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M2 Proton Channel from Influenza A: Example of Structural Sensitivity to Environment

J. Paulino[1,2], A. Wright[1,2] and T. A. Cross[1,2,3]
[1]Institute of Molecular Biophysics, Florida State University, Tallahassee, FL, USA
[2]National High Magnetic Field Laboratory, Florida State University, Tallahassee, FL, USA
[3]Department of Chemistry and Biochemistry, Florida State University, Tallahassee, FL, USA

Definition

Numerous structures of the tetrameric M2 proton channel have been published. Each monomer has a single transmembrane helix that shelters a pair of functional residues, His37 and Trp41. These residues are uniformly shown to be facing the interior of the tetrameric bundles in all of the structural models. In addition, there is an amphipathic helix that interacts with the lipid interface in some of the structures. This helix facilitates the proton conductance activity of the protein. M2 Structures obtained from multiple technologies in a variety of environments are discussed.

Introduction

Here, we review and analyze a selection of the multiple solution and solid-state NMR structural endeavors as well as an X-ray crystal structure of the M2 protein from influenza A virus, an important drug target for influenza infections (Holsinger and Lamb 1991; Pinto et al. 1997; Sakaguchi et al. 1997). A little more than 100 years ago, the great influenza epidemic resulted in tens of millions of deaths worldwide. A more devastating pandemic than the current (as of January 2022) covid crisis. Today, in addition to vaccines that are available to prevent the spread of influenza infections, there are also pharmaceuticals, some of which target the M2 protein. However, this virus is constantly evolving, and part of this evolutionary process are mutations to the M2 protein including the S31N mutation and more recently the V27A mutation that made the antiflu drugs amantadine and rimantadine ineffective (Bright et al. 2006). Consequently, the atomic resolution structural characterization of the M2 protein has been an important and ongoing structural biology endeavor. Furthermore, characterizing the native structure is not only an academically satisfying achievement but it is also critically important, as drugs are often designed, and developed based, in part, on the protein structure that the drugs target.

The tetrameric M2 protein of the influenza A virus appears to have at least two functions. The most well characterized of the M2 functions is as a facilitator of selective proton conductance

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across the cell membrane of the host cell (Sakaguchi et al. 1997; Sugrue and Hay 1991). For this function, a set of four His_{37} residues in the midst of the transmembrane domain (residues 22–46) are responsible for shuttling protons from the N-terminal side of the membrane to the C-terminal side. Hydrogen bonds that play a critical role in facilitating this transport have been identified between two of these His_{37} residues (Fu et al. 2020; Movellan et al. 2020). On the C-terminal side of the His_{37} tetrad is the Trp_{41} tetrad that acts as a gate for proton conductance. The second function of the M2 protein is associated with viral budding. The frustrum shape of the tetramer is derived from the penetration of an amphipathic helix (residues 47–62) into the membrane on the cellular interior (Sharma et al. 2010). It is important to recognize that the amphipathic helices have a large hydrophobic surface and consequently not only the lipid headgroups are forced away from the TM helices, but these amphipathic helices penetrate well into the hydrophobic domain of lipid bilayers generating the frustrum shape. Given the large number of these tetramers in the membrane at the site of viral budding, dramatic membrane curvature is achieved, facilitating the budding process for the virus.

Membrane protein structural biology includes a range of technologies from X-ray crystallographic methods to NMR spectroscopy. The crystallization of small helical membrane proteins is challenging and can lead to structural distortions induced by crystal lattice contacts and a weak model of the membrane environment in the form of detergents. The larger the membrane protein, the more opportunities exist for crystal lattice contacts that do not distort the overall structure in the protein. The solubilization of membrane proteins in a detergent environment for solution NMR (sNMR) can also lead to structural distortions as detergent micelles generate a relatively weak hydrophobic environment. Lipid bilayers can be used for solid-state NMR (ssNMR) spectroscopy, but it is essential to use an adequate lipid environment to avoid distortions.

