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Structural and Dynamic Mechanisms for the Function and Inhibition of the M2 Proton Channel From Influenza A Virus

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Abstract

The M2 proton channel from influenza A virus, a prototype for a class of viral ion channels known as viroporins, conducts protons along a chain of water molecules and ionizable side chains, including His37. Recent studies highlight a delicate interplay between protein folding, proton binding and proton conduction through the channel. Drugs inhibit proton conduction by binding to an aqueous cavity adjacent to M2's proton-selective filter, thereby blocking access of proton to the filter, and altering the energetic landscape of the channel and the energetics of proton-binding to His37.

Introduction

Protein function requires correct folding into a native ensemble of three-dimensional structures with finely orchestrated dynamic interconversion between conformational substates. Thus, protein sequences reflect a compromise between stability and function [1,2]. The thermodynamics of folding have been studied for very few membrane proteins, and the relationships among stability, dynamics, and function remains largely unexplored for this class of proteins [3,4]. Here, we examine these relationships for the M2 proton channel [5] in comparison to the potassium channel KcsA [6–8], focusing on the interplay between folding energetics, permeant ion binding/translocation, and inhibition by binding of pore-blocking drugs.

M2 is a multi-functional, modular protein

The M2 protein, which was discovered as the target of the anti-influenza drugs amantadine and rimantadine [9–11], has multiple functions [5,12,13] associated with different regions of the sequence of this short 97-residue protein. Influenza viruses gain access to cells via receptor-mediated endocytosis, which places the virus within an acidifying endosome. M2 facilitates diffusion of protons into the interior of the endosomally-entrapped virus as the endosome matures, leading to uncoating of the viral RNA from the matrix protein M1 [14]. M2 is also important for delaying acidification of the late Golgi in some strains of the virus [15,16].

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M2's functions are compartmentalized into parsimonious sequences, consisting of:

- A. Residues 1–24 comprise a short unstructured N-terminal region important for incorporation into the virion [17] in influenza A virus, but entirely missing in influenza B virus [18,19].
- B. Residues 25–46 encompass the transmembrane (TM) helix that is necessary and sufficient for tetramerization, proton conductance and drug-binding [20–23]. Drug-resistant mutations map to pore-lining residues of this TM helix (particularly S31N, V27A and L26F) [24–26]. A secondary binding site on the outside of the TM helices is observed when the drug is present at very high concentrations in micelles [27] or bilayers [28], but electrophysiological studies and the drug sensitivities of reverse-engineered viruses showed that this site does not contribute to the pharmacological inhibition of the channel [24–26]. Much work on the structure and function of M2 has been conducted on fragments spanning from residues 22–46, or closely related sequences, which we call M2TM.
- C. Residues 47–61 defines a cytoplasmic amphiphilic helix involved in cholesterol-binding [29], membrane localization, budding and scission [12]. This sequence is not required for channel formation or drug-binding [20,25,29]. Together, the TM and cytoplasmic helices are often studied as a single peptide (approximately residues 20 – 60), defined as M2TM+cyto peptides.
- D. Residues 61–98 comprise a disordered tail that interacts with the matrix protein, M1 [30].

Structure determination of M2's membrane-interacting domains

The membrane-interactive domains of M2 were identified by limited proteolysis [31], which identified both M2TM+cyto as a meta-stable product, as well as M2TM as a final cleavage product (unpublished result). This, and related studies [20,32,33], showed that the TM domain was both necessary and sufficient to form tetramers and bind amantadine in micelles and bilayers. M2TM has also been the subject of numerous studies using optical spectroscopy (IR [34], Raman [35], CD [32], fluorescence [36], solid state NMR (SSNMR) [23,37–39], X-ray crystallography [40,41], isothermal calorimetry [20], analytical ultracentrifugation [42,43], and surface plasmon resonance [44]) to examine pH activation and drug-binding. Recently, a second series of structural studies focused on the M2TM+cyto fragment, which also includes the cytoplasmic amphiphilic helix [27,45–47].

Before discussing the structural basis for M2's functions, it is first important to consider the various models and structures proposed for M2 using different techniques, their resolution, and the degree to which the presence or absence of the C-terminal cytoplasmic helix might influence the structure of the pore-forming tetramer. We compare the findings to the potassium channel KcsA [6–8], which has two cytoplasmic domains, consisting of an N-terminal membrane-interactive helix as well as a C-terminal helical bundle domain. Almost all high-resolution structural work on KcsA has been accomplished with constructs lacking its N-terminal and C-terminal cytoplasmic helices. Lower resolution site-directed spin label EPR studies and a 3.8 Å crystal structure [48] showed that the N-terminal cytoplasmic domain formed a helix bound to the membrane surface, while the C-terminal cytoplasmic domain forms a water soluble four-helix bundle [49]. The presence of the C-terminal four-helix bundle has little effect on the channel pore, except to close one end of the bundle slightly more tightly near the activation gate. This is consistent with the fact that the basic structural features, conductance mechanism, and gating mechanism inferred from the structure of KcsA has proven to be general across many mammalian K⁺ channels. These proteins have very similar channel-forming regions and differ most markedly in their

