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The Flu's Proton Escort

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Abstract

A flurry of structural data provides sometimes conflicting insights into the M2 proton channel.

The influenza A virus, which causes seasonal flu, poses a major threat to human health. One recent focus of research has been the M2 protein, a small membrane protein that enables hydrogen ions to enter the viral particle (1, 2); this “proton channel” plays a critical role in enabling the virus to infect cells and replicate and in other processes (3, 4). In recent flu seasons, a mutation in the M2 protein has rendered the virus resistant to two common antiviral drugs, amantadine and rimantadine. Efforts to develop new antiviral drugs would benefit from a better understanding of M2's structure and how drugs act on the protein, but recent studies have often produced conflicting results. This trend continues with two papers in this issue, by Sharma *et al.* (5) on page 509, and Hu *et al.* (6) on page 505. There are, however, possible explanations for the apparent inconsistencies in these and other recently reported structures (7, 8).

The M2 proton channel mediates the acidification of the interior of endosomes, intracellular compartments created by host cells. The pH change enables viral RNA to escape into the cell and replicate. M2 has a 40-residue region that interacts with membranes (9) consisting of a transmembrane helix (which mediates tetramerization, drug-binding, and channel activity), followed by a basic amphiphilic helix important for budding of the virus from cellular membranes and release (scission) (4, 10). Research on M2 has been marked by debate since 2008, when investigators reported structures with anti-flu drugs bound at entirely different sites (11,12). Even as this debate is settling in favor of a pharmacologically relevant site in the pore (13, 14), new papers (7, 8), including the two in this issue, stoke new controversy concerning the mechanism of proton conduction, and the structure of the cytoplasmic helix. Sharma *et al.* combine oriented solid-state nuclear magnetic resonance (SSNMR) measurements with previous electron paramagnetic resonance (EPR) measurements (15) to determine a backbone structure of the 40 residue region in phospholipid bilayers, resulting in a structure that differs significantly from recent solution NMR structures (7, 12). Hu *et al.* use magic angle spinning SSNMR to specifically probe the His residues that mediate proton translocation. Their high-resolution data are in good agreement with other recently published structures (7, 8, 12, 16) but contradict many conclusions of Sharma *et al.* Here we offer explanations for the apparent paradox and relate the structures to the mechanism of proton transduction and vesicle budding/scission.

The EPR/SSNMR structure places the cytoplasmic helix at the C-terminal end of the bundle near the headgroup region of the bilayer (see the figure). By contrast, in the solution structure (12) the cytoplasmic helix forms a tetrameric bundle extending into the cytoplasm beyond the end of the TM domain (see the figure). The resolution of the solution structure is

greater than that of the EPR/SSNMR, which was computed without the long-range distance and side-chain dihedral restraints used in computing previous solution and SSNMR structures (12, 16). Nevertheless, the structural differences appear larger than can be attributed to coordinate error and represent different conformational forms.

It would be premature to dismiss either as irrelevant, given that M2 is a highly dynamic protein with manifold conformations and functions (3, 4). Nevertheless, the EPR/SSNMR structure is more consistent with M2's ability to promote membrane budding and scission. M2 localizes to the neck of a budding vesicle, a region of extreme curvature that topologically resembles a donut-hole. Such saddle-shaped surfaces have negative Gaussian curvature characterized by orthogonally directed negative and positive local curvature. The EPR/SSNMR structure positions the M2 cytoplasmic helix appropriately to promote negative Gaussian curvature by interacting with phospholipid headgroups (17). Future questions will likely focus on how the TM domain modulates the cytoplasmic helix, which has considerable activity as an isolated peptide.

The highest resolution studies of the channel-forming pore of M2 were determined using the TM peptide (M2TM), which is sufficient for proton channel formation. Electrophysiological measurements of a similar TM construct expressed in *Xenopus* frog oocytes showed channel activity within a factor of two of the full-length protein (18). To determine whether the cytoplasmic peptide interactions seen in the EPR/SSNMR and solution NMR structures are important for proton channel activity, Rossman *et al.* simultaneously replaced all five hydrophobic residues along one face of the cytoplasmic helix (F47, F48, I51, Y52, F55) with Ala in the full-length protein (4, 10). As expected, the surface expression level, proton flux, pH-activation, and drug-binding of this quintuple mutant were indistinguishable from wild type, while it had greatly diminished scission activity. Thus, although the detailed interactions of the cytoplasmic helix seen in the EPR/SSNMR structure might relate to scission they do not influence channel activity.

