyristolphosphatidylglycerol (DMPG) lipid (Avanti Polar Lipids) was used to reconstitute the protein in a 1:30 protein to lipid molar ratio. First, lipid mixture was re-suspended in 40 °C Tris, pH 8.0 buffer (1 mL for every 10 mg of lipid mixture) and subjected to vortex mixing (VWR) to obtain homogenized suspension. Then OG was added from a stock solution (~ 15% wt/vol) to a final OG concentration is 3% (wt/vol). Once the lipid mixture becomes clear due to the formation of mixed micelles, the mixture was transferred to a FalconTM 50mL conical tube (Fisher Scientific) and kept in an orbital shaker for 30 mins at room temperature for completion of lipids solubilization. The protein mixture was added to the lipid mixture in the orbital shaker and mixed for an additional ~ 3 hours at room temperature. When cholesterol was added (Sigma-Aldrich) a molar ratio of 1:6 (cholesterol:other lipids) was used. The protein-lipid mixture was transferred to a pre-soaked dialysis bag (SpectraPor) having a molecular weight cut-off of 3.5
kDa, and dialyzed against 2 L of buffer (20 mM HEPES for pH 7.5 or 20 mM citrate buffer for pH 5.0) at 37 °C to remove detergent. The external buffer was changed twice a day and typically carried out for four days. Removal of detergent was confirmed by HPLC using evaporative light scattering detection (ELSD)\(^2\). For amantadine binding experiments, the drug was added after the removal of detergents to the resulting proteoliposome suspension at a 1:2 molar ratio of channel:drug. The resulting proteoliposome preparation was bath sonicated for ~5 min to make the liposomes homogeneous followed by ultracentrifugation at 354,000 g at 8 °C for 5 h. Then this pellet was transferred to a 98% hydration chamber containing saturated potassium sulfate until the excess buffer is removed.

- **Magic Angle Spinning NMR experiments**

  M2 proteoliposomes were packed into 3.2 mm thin walled MAS rotors (Revolution NMR) as reported\(^3\) (Das et al., 2013). The ssNMR experiments were acquired on a Bruker Avance 600.1 MHz (14.1 T) NMR spectrometer at 262 K. Home built Low-E triple resonance probes, which minimize rf-induced sample heating, were utilized\(^4,5\) for all experiments. Different mixing times (300-500 ms) were used for the \(^{13}\)C-\(^{13}\)C dipolar assisted rotational resonance (DARR) experiments. Spinning rates from 9 to 12.2 kHz were used for different samples to avoid spinning sideband overlap with the potential crosspeaks. 200 to 512 t1 increments were used for the DAAR experiments with 224-512 transients for each t1 point. Topspin (Bruker) was used to process all data using Gaussian window functions (LB=−50 Hz and GB=0.12) in both dimensions. All \(^{13}\)C chemical shifts were externally referenced using \(^{13}\)C-CO labeled glycine and setting the carbonyl carbon resonance to 178.4 ppm relative to TMS.
Supplementary Figures and Tables

**Figure S1:** 2D DARR spectra of M2FL with individually $^{13}$C labeled amino acids. Sample pH is at 7.5 and the temperature is 262 K. MAS frequencies: 12.2 kHz for Ile, Leu; 10.8 kHz for Val; 9.0 kHz for Phe. Natural abundant $^{13}$C lipid signals appear at 16 ppm, 27 ppm and 35 ppm. Note multiple crosspeaks, between lipid C2 at ~ 35ppm to amino acid residues, in all spectra can be observed. Amino acid residues located in the helical interface can be expected to contribute to these signals.
Figure S2. Structural influences of pH on M2FL. Spectral slices obtained at 262K from $^{13}$C-$^{13}$C DARR spectra of $^{13}$C amino acid labeled mixed M2 samples at pH 7.5 and 5.0. A) Spectral slices through Val-Cα frequency (66.8 ppm) using a mixing time of 500 ms and 10.8 kHz spinning rate. Dashed line is at Leu-Cα frequency (~58 ppm) B) Spectral slices through Ala-Cα frequency (55.8 ppm) using a mixing time of 300 ms and 12.2 kHz spinning rate. Dashed line is at the Ile-Cγ1 frequency (~27 ppm) C) Spectral slices through Leu-Cα frequency (58.0 ppm) using a mixing time of 500 ms and 12.2 kHz spinning rate. Dashed line is at Ile-Cα frequency (65 ppm). D & E) Spectral slices through Leu-Cγ frequency (26.6 ppm) using a mixing time of 500 ms and 9.0 kHz spinning rate. Dashed lines are at Phe-Cγ (~138 ppm) and Phe-Cδε (~130 ppm) frequencies.
Figure S3. Structural influences on M2FL upon AMT binding. Spectral slices obtained at 262K from $^{13}$C-$^{13}$C DARR spectra of $^{13}$C amino acid labeled mixed M2 samples with and without AMT at two pH values. A & B) Spectral slices through Val-Cα frequency (66.8 ppm) using a mixing time of 500 ms and 10.8 kHz spinning rate. Dashed line at Leu-Cα frequency, ~58 ppm. C) Spectral slices through Ile-Cα frequency (65.7 ppm) using a mixing time of 500 ms and 12.2 kHz spinning rate. Dashed line at Leu-Cα frequency ~58 ppm. D & E) Spectral slices through Leu-Cγ frequency (26.6 ppm) using a mixing time of 500 ms and 9.0 kHz spinning rate. Dashed lines are at Phe-Cγ frequency ~138 ppm and Phe-Cδε frequency ~130 ppm.
Figure S4: Influence of cholesterol on AMT bound M2FL at pH 7.5. Slices from 2D DARR spectra of $^{13}$C Phe:$^{13}$C Leu mixed sample of M2FL in lipid bilayers at 262K with and without cholesterol and AMT bound. Slices through Leu-$\gamma$C at 26.6 ppm. Dashed lines are at Phe C$_{\gamma}$ (~138 ppm) and Phe C$_{\delta \epsilon}$ (130 ppm). Spectra obtained with 500 ms mixing time and 9.0 kHz spinning rate.
Table S1. Influence of AMT binding to the observation of intermonomer distance crosspeaks.

<table>
<thead>
<tr>
<th>Residues Involved</th>
<th>pH</th>
<th>Without AMT</th>
<th>With AMT</th>
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<tr>
<td></td>
<td></td>
<td>2L0J (Å)</td>
<td>Crosspeaks Observed</td>
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<tr>
<td>Leu$<em>{26}$ C$</em>\alpha$ - Val$<em>{28}$ C$</em>\alpha$</td>
<td>7.5</td>
<td>8.1-8.2</td>
<td>-</td>
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<td>+</td>
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<td>7.5-8.1</td>
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<tr>
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<td>7.7-8.2</td>
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<tr>
<td>Leu$<em>{46}$ C$</em>\gamma$ - Phe$<em>{54}$ C$</em>\delta\epsilon$</td>
<td>7.5</td>
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<tr>
<td>Leu$<em>{59}$ C$</em>\gamma$ - Phe$<em>{48}$ C$</em>\delta\epsilon$</td>
<td>7.5</td>
<td>5.2-6.7*</td>
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<tr>
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<td>+</td>
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<td>Leu$_{C\gamma}$ - Phe$C\gamma$</td>
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<td>+</td>
<td>+</td>
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</table>

* Measured range for three of four residue pairs in 2L0J

Supplementary References

(3) Das, N.; Murray, D. T.; Cross, T. A. Nat. Protoc. 2013, 8, 2256.