These three technologies have been aggressively used to characterize the M2CD structure of this important drug target. NMR spectroscopists are interested in developing samples with high sensitivity, be it for sNMR or ssNMR spectroscopy. For such spectroscopic endeavors, the less sample volume that is occupied by the membrane mimetic and water, the higher the spectral sensitivity and less spectrometer time that is needed for the structural characterization. However, the native structure of these proteins is dependent on an adequate mixture of hydrophobic and hydrophilic environments to solubilize and stabilize the native and functional conformation of a membrane protein. These spectroscopic, crystallographic and sample preparation challenges have been played out in the structural biology literature of the M2 protein. For crystallography, it is essential that crystal contacts be formed so that a stable crystal lattice is formed; such contacts and their interactions, however, can lead to structural deformations, which is why crystal structures of larger protein targets are more common.

Cellular membranes are packed densely with proteins suggesting that extensive lipid or detergent environments may not be essential for achieving a structurally homogeneous protein preparation. For the spectroscopists, if spectra can be obtained in a reasonable amount of time, this opens opportunities for additional spectral collection to pursue a variety of experimental conditions that would otherwise not be possible due to the limited availability of experimental time. While disruptive protein interactions do not occur between various membrane proteins in native membranes, this does not mean that a single type of membrane protein can be incorporated into a model membrane at high concentration without the possibility of inducing structural deformations. This is an important and critical challenge for both spectroscopists and crystallographers of small membrane proteins.

The Membrane Protein Environment

The cell membrane environment is composed of many membrane proteins and a broad spectrum of lipids generating a hydrophobic hydrocarbon interior that has a thickness range upwards of ~27 Å.

This hydrocarbon layer is bounded by the glycerol backbone that tethers the fatty acyl chains to the phosphate and lipid head group. While water molecules rarely transit across membranes, they do penetrate the headgroup region to associate in a stable fashion with the glycerol backbone that links the acyl chains to the head groups (Watanabe et al. 2019). The phosphate and head groups of the lipids also support many more water molecules. Consequently, in native membrane environments, there is a stable water gradient from the lipid headgroup region to the glycerol backbone and into the hydrocarbon region of the bilayer. In this latter region of the membrane, the dielectric is very low. While occasional water molecules pass through the hydrocarbon layer, it has a negligible effect on the dielectric, and from the perspective of sample preparation, the water content of the hydrocarbon layer can be ignored.

The transmembrane domains of proteins are either α-helical or have β-strands that form a barrel type structure and due to the low dielectric environment, the backbone dynamics are typically very limited (Zhou and Cross 2013). The M2 transmembrane domain is α-helical dominated by large hydrophobic amino acids that interface with the fatty acyl chains, such as: leucine, valine, and isoleucine. These residues shield the hydrophilic amide linkages of the polypeptide backbone from the lipid environment. In addition, these sidechains are often highly dynamic as they exist in a liquid crystalline environment favoring rotameric states about the covalent bonds and generating almost a fluid-like interaction surface with the dynamic fatty acyl chains. This borders on a liquid-liquid interface but, the dynamics of both the protein and fatty acyl chains have significant limitations. For the amino acids and lipids, the dynamics favor rotameric states about the C-C bonds. The result is an effective packing of the protein surface with fatty acyl methylene groups.

The head groups of a typical lipid, such as phosphatidylcholine, have a negatively charged phosphate and a positively charged choline group. Typically, these headgroups have an extended structure due to the close packing of the lipids resulting from the very limited average cross-sectional area of the fatty acyl chains. The result is a well-hydrated and relatively thick lipid headgroup region that provides a hydration gradient and electrostatic interaction opportunities for the hydrophilic surfaces of transmembrane proteins. This relatively thick head group region also provides a dielectric gradient from the very polar aqueous environment to the nonpolar hydrocarbon region of the membrane interior.