pendant cytoplasmic domains, which help modulate the energetics and kinetics of interconversion of the conformational states underlying gating. Crystallographic structures of the membrane-spanning domains have been invaluable for defining these conformational states, while functional measurements and other lower-resolution methods, such as fluorescence spectroscopy, EPR, solution and solid-state NMR, are indispensable to assign these conformations to discrete functional states and to map their populations and kinetics of interconversion.

The structures of M2TM have been extensively studied by X-ray crystallography and SSNMR. The highest-resolution structure is a 1.65 Å crystal structure [41] of the TM domain (M2TM) from micelles (Figure 1a), which complements earlier crystallographic structures at intermediate resolution (2.0–3.5 Å) [40]. The 1.65 Å-resolution structure precisely pinpoints water molecules and sidechains that form a pathway for proton translocation. Functional water molecules are often very well-ordered, and this structure abounds with waters whose thermal factors are on par with the most ordered backbones. As further discussed below, the most important residues required for proton-channel function are His37 and Trp41, which associate to form a “His-box” and a “Trp-basket” (Fig. 1a). In the crystal structures of M2TM obtained under other conditions [40], the bundle is more splayed at the cytoplasmic end, partially or fully disrupting the His-box.

M2 has been studied by SSNMR in aligned phospholipid multilayers [50–52] to evaluate the orientation of individual amides via a ^{15}N -based two-dimensional experiment, PISEMA, in which the N-H dipolar coupling and the ^{15}N chemical shift anisotropy are correlated [38]. This method defines the crossing angle, kinking and rotation of a monomeric helix relative to the membrane normal. However, many structures of the *tetramer* can be devised that are consistent with these main-chain restraints, so early SSNMR models of M2TM (2H95, 1NYJ, 2KAD) have now been superseded by more recent, high-resolution crystallographic (3LBW), SSNMR (2KQT, 2L0J) and solution NMR structures (2RLF). Hong and coworkers recently used experimental side chain dihedral restraints and REDOR distance measurements together with the previous dipolar coupling data of Cross and coworkers [51] to obtain a well-defined structure for the M2TM-amantadine complex [28] (2KQT), which is within 1.0 Å r.m.s.d. of the backbone structure of the high-resolution crystallographic structure of M2TM (3LBW) [28].

The M2TM+cyto fragment was recently studied by solution [27,46] and solid-state NMR (SSNMR) [45,47]. The solution structure is at moderate resolution, being defined by 20 inter-monomer NOEs (0.47 per residue), 23 dihedral restraints, and 27 residual dipolar couplings [27] (Figure 1b). The SSNMR structure (2L0J) [47], based on only backbone orientation and membrane depths for the C-terminal helix from EPR studies [53] (Figure 1c), was computed by MD calculations, using these restraints plus reasonable (but nevertheless hypothetical) distance restraints [47]. The solution and SSNMR structures are in reasonable agreement in the TM region; they feature left-crossing transmembrane bundles that place His37 and Trp41 in the pore. However, the cytoplasmic helices differ significantly. In the solution structure [27], it forms a tetrameric bundle extending into the cytoplasm beyond the end of the TM domain (Figure 1b), while the SSNMR structure places the cytoplasmic helix against the C-terminal end of the bundle exposed to the headgroup region of the bilayer [47] (Figure 1c).

One may ask “What is the role of all the detailed interactions formed by the cytoplasmic helices in these structures? Surely, they *must* be essential for proton channel activity!” To answer this question, the five hydrophobic residues (F47, F48, I51, Y52, F55) in the cytoplasmic helix involved in these interactions were simultaneously changed to Ala in the full-length protein [12,20,29,54]. The surface expression level, proton flux, pH-activation,

and drug-binding of this quintuple mutant were indistinguishable from WT, the only change being its inability to promote virus budding and vesicle fission [12,20,29,54]. Thus, the interactions of the cytoplasmic domain in these structures have a subtle effect (if any) on the proton channel activity of the TM domain. They also have little effect on the structure of the TM domain; the overall backbone structures of M2TM are in good agreement with the corresponding TM domains seen in structures of M2TM+cyto constructs, showing small (RMSD 1 to 2 Å) differences within the range associated with subtle changes in bilayer composition [23,55–57]. Thus, studies with the TM construct, which have been conducted in much greater resolution, should provide relevant insight.

