The M2 Proton Channels of Influenza A and B Viruses*

Published, JBC Papers in Press, December 30, 2005, DOI 10.1074/jbc.R500020200

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Influenza virus and influenza B virus are two different enveloped, negative stranded RNA viruses that cause epidemic infections. The virion (virus particle) of each virus contains a small integral membrane protein (A/M2 and BM2, respectively) that functions as a proton channel and is essential to viral replication. These proton channels are of interest because they are among the smallest bona fide ion channel proteins (with the properties of ion selectivity and activation), and they succeed in accomplishing the same function with only a meager similarity in primary amino acid sequence. This similarity is contained in a single turn of the transmembrane helix that appears to impart the channels with their key proton transport properties. One of these proteins, the A/M2 protein from influenza A virus, is the target for action of the antiviral drug amantadine. Thus, these proteins are also important because they are important therapeutic targets (1).

The proton channels of both viruses must function for viral replication to occur (Fig. 1) (2, 3). Both viruses enter the infected cell by endocytosis, and the interior of the virion must become acidified while it is contained in the endosome as a prerequisite for uncoating (release of genetic material to the cytoplasm) (4–6). The proton channels serve this acidification function. This review will discuss the mechanisms for proton selectivity, for turning-on (activating) and for inhibiting these proton channels.

Basic Biochemical Properties of the Proteins

Both A/M2 and BM2 proteins are homotetrameric, type III integral membrane proteins containing a small N-terminal ectodomain, a single transmembrane domain, and C-terminal cytoplasmic tail. The transmembrane domain acts both as a signal sequence and a membrane anchor during protein synthesis. The predicted membrane-spanning domains of A/M2 and BM2 are 20 amino acids long, and the N-terminal domain of the BM2 protein (7 residues) is shorter than that of the A/M2 protein (23 residues). Long cytoplasmic C-terminal domains characterize both the A/M2 protein (53 residues) and the BM2 protein (82 residues). The only homology between the amino acid sequences of these two proteins is found in the HXXWX motif of the inner membrane-spanning residues; this motif proves to be critical to the ion channel activity (see below). Both A/M2 and BM2 behave as homotetramers in cross-linking experiments (6–9), and the active oligomeric state of the A/M2 protein was demonstrated to be a tetramer (10). Post-translational modifications occur to the A/M2 protein but do not seem to be important for ion channel function (11). Thus, the flux of protons across the membrane must occur within the pore formed by the four identical subunits of the transmembrane domain of the protein, and protons interact with the amino acids that form the lining of the pore.

Functional Mechanism for Proton Transport

Both ion channels are very selective for protons (12–16), and their selectivity depends on a histidine residue in the transmembrane domain. Ion selectivity measurements have been made using in vitro expression systems (12, 16–19) and by reconstitution of ion channel activity from recombinant protein in bilayers (20, 21) or liposomes (15). The high proton selectivity of the channel is lost when transmembrane domain His37 is replaced with glycine, alanine, glutamic acid, serine, or threonine (22, 23), making the mutant channel capable of transporting Na+ and K+ as well. The ion selectivity of histidine substitution mutant proteins is partially restored by adding imidazole buffer to the solution bathing the expressing cell (23). Thus, the imidazole side chain of histidine plays an essential role in the specificity for proton transport.

The mechanism for transport of protons through the aqueous pore of the channel has not been established with certainty, but two observations are informative. First, the specific activity (single channel conductance) of the wild-type A/M2 ion channel is very low (it transports roughly 10⁴ protons per tetramer per second at pH 5.7, the pH found in endosomes) (15, 19). Second, the kinetic isotope effect measured when deuterium replaces hydrogen shows that this replacement results in a decrease in conductance by an amount greater than the ratio of diffusion coefficients of the two isotopes. These observations suggest that bulk transport of hydronium ions is not responsible for proton transport (19). Two other mechanisms have been suggested for proton transport. First, imidazole might serve as a “relay” molecule (Fig. 2), binding protons presented from one end of the channel and releasing them to the other end by dissociation; this mechanism might be assisted by autoionization of imidazole (24). Second, short-lived proton “wires” might open to allow shuttling of protons from one water molecule to another in the pore, without the water molecules themselves moving (19). Energy minimization simulations support the former model (25) and molecular dynamic simulations support the latter model (26). Thus, the exact mechanism for transport of protons with high selectivity is not known.