Detergents are sometimes used to solvate membrane proteins, such as in the process of membrane protein purification or the preparation of membrane protein crystals. Three broad classes of detergents (ionic, nonionic, and zwitterionic detergents) are used for this purpose (Krueger-Koplin et al. 2004). Detergents do not form bilayer structures, but they do form micelles, which is a single layer of detergent molecules wrapped into a sphere or disk-like structure that has a hydrophobic core and a hydrophilic surface. The advantage in preparing membrane protein samples in such an environment is that these structures tumble rapidly in solution and hence give rise to high-resolution sNMR spectra if the protein is not too large. However, the hydrophilic surface is typically much thinner than that of lipids and consequently, it does not have the hydration gradient typical of phospholipid head groups. In addition, water penetration into the micelle is much greater than that into the hydrophobic lipid bilayer environment. However, this detergent environment is often used to solubilize a monomer or single oligomeric states of a membrane protein, such that structures can be characterized by high-resolution sNMR spectroscopy (Wang et al. 2013).

The M2 Proton Channel

Here, we discuss structural studies of what is referred to as the conductance domain (M2CD; residues 24–62) of the M2 protein – that portion of the protein that is not natively unfolded. The full-length protein (residues 1–97) has a segment at both the N- and C-termini that is natively unfolded. M2CD structures of the influenza A virus have been obtained from sNMR and ssNMR spectroscopy as well as X-ray crystallography. Here, we exclude from this discussion the numerous structures of the isolated

transmembrane domain (M2TM). While this domain has reproduced the ion conductance properties reasonably well in lipid bilayer preparations, the M2TM structures have a broad range of helical tilt angles sometimes leading to fenestrations into the pore from what would be the hydrophobic core of the lipid bilayer that consequently fails to reflect the native structure.

An initial structural study of the full-length M2 protein in lipid bilayers was performed in 2003 using amino acid specific labeling for ssNMR that generated a limited data set for the tilt of the TM and amphipathic helices, the only structured regions of the protein. The TM helix was shown to have an approximate tilt of ~25° and the amphipathic helix a tilt of ~80° from the bilayer normal (Tian et al. 2003). Furthermore, it was shown by hydrogen-deuterium exchange that the slowest exchanging amides in the entire protein were not in the TM helix, but in the amphipathic helix, documenting that the hydrophobic surface of the amphipathic helix penetrates substantially into the hydrophobic domain of the lipid bilayer.

M2CD (residues ~20–62) structures have been characterized in multiple labs, by ssNMR, sNMR, and X-ray crystallography (Andreas et al. 2015; Pielak and Chou 2010; Pielak et al. 2009; Schnell and Chou 2008; Sharma et al. 2010; Thomaston et al. 2020). The fourfold symmetry of the M2 structures has been well-documented by solution and ssNMR (Schnell and Chou 2008; Sharma et al. 2010) as well as X-ray crystallography (Thomaston et al. 2020), except for the functionally active His_{37} and Trp_{41} residues that have critical functional roles (Can et al. 2012; Sharma et al. 2010). There has been an exception to this observation; the PDB file 2N70 structure, an S31N M2CD structure characterized by magic angle spinning ssNMR (Andreas et al. 2015) showed twofold symmetry, a dimer of dimer structure.

We have chosen to discuss a small but representative set of the M2CD structures as outlined in Table 1 and displayed in Fig. 1. These structures span the primary structural characterization tools of solution and solid-state NMR as well as X-ray crystallography. The 2L0J structure (Fig. 1b) is a WT structure characterized in a lipid bilayer environment primarily by OS ssNMR spectroscopy of uniformly aligned samples of M2 in extensive lipid bilayers aligned between thin glass plates. The fourfold symmetric structure has the TM helices (D24-L46) packed, such that there is a narrow pore at the N-terminus or what would be the cellular exterior. The helices flare somewhat toward the C-terminus of the TM helices to accommodate the dynamics of the functional H37 and W41 residues that facilitate the proton conductance activity of this protein. The amphipathic helices (residues F47-G62) that follow the TM helices on the cellular interior form a four-helix framework in the lipid interface stabilizing this flaring of the TM helices leading to the generation of a frustrum-shaped structure. This shape is critical for facilitating the viral budding process as the viral particle "pinches-off" from the cellular surface, requiring a highly curved membrane surface.