The structures of the longer M2TM+cyto constructs help inform the mechanisms of vesicle budding and scission. In a budding virus, M2 localizes to the neck of the budding membrane at 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 cytoplasmic helix is rich in Arg and hydrophobic residues that can promote negative Gaussian curvature [58], and in the SSNMR structure the hydrophobic and positively charged sidechains of the M2 cytoplasmic helix are well positioned to promote negative Gaussian curvature by interacting with phospholipid headgroups. The cytoplasmic helices in the solution NMR structure [27] are less well oriented for this function, but this conformation might serve another functional role. A longer helical bundle is observed in the corresponding BM2 protein from influenza B virus [59]. Also, the C-terminal cytoplasmic helical bundle in KcsA has recently been suggested to dissociate, and its individual helices interact with the membrane during gating [60]. Thus, the full functional role of the M2's cytoplasmic helix remains a fruitful area for further investigation.

TM structures and dynamics

NMR studies on M2TM and M2TM+cyto have shown that the TM helical motions are strongly modulated by pH, being greatest at acidic pH where the protein functions. The pH-dependent broadening of peaks in the amide region of the solution NMR [27], aligned SSNMR [61], and magic angle spinning SSNMR [62] spectra are indicative of backbone conformational fluctuations in the microsecond to millisecond time scale, and the critical residues His37 and Trp41 also show pH-dependent motions in the microsecond regime [23,27,63]. SSNMR is particularly well suited to examine dynamics of M2TM and M2TM+cyto, because one can access both the slow-exchange to the rapid exchange regimes on the same sample by simply altering the temperature [64]. It also allows exploration of different bilayers to evaluate their effects on dynamics [56,62].

Conformation and dynamics of M2 are also modulated by the chain length of the lipid; short chain lipids tend to increase the crossing angle of the helix relative to the membrane normal [55], allowing a better match between the hydrophobic width of the peptide and the bilayer. Hong and coworkers have used cholesterol-rich bilayers to freeze out rapid uniaxial rotation and minimize contributions from exchange-broadening over a wide range of temperature [56]. The addition of cholesterol is also interesting because it is required for maximal proton channel activity [65,66] and is known to stabilize the tetramer [67]. At ambient temperature, M2TM has two conformations at pH 7.5, and a third conformation at pH 4.5 [62]. The binding of drugs and the composition of the surrounding membrane bilayer also has a large effect on the fraction of each conformational form [62].

The M2TM+cyto construct has also been examined in bilayers by SSNMR. Cross and coworkers used a membrane rich in the non-bilayer-forming phospholipid DOPE (DOPC/DOPE 4:1) to achieve good reconstitution and alignment in multilayers [47]. Two

conformations exist in these preparations, based upon doubling of many of the peaks, including S31 (see Figure S3 of the supplementary material from [47]).

The multi-conformational behavior seen in these studies is similar to that seen in parallel solution NMR and SSNMR studies of KcsA, which has multiple structures associated with its gating between resting and conducting states. The opening of the “activation gate” occurs on the low ms time scale [68], and involves large changes in packing of the pore-forming helices, resulting in a 20-Å increase in the diameter of the cytoplasmic section of the pore that leads to the selectivity filter [69]. The structures of the open and closed form of this activation gate, and possible intermediates along the trajectory, have recently been elucidated by X-ray crystallography in KcsA [69], as well as the Na/K channel [70]. The opening motion involves bending and rigid-body tilting of the pore-forming helix (Figure 2a). The use of a minimal construct, lacking the all of the cytoplasmic domains was key to obtaining the full family of conformational states [71]. These truncations removed short segments that, in earlier studies, had engaged in crystallographic packing interactions that otherwise interfered with the marginally stable open activation gate.

Figure 2b shows the corresponding ensemble of the TM regions of all recent structures of M2 obtained by X-ray crystallography, solution NMR, and SSNMR. As for KcsA, the structures fall along a smooth trajectory (Figure 2a). The structures are related by a rigid-body tilt of the pore-forming helix with a pivot point near the top of the bundle, causing variable dilation of the C-terminal end of the bundle near His37 and Trp41. A slight (up to 12°) helical bend minimizes the divergence of the helices in the most bent structures. Both motions are supported by SSNMR studies of M2TM in bilayers [23,51]. The concerted nature of the motions suggests movement along a smooth and functionally relevant energy landscape.

There is a general trend towards greater dilation of the C-terminus proximal to His37 under experimental conditions with increased protonation of this residue [40], although the protonation states are not unambiguously defined. Thus, it is reassuring that MD simulations from the groups of Klein [41,71,72] as well as Cross, Zhou and their coworkers [73,74] showed the same trend as the charge state of the four His residues was increased. A small constriction at the N-terminal Val27 valve [40,41,71–73] accompanies the dilation with increased hydration of His37. Thus the weakening of packing at the C-terminus appears to be compensated by improved packing near the N-terminal end of the bundle. The functional significance of this compensatory motion will be discussed below.