Mechanism for Opening of the A/M2 Ion Channel

The A/M2 channel does not conduct protons under all conditions; to do so the pH of the medium bathing the N-terminal ectodomain, pH<sub>ext</sub>, must be lowered below pH 7. This ability to open and close is dependent on the action of a single transmembrane domain residue, Trp<sub>41</sub>. Two observations suggest that the channel is closed when pH<sub>ext</sub> exceeds pH 7.5 and is opened when pH<sub>ext</sub> is lower than pH 6.5. First, oocytes that express the channel become rapidly acidified when they are bathed in solutions of low pH, but upon restoration of pH<sub>ext</sub> to its normal value their internal pH recovers only very slowly. This restoration of pH occurs much more slowly than the re-alkalization of cells treated with the protonophore carbonyl cyanide p-trifluoro-methoxyphenylhydrazone (27), suggesting efflux of protons from M2-expressing cells is impaired by elevated pH<sub>ext</sub>. Second, cells expressing A/M2 protein that are injected with acid (e.g. 1 M HCl) while pH<sub>ext</sub> is above pH 7.5 do not experience an efflux of protons, but when pH<sub>ext</sub> is low, efflux of protons can be brought about by applying a positive voltage to the inside of the cell (12, 14, 17, 19). Thus, high pH<sub>ext</sub> closes the channel and low pH<sub>ext</sub> opens (activates) the channel.

Several lines of evidence point to Trp<sub>41</sub> as the key residue in opening and closing the channel pore (27) (1). For the wt channel, outward currents are not observed when pH<sub>ext</sub> is high, regardless of the means taken to establish an outward electrochemical gradient for protons. Unlike the case for the wt ion channel, it is possible to observe outward proton currents under these conditions for mutant ion channel proteins in which Trp<sub>41</sub> is replaced with amino acids having a small side chain (2). It is possible to improve the closing of a mutant ion channel in which Trp<sub>41</sub> is replaced with Cys by placing a functional group resembling the Trp side chain on the Cys sulfur atom (3). Cu(II) injected intracellularly is able to coordinate with His<sub>37</sub> in a mutant ion channel in which Trp<sub>41</sub> is replaced by Ala but is not able to coordinate with His<sub>37</sub> when injected into cells expressing the wt channel. An explanation for these observations is that the bulky indole side chain of Trp<sub>41</sub> interferes with the passage of protons when pH<sub>ext</sub> is high (27). This interpretation is supported by oxidative disulfide cross-linking analysis showing that a structural rearrangement occurs in this region of the protein when pH<sub>ext</sub> is altered (28). Furthermore, resonance Raman spectroscopy has shown that pH-dependent interactions occur between His<sub>37</sub> and Trp<sub>41</sub>, perhaps between protonated imidazole and the pi electrons of indole (29). Thus, opening and closing of the channel depends on pH<sub>ext</sub> and probably involves structural alterations that encompass Trp<sub>41</sub>. It is noteworthy that the key functional elements of the channel, His<sub>37</sub> and Trp<sub>41</sub>, are contained in a single turn of the transmembrane helix of this very compact channel.

* This minireview will be reprinted in the 2006 Minireview Compendium, which will be available in January, 2007. This work was supported by Research Grants R01AI-31882 (to L. H. P.) and R37AI-20901 (to R. A. L.) from the NIAID/National Institutes of Health. This is the second article of three in the Virus-Host Interaction Minireview Series.

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3 The abbreviation used is: wt, wild-type.
Identification of the Pore-lining Residues of the A/M2 Ion Channel