The 2RLF structure (Fig. 1a) is a solution NMR structure of the WT M2CD that has fourfold symmetry. The TM helices (P25–L46) have multiple fenestrations at the helix-helix interfaces, but the polar residues are all oriented in toward the pore. Here, the detergent micellar environment does not support the C-terminal amphipathic helices in a layer at the C-termini of the TM helices; instead, the amphipathic helices (residues 52–58) form a weakly associated water-soluble bundle of short helices removed from the bilayer surface through the disordered residues F47–I51. These amphipathic helices have their hydrophobic residues oriented toward the center of the bundle that appears to be an aqueous pore. However, it is possible that the hydrophobic residues of the amphipathic helices (I51, F54, and F55) may have its own detergent micelle to solubilize these hydrophobic residues that would otherwise be exposed to the aqueous environment.

The V27A mutant M2CD structure, 6OUG is a tetramer with a bound inhibitor in the pore, spiro-adamantyl amine, near residue A30 (Fig. 1c). The TM helical structure runs continuously from P25 to Y52. Consequently, the amphipathic helix has become an extension of the TM helix, as opposed to being essentially orthogonal to the TM helices in the modeled bilayer interface. Presumably, this

M2 Proton Channel from Influenza A: Example of Structural Sensitivity to Environment, M2 Proton Channel from Influenza A: An example of structural sensitivity to environment – The six Influenza A M2 Protein Structures deposited in the Protein DataBank identified by their PDB file name, amino acid sequence, structural symmetry and experimental technique used to characterize their atomic resolution structure, as well as a literature reference for each structure

PDB file	Sequence	Symmetry	Notes	Reference
2RLF	WT	4-fold	sNMR	(Schnell and Chou 2008)
2L0J	WT	4-fold	OS ssNMR	(Sharma et al. 2010)
6OUG	V27A	4-fold	X-ray crystallography	(Thomaston et al. 2020)
2KWX	V27A	4-fold	sNMR	(Pielak and Chou 2010)
2N70	S31N	2-fold	MAS ssNMR	(Andreas et al. 2015)
2KIH	S31N	4-fold	sNMR	(Pielak et al. 2009)