The crystal structures lie within various portions of the ensemble (Figure 2b), ranging from the best-packed structure to one in which the helices diverge maximally near the C-terminus. Thus, it is possible that the micelle environment exaggerate the motions that occur in bilayers [57]. However, TM bundles are often not as uniformly well-packed throughout the entire length of a bundle as in water-soluble proteins, so divergence is not necessarily artificial. Gaps in the packing of TM helices are quite frequent, particularly in channels and pores [75], and tend to locate near Trp residues, precisely as found in the M2 structures. Moreover, Cross and coworkers point out that these divergent crystal structures are in good agreement with their own SSNMR data [76], and MD simulations in phospholipid bilayers (see Figure S4 of [47]). Also, while detergents penetrate between the helices near their C-termini in the crystallographic structures, similar gaps are filled by phospholipids or cholesterol in other membrane proteins [77,78]. It is thus interesting to note that cholesterol is required for efficient proton channel activity [65,66]. In summary, these structures show that M2 moves along a smooth trajectory by rigid-body tilting and slight bending near Gly34.

Mechanism of proton conduction through M2

The biochemical mechanism of proton conduction through M2

The mechanism of M2 proton transport has been thoroughly studied in oocytes, mammalian cells, and vesicles [79–81]. The channel has very high proton-selectivity, although protons are at 10^4 to 10^6 -fold lower concentration than other ions, such as K^+ , at the pH where M2 functions. M2 has minimal conduction at high pH, because the permeant ion (proton) is at low concentration, and also because protonation of His37 is required to activate the channel for conduction [82]. His37 is required for proton-selectivity, and mutants in which this residue is changed to other side chains form less selective ion channels [83]. M2 is arranged with its N-terminus facing the outside of the virus.

M2 from the highly studied A/Udorn/72 and Weybridge influenza A strains show the interesting property of having greater proton flux when the pH on the N-terminal side of the channel (pH_{out}) is lower than pH_{in} , *versus* when the gradient is reversed [83,84]. This property requires the presence of Trp, or another electron-rich natural or an unnatural aromatic amino acid sidechain at position 41, one turn down the helix from His37. However, this His-Xxx-Xxx-Xxx-Trp motif alone is insufficient to impart this asymmetric pH-dependence of the conductance. In fact, M2 from the Rostock strain of the virus, whose sequence shares this invariant motif, has just the opposite behavior. M2 from the Rostock strain has greater outward conductance when the pH_{out} is high and pH_{in} is low than the corresponding inward conductance with the reverse gradient [84]. Two single-residue mutations are necessary to switch from Weybridge or (Udorn-like) to a Rostock-like phenotype: N44D and V27I [84]. In structures of Udorn M2 channels, Val27 forms a narrow valve that controls entrance of protons [71,72], while D44 is indirectly hydrogen-bonded to the indole NH of Trp41 via a water cluster at the exit of the channel (Figure 3). D44 is further held in place by a salt bridge to R45 from a neighboring chain [40,41,47]. Thus, D44 and V27 act as gate-keepers at opposite ends of the channel. Mutating Asp44 to Asn might weaken its interactions with Trp41, Arg45, and the exit water cluster, facilitating proton transfer from His37 to the interior of the virus. Indeed, replacing Asp44 with Asn or a variety of other sidechains increases the rate of proton flux through the Weybridge or Udorn M2 proteins [24]. However, the full Rostock phenotype also requires mutation of the other V27 to Ile. This requirement for end-to-end cooperation is reminiscent of the finding of changes occurring throughout the channel when drugs bind to a specific location within the pore [45], as well as the crosstalk between the occupancy of ions in the selectivity filter of KcsA and the opening/closing of the activation gate on the other side of the membrane [69].

The functional richness of M2's asymmetric conductance is amplified by a recent conductance study of the full-length protein unidirectionally oriented in phospholipids vesicles in the native topology [65,66]. Building on previous studies [86], robust and reproducible reconstitution required careful consideration of phospholipid composition, particularly the inclusion of cholesterol [66]. This system allowed examination of a question critical to the biological function of M2; how can protons accumulate in the viral interior without developing a large electrical potential that impedes further inward proton translocation? If M2 were *perfectly* selective for H^+ (assuming no other conductance is present), then as protons flow into the virus, a large electrical potential would rapidly ensue, prohibiting inward diffusion of protons before the interior and exterior pH are equilibrated. It proved difficult to address this question electrophysiologically due to the problems of maintaining low inner and outer pH for extended periods [83,84]. Recordings in which the exterior is acidified showed that as the interior became more acidic, the proton flux decreased more than could be accounted for by a change in the chemical potential. Thus, the combination of low interior and exterior pH appeared to diminish selective proton flux. By contrast, manipulation of interior and exterior pH in vesicle systems is much easier, and it