The structures of M2TM (8, 11, 16,) and M2TM+cytoplasmic constructs (15, 12) together with papers in this issue, suggest a unified mechanism for proton conduction (see the figure). Protons enter the channel through a narrow opening, the Val²⁷-valve into an aqueous pore leading to His³⁷. Four His³⁷ and Trp⁴¹ side-chains associate in an interaction that also inhibits reverse flow of protons out of an acidified virus. The His³⁷ tetrad is primed for conduction by binding two protons with high affinity (a negative log of dissociation constant or pK, value of ~8) (19). The affinity (pK~6) for the third proton appears evolutionarily tuned to match the pH of an acidifying endosome (20). In the shuttle mechanism (21), binding of the third proton from the exterior induces loss of a proton to the internal side, regenerating the +2 state.

The high-resolution crystal structure suggests that incoming protons are statistically delocalized in an imidazole/water cluster (8) (see the figure) with some parallels to the storage of electrons in bio-inorganic clusters. Addition of the third proton increases the hydration of His³⁷ (6), and induces dynamics on at a rate more rapid than that of proton flux (6), thereby promoting proton release into the viral interior. Finally, computation (22) and the experimental structures suggest that as the His³⁷ tetrad is increasingly protonated, the cytoplasmic end of the bundle dilates and the exterior-facing Val²⁷-valve constricts. This transporter-like mechanism also explains the greater rate of conductance when the pH is low outside but high inside, versus the opposite situation. So long as the His residues are deprotonated by high pH outside they remain kinetically protected by Trp⁴¹ from the low pH inside. This mechanism is also consistent with the low-level counter-flow of alkali metal cations, which accompanies proton flux in sealed vesicles to maintain electrical neutrality (23).

While these fundamental steps in conduction are becoming clear, significant questions remain. For example, in several structures, the four His³⁷ residues form a square His-box (see the figure); the imidazole side chains are connected via hydrogen-bonds to intervening water molecules also seen in Hu *et al.*'s SSNMR studies. By contrast, Sharma *et al.*, propose a different geometry that is inconsistent with Hu *et al.*'s direct measurements of side-chain geometry at both low and high pH. However, their structural data are limited to backbone orientational restraints, which do not match the resolution of a 1.65 Å x-ray structure (8) or direct side-chain distance and dynamic measurements (6).

Other remaining mechanistic questions include: whether the large-scale transporter-like backbone motions accompany the transport of each proton through M2 (which is consistent with its transporter-like maximal turnover of about 100 protons/s), and whether a permeating proton is shuttled on and off an individual His³⁷, or rapidly delocalized throughout the water-cluster/His-box (8). It has also been proposed that a permeating proton might bypass His³⁷ altogether and travel via a chain of water molecules (24). Clearly, biophysicists will remain busy resolving these issues, illuminating interactions that can be mined for the design of new generations of anti-flu medications.

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Figure 1. Flu in detail

The high-resolution crystal structure (8) of M2TM (**A**) is similar to spectroscopic structures of longer (**B**) constructs solved by SSNMR (5), (red), solution NMR (12) (light blue) or EPR (15) (yellow). The high-resolution structure (A) at intermediate pH shows a proton-conduction path consisting of layers of water molecules interleaved between the pore-lining side chains. The His³⁷ residues polarize intervening water molecules, encouraging entry of protons from the exterior. Water molecules are well positioned to increase the basicity of the N atoms of His³⁷ by simultaneously forming hydrogen bonds to the carbonyl of Gly³⁴. Interactions between N and the electron-rich face of Trp⁴¹ further stabilize the His-box. In the doubly charged state, this interaction is mediated by an intervening dimer of water molecules, which are weakened or converted to direct side-chain–side-chain interactions (8) in the neutral state. Additional water clusters toward the interior provide indirect charge stabilization and complete an exit pathway. (C) Low protonation (PDB ID codes: 2KQT, green; 3LBW, purple) (11, 16) versus (D) greater protonation (PDB ID code 3BKD, blue) (8) show that increasing protonation of the His box/water cluster causes the pore to contract on the exterior and expand near the interior, promoting movement of protons past the Trp⁴¹ gate into the interior of the virus (11). The structure of the C-terminal amphiphilic helix of Sharma *et al.* seen in (E) differs markedly from that of Schnell and Chou (12) in (F) but resembles the EPR model of Nguyen, Howard, and co-workers (G) (15).