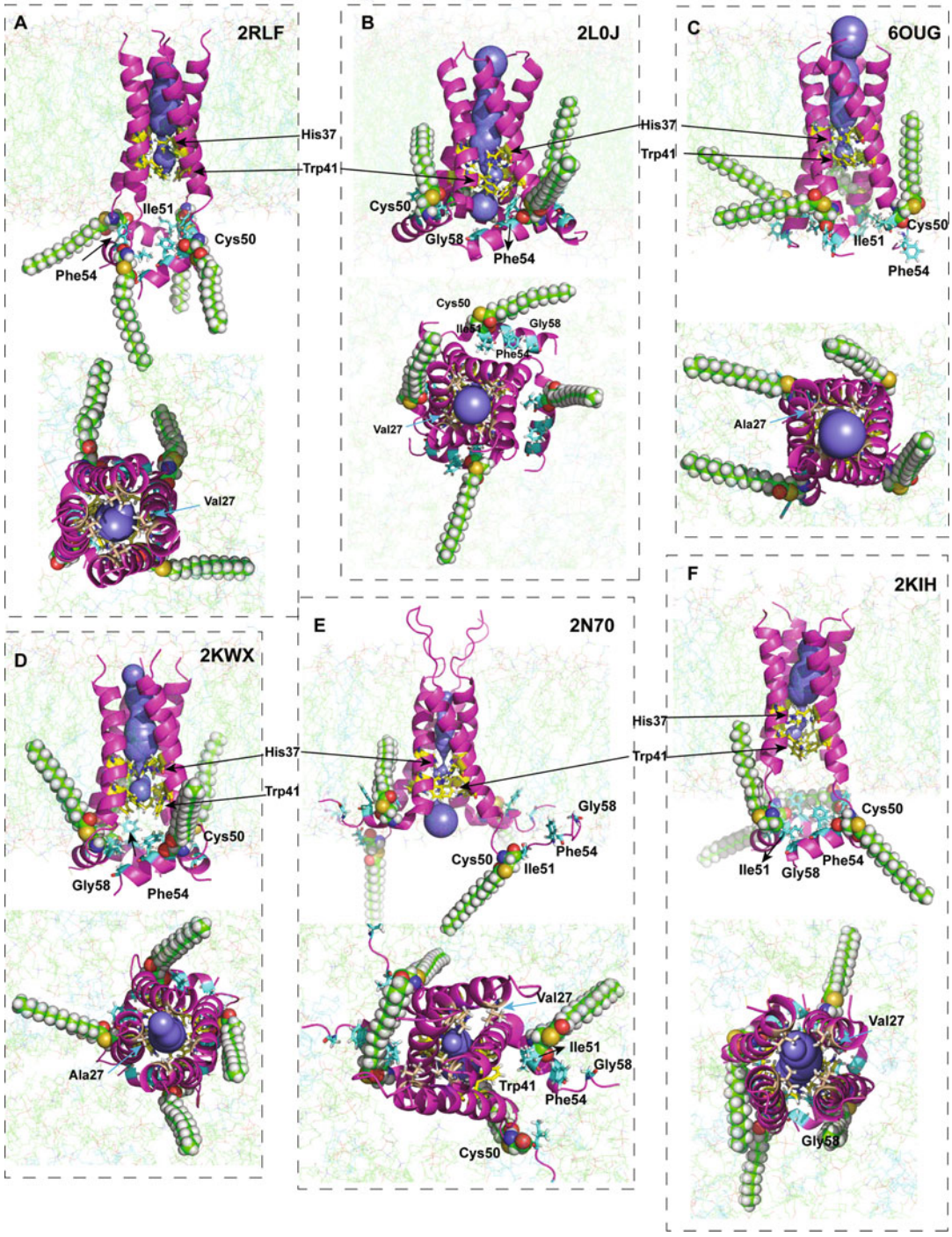
reflects the difficulty of generating a hydrophobic/hydrophilic layered environment in the crystal lattice. Despite this non-native aspect of the structure, the transmembrane domain is well packed at the N-terminus and at the level of W41 sites responsible for gating proton conductance through the pore.

The 2KWX solution NMR structure (Fig. 1d) is also a V27A M2CD structure. The TM helix runs from D22 to L46. The fourfold symmetric structure also displays amphipathic helices (AH) from residues S50 to L59. Consequently, residues 47–49 form a loop reducing the length of the AH compared to that in the 2L0J structure. The TM helix has a significant curve in the plane formed by the TM and AH helices of the same monomer. This relatively tight packing of the four-helix bundle results in limited space for H37 and W41 dynamics associated with their functional activities. Once again, the tether from the TM to AH is significant resulting in a much shorter AH and consequently the C-terminal ends of the TM helices are not splayed so far apart.

The KIH structure is a solution NMR structure of the S31N M2CD construct (Fig. 1e). This structure has fourfold symmetry for the TM helices (P25–R45), but there are multiple large fenestrations at the helix-helix interfaces. However, the polar residues are all oriented in toward the pore. The detergent micellar environment does not support the C-terminal amphipathic helices in a layer at the C-termini of the TM helices; instead, the amphipathic helices (residues 51–58) form a water-soluble bundle of short helices removed from the bilayer surface through disordered residues L46–S50. These amphipathic helices appear to have their hydrophobic residues oriented toward the center of the bundle suggesting that these helices may also have a separate detergent micellar environment.

The 2N70 structure mentioned above is unique in that it forms a dimer of dimer structure (Fig. 1e). The TM helices extend from P25 to L46 in all four monomers, and in two of the monomers (A, C) on opposite sides of the tetramer, an amphipathic helix exists between residues F47–F55, leaving residues R18–D24 and residues E56–K60 disordered in all four helices. In addition residues F47–K60 are disordered in just two of the helices (B, D). The TM helices are nearly linear structures; however, the packing of the AC helices at the N-termini has closed the aqueous pore through residue N31. Furthermore, I35 and L38 appear to be pinning the functionally critical His37 residue against the helical backbone, such that, without movement of the helical backbone, the His37 residues are not free to function. Consequently, this dimer of dimer structure does not represent a functional structure.