was demonstrated that the rate of proton flux was greatest with low pH_{out} and high pH_{in} as in classical experiments [65,66]. However, as the pH_{in} decreases, the proton channel activity of M2 is inhibited. Remarkably, this results in a parallel decrease in its ion selectivity to allow K^+ to occasionally flow outward to maintain electrical neutrality. Additionally channel remained proton-selective; note that even a small decrease from the initial selectivity of 10^6 with the low pH_{out} , high pH_{in} can lead to significant cation flux, because K^+ is present at 10^5 -fold higher concentration than protons. This reciprocal inhibition of proton flux and activation of cation flux with decreasing pH_{in} first allows accumulation of protons in the early stages of acidification, then trapping of protons within the virus when low pH_{in} is achieved. Moreover, these findings are in agreement with M2's recently demonstrated role in activating the inflammasome by disrupting ionic balance in the Golgi [85].

The pH-dependence and magnitude of the proton conductance appears to be optimized to provide the correct degree of proton flux necessary for activity [24] without incurring toxicity to the host cell. Thus, while a large number of mutations to the channel give rise to channels that function *in vitro*, they have systematically altered conductance characteristics [24]. Only a handful of mutations are functional in viruses that are transmissible between humans, birds, or swine, and these tend to be the same mutations that give channels with properties very similar to WT. Residues that are essentially invariant are G34, H37, W41, and A30 [86]. V27 is occasionally mutated to Ala or Ile, S31 is frequently mutated to Asn, and D44 to Asn [87–91]. The D44N mutation, which increases proton flux 3 to 8-fold, is found in viruses in which it is important to maintain neutrality in the late Golgi [92].

The rate of conduction of protons through M2 shows a sigmoidal dependence on pH, reaching a maximal rate of conductance at low pH [20]. Although the absolute flux rate is slow (reaching 100 to 1000 protons/sec at low pH), the channel operates within an order of magnitude of the diffusion-controlled rate at slightly acidic pH [79]. At subsaturating ion concentrations, the flux rate of ions through a pore generally scales with the ion concentration on the side from which it is diffusing multiplied by a second order rate constant. (Ions can flow in both directions, so a net flux of a given species is observed only in the presence of a TM chemical or electrical gradient) [6]. The rate constant for conduction of protons through an aqueous pore of the approximate dimensions of M2 is about $10^8 \text{ M}^{-1}\text{sec}^{-1}$ [82]. This value is about two orders of magnitude lower than in bulk water, reflecting the restricted dimensions and length of the pore. Thus, at pH 6, the rate of proton conduction would be about 100 sec^{-1} ($10^8 \text{ M}^{-1}\text{sec}^{-1} \times 10^{-6} \text{ M}$ proton concentration) [82], similar to the value seen at this pH. Thus, near the endosomal pH range over which the channel functions, M2 is a “slow” channel only because of the low concentration of permeant ions. As the pH is lowered, however, the rate does not increase linearly with the proton concentration, but instead levels off at low pH with a midpoint near 6.

Two mechanisms were suggested for the sigmoidal shape of the pH profile. One suggested that protonation of His37 residues leads to opening of the pore [93–97] to allow proton diffusion via chains of hydrogen-bonded “water wires”. However, this mechanism has not been shown to quantitatively account for the full electrophysiological current voltage curves available for M2 or its saturation at low pH [83,84,98,99]. A second, “shuttle” model [79,100,101], instead posits that His residues along the conductance path are protonated and deprotonated as protons pass through the channel. The rate saturates when the conducting His37 residue is fully protonated [83,99], and the rate-limiting-step at low pH becomes deprotonation of His37 (assisted by coordinated motions of Trp41). Interestingly, two of the structural models predicted in the course of developing the shuttle and gated pore models were shown to be highly similar [102], and both were within approximately 1.5 Å r.m.s.d. of

the high-resolution crystal structure [41]. Such fine-grained features as the geometry of the His-box and Trp41 basket were correctly predicted in the shuttle model [100].