Because protons must pass through the membrane via the pore of an ion channel, it is very important to identify the residues that line the pore and to obtain a rough idea of the secondary structure of the transmembrane domain. The former has been done for the A/M2 ion channel by combining cysteine scanning mutagenesis and water-soluble sulfhydryl-specific reagents that attach a large hydrophobic adduct to cysteine residues (30). If the conductance of a mutant channel with a cysteine residue in a particular location is diminished by the reagent, then it is concluded that the sulfhydryl moiety of the cysteine faces the aqueous pore at that location. The conductances of cysteine mutant proteins A30C and G34C were diminished when the reagent was applied to the medium bathing the N-terminal ectodomain. The conductance of the cysteine mutant protein W41C was decreased when the reagent was injected into the cytoplasm but not when applied to the bathing medium. Moreover, the G34C mutant protein was not affected by cytoplasmic injection of the reagent. These results are consistent with His37 forming a barrier to large molecules and are also consistent with the proposed role for Trp41 as a gate capable of closing the channel. Cysteine scanning mutagenesis was also used together with oxidative disulfide cross-linking (28) to show that residues 27, 30, 34, 37, and 41 formed dimers most rapidly. Most interesting, however, was the finding that when oxidation was performed at low pHout (pH 5.2) cross-linking was much lower for residues 40, 42, and 43 than when performed at neutral pHout (pH 7.4), suggesting that a pH-dependent alteration of conformation occurs in this region of the molecule. The secondary structure of the transmembrane domain of the A/M2 channel has been studied with solid state NMR spectroscopy of the transmembrane peptide (31–34) and the full-length A/M2 ion channel protein (35, 36). These studies concluded that the orientation of the helical bundle forming the transmembrane domain has a tilt angle of 25 degrees and that transmembrane domain Trp of one subunit interacts with His of an adjacent subunit (34). Furthermore, hydrogen-deuterium exchange measurements showed that residues in the transmembrane helix exchange more rapidly than residues located in the cytoplasmic domain, consistent with the presence of an aqueous pore. Thus, the aqueous pore of the A/M2 ion channel is formed by Ala30, Gly34, His37, and Trp41, and these residues are found on a transmembrane helical bundle having a tilt angle of ~25 degrees.

The Cytoplasmic Tail of the A/M2 Ion Channel

The cytoplasmic tail is the largest domain of both the A/M2 and BM2 proteins, and this domain has been found to be essential for the function of the
A/M2 channel. Truncation of the A/M2 channel at residue 61 or shorter resulted in mutant ion channels with activity that could not be sustained (37). Solid state NMR experiments have provided the first direct structural information about the cytoplasmic domain for residues 45–62 (36). These studies indicated that an amphipathic helix is found in this region, associated with the inner membrane leaflet. This amphipathic helix was found in the same region of the cytoplasmic tail that had been found to be essential for ion channel activity (37). Thus, even though ion channel activity has been demonstrated with only transmembrane peptides (38), the cytoplasmic tail certainly plays an essential role in the function of the wt ion channel protein.

Summary of Structure-Function Relationship of the A/M2 Ion Channel

The available biochemical and solid state NMR data indicate that the transmembrane domain of the A/M2 protein is comprised of a four-helix bundle with a tilt of about 25 degrees. These experiments are also consistent with the functional studies that showed that these residues Val37, Ala39, Gly43, His47, and Trp41 line the aqueous pore. Furthermore, these results indicate that His47 forms a barrier to large molecules and that Trp41 functions as a gate that closes with high pH

Aquapor amphipathic helix. Functional studies indicate that the portion of the cytoplasmic domain nearest the membrane is important for normal ion channel function, and solid state NMR studies indicate that this domain brings it into close proximity to the inner membrane leaflet. The most remarkable aspect of this channel is the observation that much of its functionality is provided by the His47 and Trp41 residues found in one turn of the transmembrane helical bundle.

Inhibition of the A/M2 Ion Channel

This topic is important because antiviral drugs that inhibit the M2 ion channel also inhibit replication of the influenza A virus, and understanding the mechanism of inhibition would be helpful in developing better inhibitors. The antiviral drug amantadine and its derivative rimantadine inhibit the replication of the influenza A virus but not influenza B virus (39). Amantadine inhibits the A/M2 ion channel (12, 17, 40) but not the BM2 channel (16), which is consistent with amantadine inhibiting influenza A virus but not influenza B virus replication. Several lines of evidence indicate that inhibition of viral replication by amantadine results from the inhibition of A/M2 proton channel activity. The first evidence comes from amantadine-resistant escape mutant viruses in which the site of the mutation was mapped and found to lie in the A/M2 transmembrane domain (41). When these escape mutation proteins were expressed in oocytes (17, 42) or mammalian cells (12, 18) their currents were found to be insensitive to amantadine. The second line of evidence comes from consideration of the virus life cycle. Viral uncoating requires acidification of the virus prior to fusion with the endosomal membrane (43–45), and the M2 protein is capable of providing the needed acidification when the virion is contained in the endosome. The viral genome encodes three integral membrane proteins (46), and only A/M2 is capable of proton transport. Thus, inhibition of the essential proton transport function of the A/M2 ion channel results in inhibition of replication of the virus.