In recent results, structural data obtained from oriented sample (OS) ssNMR in the presence of excess lipid showed for S31N M2CD a single resonance for each amide ^{15}N labeled site in the TM helix and for each labeled site in the amphipathic helix sequence, confirming the fourfold symmetry of S31N M2CD (Wright et al. 2022). In addition, single ^{13}C and ^{15}N resonances from MAS ssNMR spectroscopy of S31N M2CD has shown single resonances for all backbone sites except for His_{37} and Trp_{41} where small



M2 Proton Channel from Influenza A: Example of Structural Sensitivity to Environment, Fig. 1 Side and end views (N-terminal side) of six tetrameric M2CD structures (fuschia ribbon) superimposed on a lipid bilayer (fine green lines) for each of the six M2CD structures identified in Table 1. The pore is illustrated in purple. Palmitylation of Cys50 of the amphipathic helix is shown (green carbon spheres). Some the key residues are highlighted in yellow and labeled

differences were observed generating a local dimer of dimer structure due to the packing of the bulky sidechains in the pore consistent with the WT structure (Can et al. 2012) (Fig. 1f). Importantly, the residues on either side of His37 and Trp41 showed essentially fourfold symmetric ^{13}C and ^{15}N chemical shifts. Consequently, the four-helix bundle has nearly complete fourfold symmetry.

The local dimer of dimer situation for His37 and Trp41 arises via hydrogen bonding between a pair of imidazole rings of the His_{37} residues (Fu et al. 2020; Movellan et al. 2020) and also by the packing of the Trp41 indole rings that generates a gate for proton conductance. The function of the imidazole sidechains is critically dependent on the orientation of the sidechain that accepts a proton from the N-terminal side of the membrane resulting in the formation of a hydrogen bond with a second imidazole sidechain. This arrangement appears to involve His37 residues that are not nearest neighbors, i.e., from monomers A and C or monomers B and D. As a result, only one pair of hydrogen-bonded imidazoles are possible due the steric hindrance formed by the hydrogen-bonded pair. Similarly, there is a break in the fourfold symmetry of the Trp_{41} indole sidechains associated with the open and closed states of the pore. These sidechain orientations and dynamics have not been fully described in the literature; however, it is clear that considerable space is needed for their function and hence the frustrum shape of the M2 structure that leads to a significant spatial volume for indole and imidazole dynamics on the C-terminal side of the membrane. Importantly, the helical backbone structure based on OS and MAS ssNMR spectroscopy shows a nearly uniform helical structure with fourfold symmetry throughout the transmembrane structure (Can et al. 2012; Sharma et al. 2010).

Characterizations in a Lipid Environment: How Much Lipid Is Needed?

Every structural biologist's goal is to characterize a protein structure that reflects a native state and informs the community interested in the protein. The protein content of native membranes can be very high, suggesting that it might be possible to achieve high concentrations of a single type of membrane protein in a lipid bilayer. Therefore, it is important to understand the limitations as to how much protein can be packed into a sample tube and how much lipid is needed to maintain the native membrane protein structure. Without enough lipid, hydrophobic membrane protein surfaces can pack together. Such interactions have led to non-native structures, as in the case of the 2N70 structure. Tetramer-tetramer crosspeaks in the NMR spectra assigned to V27'g1–H37'e1 and V27/28'g1–W41d1 were observed reflecting an antiparallel packing of the tetramers and resulting in the dimer of dimers structure. This would also help to account for the fact that only two of the amphipathic helices are properly folded in what would be the lipid interface, those that were parallel to the string of tetramers. The other pair of amphipathic helices that would be perpendicular to the string of tetramers appears to have been displaced from the lipid interface by the N-terminus of the neighboring tetramer.