The pKa values of His37 in the tetramer have been determined by SSNMR [39]: the first two protonations ($pK_a = 8.2$) are surprisingly high, the third $pK_a = 6.3$ matches the midpoint of the conductance curve, and the fourth pK_a is ≤ 5 [39]. Thus, the third pK_a was found to be the “conducting pK_a ”, and the shuttling of protons through M2 appears to occur via an alternation of the +2 and +3 states. This situation resembles that in the potassium channels, whose conducting state has a selectivity filter with four K^+ -binding sites but stably binds only two potassium ions at a time, oscillating between 1–3 and 2–4 configurations with nearly equal occupancy [103–107]. These K^+ ions are bound relatively tightly, with a dissociation constant about two orders of magnitude lower than that of the physiological ion concentration [103]. Thus, the binding of the first two ions stabilizes the overall structure and provides a strong driving force to induce a conformation that is selective for binding potassium over sodium ions. Movement of a third K^+ into the filter from a site in the aqueous cavity just below the selectivity filter leads to repulsive of an ion into the cellular exterior. Thus, although the structural details are very different, the negative cooperativity between two tight-binding sites and a third weak site provides both high selectivity as well as rapid ion diffusion in both KcsA as well as M2 [108].

Structural mechanism for proton storage and transport through M2

The above observations suggest a potential mechanism for the conduction of M2 that is compatible with the high-resolution structure of the +2 state as well as the structural ensemble seen for the combined structures (Figure 2b). Protons enter the channel through the Val27 valve via transiently populated water molecules via a Grotthuss mechanism [41,73,94,95,97,109–112]. The Val27 gate might constrict in the +3 and +4 states, possibly minimizing loss of a proton to the exterior when His37 reaches the +3 state [71,72].

Once inside the pore, the excess proton passes through an area of disordered solvent and reaches an “entry cluster” (Figure 3) of water molecules that form tight hydrogen bonds with His37, as shown by SSNMR [63]. In the high resolution structure of G34A, four water molecules form a tight simultaneous interaction with His34 and the strongly dipolar carbonyl of residue 34 stabilizing charge in the His-box, and contributing to the strong basicity of the N^δ of His37 [63]. Two additional waters completely associate with the His-bound waters creating an “entry cluster” that bears a striking resemblance to the gas phase structure of hexa-water with a bound excess proton [113]. In the WT structure with Gly at position 34 it is possible that the cluster expands, providing additional opportunities for charge stabilization.

The His residues engage in a His-box interaction (Figure 3) similar to aromatic boxes [114]. There is no direct H-bonding between the imidazoles; instead they are connected via the “entry cluster” and a bridging dimer that cap the top and bottom of the His-box (Figure 3). The water dimer connects the four N^ϵ nitrogens of the His-box, and is well situated to mediate a π -cation interaction to Trp41 basket (Figure 3) [35]. As mentioned above the indole NH of Trp41 interacts with Asp44 via an “exit cluster” of water molecules (Figure 3). Thus, diffusing protons are stabilized by a very extensive network of hydrogen-bonded, π -cation and dipolar interactions, in effect dispersing the effective charge to the outside of the channel.

The basic arrangement of the His-box is also seen in the solution NMR structures [27] as well as a high-resolution SSNMR structure of the amantadine complex [28]. The direct interaction of the His37 N^δ atom with water molecules has been seen at both low and high pH by SSNMR [63]. On the other hand, the water molecule at the N^ϵ of His37 was found to

be present only at acidic pH, consistent with a role in stabilizing charge via π -cation interactions.

An alternate orientation of the His residues has, however, been suggested, in which the His37 residues are not hydrogen-bonded to water, but rather to one another in a low-barrier hydrogen bond (LBHB) via an imidazole-imidazolium interaction [39,115]. The hallmarks of this interaction is a close approach of the two heavy atoms, resulting in a low (or no) barrier for transfer of the proton from one atom to another [116]. Because of the ultra-rapid exchange of the proton, the two heavy atoms engaged in the hydrogen bond give rise to a single resonance roughly midway between chemical shift in imidazole-imidazolium pairs generally seen for the donor and acceptor in the ^{15}N NMR spectrum at 270 K and above [115]. At lower temperatures, two peaks of equal intensity can be observed for dimers that deviate from true symmetry, although the exchange in the dimers is rapid throughout the temperature range [115]. The His37 residues of M2TM have also been proposed to form a direct LBHB in the +2 state, although the interpretation was ambiguous due to exchange broadening, which obscured key diagnostic peaks [39]. The chemical shifts assigned to imidazole-imidazolium interactions in this study might instead arise from interactions between His37 and water molecules [63]. Moreover, exchange broadening is generally indicative of microsecond to millisecond processes with significantly larger energetic barriers than that associated with a LBHB, so its presence is not good evidence for a LBHB. A second argument for a LBHB came from quantum mechanical (QM) calculations in which the imidazoles were found to form stable pairwise interactions [47]. This computed geometry was used to guide the MD simulation of the aligned SSNMR structure of M2TM+cyto [47]. However, when water was included in more extensive QM simulations [41,109], the imidazoles were instead found to interact via bridging water molecules, as in the crystal structure. The chemical shifts computed from the crystal structure (supporting information of [41]) are also in agreement with the same aligned SSNMR data [47]. Thus, the His-box is clearly formed in at least one conformational form of the channel. It remains possible that there is a direct interaction of imidazoles in some alternate conformers, but direct evidence for this interaction is not definitive. Clearly, the viral membrane mimetic bilayer, with its lack of exchange broadening from axial rotational motions, is well suited for future investigations of this issue [56].