Five observations, taken together, suggest a mechanism for inhibition by amantadine: 1) Two of the mutations that result in resistance to amantadine occur on residues that have been found by cysteine scanning mutagenesis to line the aqueous pore. Both of these mutations are to residues that are less hydrophobic (A26T and G34E) than the native residue (41). 2) Inhibition of the channel occurs more readily when pH of the bathing medium is high (40). 3) Amantadine only inhibits when it is applied to the medium bathing the N-terminal ectodomain and not when applied to the C-terminal cytoplasmic tail; injection of as much as 1 mM amantadine into cells expressing the A/M2 channel does not inhibit the channel, whereas application of 10 \( \mu \text{M} \) to the solution bathing the ectodomain inhibits fully (40). 4) Neutron diffraction studies of amantadine applied to the M2 transmembrane peptide show the compound to lie in the outer region of the membrane (47). 5) Amantadine inhibits with 1:1 stoichiometry (40). These observations suggest that amantadine acts from the outside of the aqueous pore, that the hydrophobic adamantane group interacts with hydrophobic pore-lining residues, and that perhaps the ammonium nitrogen of amantadine shares a hydrogen bond with an unprotonated imidazole histidine (19). If such hydrogen bonding occurred it would interrupt the interactions formed by the ring of His and Trp residues from adjacent subunits (34). Thus, amantadine probably resides within the aqueous pore of the channel when it inhibits but probably does not inhibit by simply blocking the pore.

Why Doesn't Amantadine Inhibit the BM2 Ion Channel?

It is useful to compare the amino acid sequences of the outer region of the transmembrane domain of these two ion channels because amantadine inhibits the A/M2 channel by acting in the outer region of the pore (see above). The pore-lining residues of A/M2 have been identified in previous studies (30, 48). It is reasonable to accept the conclusion that His37 and Trp41 of the BM2 protein are pore-lining residues because mutation of these residues causes alterations in BM2 function that are similar to those found by mutation of His47 and Trp41 in the A/M2 ion channel. Accepting these residues as pore-lining and assuming that the BM2 transmembrane domain forms a helical bundle (Fig. 3), alignment of the sequences of the two channels shows that polar serine residues occur in the BM2 channel in locations that are predicted to be pore-lining. The presence of polar residues would be expected to reduce the affinity of the hydrophobic adamantane moiety of the inhibitor. It is of interest to note that replacement of a hydrophobic residue with a polar residue occurs in an amantadine-resistant escape mutation of the A/M2 ion channel. The mutation M2-A30T is amantadine-resistant and in fact quite active (42). Thus, the reason that the BM2 ion channel is not inhibited by amantadine is probably because the BM2 channel pore region is lined with polar, and not hydrophobic, amino acids. This explanation shows that the inability of amantadine to inhibit the BM2 protein is understandable in terms of the primary amino acid sequence of its transmembrane domain.

Remaining Questions

One of the remarkable properties of the A/M2 ion channel is its high selectivity for protons, and two questions stem from this property. The first is whether selectivity is achieved by a “proton wire” mechanism or by a mechanism in which the imidazole of transmembrane His37 relays protons, binding and releasing them but acting as a barrier for other ions. In answering this question it will be of use to consider that, although the channel is not permeable to \( \text{K}^+ \) or \( \text{Na}^+ \), it is permeable to ammonium and hydroxyamine (13). The second question posed by the very high proton selectivity of the channel is “how can it transport enough protons across the virion membrane to permit sufficient acidification to allow uncoating to occur?” When protons flow into the virion they will bring with them a positive electrical charge. As the only pathway for ions to flow across the virion membrane is the M2 ion channel proton, their influx will impart a positive voltage on the inside of the virion. It takes only a few dozen ions entering the virion to make its voltage sufficiently positive to stop further proton influx, and this small number of ions seems insufficient to acidify the virion substantially. Perhaps the flow of protons occurs at the very moment of virion fusion with the endosomal membrane, and endosomal ion channels prevent formation of a positive voltage. A separate question has to do with the cytoplasmic tail of the A/M2 and BM2 proteins. The cytoplasmic tail of both of these proteins is larger than the other domains, and its full-length does not seem to be absolutely necessary for ion channel activity. Perhaps the cytoplasmic tail serves another purpose, e.g. facilitating assembly of the virion. Finally, an important challenge to chemists would be the discovery of a compound that inhibits the function of the HXXXW motif of both proteins without interfering with cellular processes.

Acknowledgments—We thank Drs. Paul Loach and R. Gundloch for reading the manuscript.