In addition to the observed crosspeaks that confirm an antiparallel structure in 2N70, there were a pair of Cα resonances observed and assigned to two Ile residues, Ile32 and Ile39 in the transmembrane helix that are shifted upfield dramatically by 5 ppm in just two of the four helices (A and C). These Ile chemical shifts lie well outside the range of helical Ile frequencies suggesting that there is a dramatic external influence other than the milieu of the lipid bilayer that is affecting these sites. These sites also have unusual chemical shifts for their Cβ and Cγ resonances. This suggests that the tetramer-tetramer packing surface includes these residues.

The spectroscopy reported above was obtained in a lipid environment; however, the protein tetramer to lipid molar ratios used was only a 1:24 ratio and consequently, for each lipid monolayer, only 12 lipids were present per tetramer (Andreas et al. 2015). It has been shown since 2015 that when the protein to lipid ratio is 1:30 (15 lipids per monolayer) for just the transmembrane helix (M2TM), the correlation time for the tetramer is reduced by

a factor of 25 compared to samples of the M2TM in a protein:lipid ratio of 1:80 (Paulino et al. 2019). Such an increase in correlation time at low lipid ratios means that the tetramers were not free to rotate as they do in a more extensive lipid environment. Certainly, more lipid rather than less is needed to permit unhindered rotational motion of the tetramer for the M2CD, and therefore avoid tetramer-tetramer interactions.

While many biochemistry textbooks describe protein structure from a given amino acid sequence as a unique structure dictated by the sequence, this concept has recently come into question. For instance, the two observed conformations of the EmrE protein that form a dimeric structure with two significantly different conformations (Morrison et al. 2012). Consequently, the concept of two different conformers within the S31N M2 tetramer (2N70) was not viewed as breaking any rules of protein structure. However, these structural differences observed in 2N70 represent a substantial distortion of the helical structure that is due to tetramer-tetramer packing that, in turn, results from an insufficient number of lipids to fully solvate each tetramer.

Summary

The WT structure (2L0J) that was characterized by MAS ssNMR in samples at a molar ratio of 1:96 is a fourfold symmetric structure with the exception of the two critical residues that line the C-terminal transmembrane pore – His_{37} and Trp_{41} (Can et al. 2012). These key functional residues are associated with proton conductance and proton gating. For the native functional state, it is clear that considerable lipid is needed and while it is not possible to quantify a specific number of lipids or molar ratio for M2 within the plasma membrane during influenza infection, we have mentioned above that for the short M2TM peptide, a 1:80 peptide to lipid ratio results in a dramatic reduction in the correlation time compared to 1:30 (Paulino et al. 2019). Clearly, much more lipid will be needed to solvate the M2CD construct due to its much larger cross-sectional area on the C-terminal side of the membrane resulting from the penetration of the four amphipathic helices into the lipid environment. This C-terminal cross-sectional area is ~1200 $Å^2$ compared to the N-terminal cross-sectional area of ~300 $Å^2$. Indeed, when inadequate lipid is present in the sample preparation, the antiparallel packing of the M2 tetramers would be favored saving the sparse lipids to solvate an extensively oligomerized structure. Strings of dimer of dimer conformers were formed in the 2N70 structure leaving less hydrophobic surface area to be solvated by the very limited quantity of lipid in the sample. Furthermore, significantly more lipid would have been needed to achieve an independent correlation time for the M2CD tetramers compared to that for M2TM. All of this suggests that multiple layers of lipid surrounding these oligomeric structures are required to achieve unhindered correlation times and to avoid the structural distortions (Andreas et al. 2015).

It is essential to evaluate samples used for spectroscopic and crystallographic structural analysis. Here, we have seen that multiple sample environments can lead to distorted structural characterizations. Such structures have the potential to be misleading for the community that depends on protein structures for pharmaceutical development. Indeed, it is important to evaluate the sample not only for the spectroscopic or diffraction quality that it generates, but for the biological and pharmacological value of the structure that it supports. It has been shown for the wild-type M2 protein that a fourfold symmetric structure is obtained when enough lipid is present (the PDB 2L0J structure).

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