In summary, the recently published high-resolution structure of M2TM, together with many other biochemical and SSNMR studies of M2TM and M2TM+cyto in bilayers, explains how protons enter and are stabilized in the channel, and provides a rationale for the high stability of the +2 state. Furthermore, upon reaching the third protonation state, the highly ordered structure of the His-box is destabilized [63], and the structure of the protein becomes more dynamic, allowing efflux of a proton past Trp41 and into the interior. The mechanism of this final step remains an important challenge. SSNMR [117] and IR spectroscopy [34] studies show an increase in hydration in the +3 state, consistent with the structural and MD studies showing dilation of the C-terminal end of the bundle [71]. Raman spectroscopy [118] shows a protonated His-Trp interaction which forms with a pKa near 6, and presumably reflects residual tertiary interactions retained in the time-averaged structure of the +3 state.

Another challenge will be to refine the original shuttle mechanism which suggested protonation and deprotonation at the N^{δ} and N^{ϵ} of the imidazole occurred by either a water-mediated tautomerization or a ring-flip as recently proposed in greater detail in [63].

Thermodynamic coupling between the free energy of tetramer assembly, proton-binding, conduction, and drug-binding

M2 is a finely tuned machine, with multiple conformational states whose stability and kinetics of interconversion are tightly controlled by pH. Thus, its sequence has evolved to mediate these processes, rather than to simply form a stable static tetramer [2]. Indeed, mutation of each of M2's pore-lining or interfacial positions to either Ala or Phe either had no effect on or enhanced the free energy of tetramerization of M2TM [43]. The only exceptions were at a single position in the pore where substitution with Phe was not sterically feasible, or at the polar residue His37, where mutation to Ala or Phe was strongly destabilizing. Thus, the primary determinant for the strong conservation of sequence in M2 reflects the need to maintain the energetic and kinetic balance between its multiple conformational sub-states. To assess how mutations affect a more restricted set of specific conformations of the tetramer, the binding of amantadine to the channel was measured to a set of variants in which the substitution was not located directly within the binding site [43]. In micelles and bilayers, all of the mutations destabilized amantadine binding or were isoenergetic.

The pKas of His37 in the tetramer are strongly perturbed, so pH should control the stability of the tetramer in a biphasic manner. The pKas of the first two protonations of the tetramer (8) are greater than that of the corresponding monomer (app. 6.5), so in the pH range of about 8 to 6.5 the stability of the tetramer should gradually increase with decreasing pH. On the other hand, because the pKa values for the third and fourth protonations of the tetramer are lower than that of the monomer, stability should gradually decrease with decreasing pH below 6. Overall, the equilibrium constant for dissociation of the tetramer (K_{obs}) at a given pH is:

$$K_{\text{obs}} = \frac{\left(1 + \frac{[\text{H}^+]}{K_{\text{mon}}}\right)^4 K_{\text{tet}}}{1 + \frac{[\text{H}^+]}{K_1} + \frac{[\text{H}^+]^2}{K_1 K_2} + \frac{[\text{H}^+]^3}{K_1 K_2 K_3} + \frac{[\text{H}^+]^4}{K_1 K_2 K_3 K_4}}$$

where K_{tet} is the dissociation constant for the neutral tetramer, K_{mon} is the dissociation constant for the protonated monomer, and K_1 through K_4 are the dissociation constants of the mono, di, tri and tetra protonated His37 in the tetramer.

Analytical ultracentrifugation showed the expected maximum in stability near pH 6.5 for both M2TM and full length M2 [20], confirming the high pKa values for the first two protonations of His37 and helping to define conditions under which the protein could be studied while retaining the tetrameric form. Ion-specific stabilization of the tetrameric form has also been observed for KcsA [119].

The binding of drugs specifically stabilizes the tetrameric form of M2, as expected from thermodynamic linkage. The structure of the physiologically relevant M2-drug complex [28,40] shows many parallels with the complex of KcsA with quaternary ammonium blockers. In both cases, the drugs are bound in an aqueous pocket, blocking access to the selectivity filter or His-box, and also affecting the thermodynamics and kinetics of conformational transitions. As discussed above, binding disrupts the energetic balance of distinct structural state, and also perturbs the pKa of His37 [120].

Recent structure-activity studies have elucidated the interactions required for high-affinity binding [121], and enabled the design of novel inhibitors that are beginning to address the problem of amantadine-resistance [122]. Because the binding site lies along the four-fold

symmetry axis of the channel, a single mutation changes four positions per tetramer, which can have a large effect on not only the ability to bind drugs but also the function of the channel [24]. Thus, only a few amantadine-resistant mutations, namely V27A, L26F, and S31N, have been observed in transmissible viruses in the past eight decades for which a genetic record is available [86,123], although other mutations can easily be observed *in vitro*. The mutations that cause the greatest decrease in inhibition, S31N and V27A, increase the polarity of pore-lining residues.

Computational investigations of amantadine/rimantadine action have involved molecular dynamics (MD) simulations [72,74,95,124], docking [125,126] and small molecule probe mapping [127,128]. Since these studies used various M2 structures/models in varying protonation states, it is not surprising that a consensus failed to emerge from these studies. Using MD, Yi et al found the aliphatic region of amantadine snuggled against the bottom of the Val 27 gate [74], while the drug was found at more C-terminal locations in other simulations [95,129]. Khurana et al [72] performed MD simulations of different protonation states of His37, using crystallographic and solution NMR structures as starting conformations. They found two distinct orientations of the drug in the pore, whose populations depended on the protonation states of His37. These orientations might correspond to the major and minor conformers of amantadine within the pore observed by SSNMR [28].

Figure 4 shows the predominant orientation of the drug [28] docked onto the 1.65 Å high-resolution structure [41]. The ammonium group projects downward towards the entry cluster of water molecules, mimicking a hydronium ion in the act of passing a proton from the central cavity to the His-box. While some rearrangement of the outermost water molecules in the cluster is required to accommodate the more extended drug, rimantadine, the four water molecules associated with the His37 residues appear well positioned to bridge between the sidechain imidazole and the drug ammonium groups. This mode of binding is also consistent with the high affinity of the recently described spiro-piperidine class of inhibitors [121], and has proved to be an excellent model for design of molecules that bind to the mutants V27A and L26F [122].

Conclusion

Recent years have seen breakthroughs in the structural biology of M2, which in turn facilitate the mechanistic analysis of proton translocation. High-resolution structures and dynamic measurements of M2 and mutants under different conditions (pH, lipid compositions etc) are needed to fully elucidate the mechanism. Mutant structures are critical for understanding drug-resistance and providing a basis for drug design. On the other hand, inhibitors developed either through design or screening can facilitate structure determination by stabilizing the M2 tetramer in specific conformational forms.

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Figure 1. Comparison of (a) crystal (PDB: 3LBW), (b) solution NMR (PDB: 2RLF), and (c) solid state NMR (PDB: 2L0J) structures. Backbone is colored in grey, while residues 49–60 are colored in yellow. Critical residues His37 (in cyan) and Trp41 (in green) are key to the extensive hydrogen bond network in the crystal structure. Water clusters are shown in pink.

Figure 2. Motions of pore forming helices of KcsA and M2 shown by overlaying KcsA and M2 structures from different sources. a) Ten structures for KcsA (residues 86–122) (PDB codes: 1R3J, 2HVK, 3EFF, 3F5W, 3F7V, 3F7Y, 3FB5, 3FB6, 3FB7, 3FB8). b) Eight structures were analyzed for M2 (residues 25–46) (PDB codes: 2RLF, 3C9J, 2KQT, 2L0J, 3LBW, and three symmetric structures derived from three different helices of the asymmetric structure 3BKD). Figures are adapted from [130].

Figure 3.

Hydrogen bonding network in the high resolution M2 crystal structure (PDB: 3LBW). Crystal waters are shown in red spheres and named entry cluster, bridging dimer and bridging cluster from Gly/Ala 34 to Asp 44. The histidine residues in the His-box stacks in an edge-to-face conformation and hydrogen bonds to crystallographically well ordered waters in both the entry cluster and bridging dimer. Trp 41 rings are stabilized in a basket-like structure via a third cluster of water molecules-bridging cluster, which bridge the indole NH of Trp41 with the carboxylate of Asp44. This structure is in the + 2 protonation state.

Figure 4.

Docked conformation of four M2 inhibitors with M2 model generated from the high resolution X-ray crystal structure of M2 (PDB: 3LBW) and SSNMR structure of amantadine bound M2 (PDB: 2KQT). Poses are shown with the amine pointing downwards towards His 37. a) Amantadine b) Rimantadine c) Spiro-piperidine d) Spiran amine. e) The structure of tetrabutylammonium (TBA) bound to KcsA (PDB: 2HVK). Note TBA displaces the K^+ hydrate near the entry of the selectivity filter, in a binding manner highly similar to the binding of M2 inhibitors (a–d) to M2, which lie proximal to or displace the top two waters in the entry cluster.