The Gate of the Influenza Virus M₂ Proton Channel Is Formed by a Single Tryptophan Residue*

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The influenza virus M₂ proton-selective ion channel is known to be essential for acidifying the interior of virions during virus uncoating in the lumen of endosomes. The M₂ protein is a homotetramer that contains four 19-residue transmembrane (TM) domains. These TM domains are multifunctional, because they contain the channel pore and also anchor the protein in membranes. The M₂ protein is gated by pH, and thus we have measured pH-gated currents, the accessibility of the pore to Cu²⁺, and the effect of a protein-modifying reagent for a series of TM domain mutant M₂ proteins. The results indicate that gating of the M₂ ion channel is governed by a single side chain at residue 41 of the TM domain and that this property is mediated by an indole moiety. Unlike many ion channels where the gate is formed by a whole segment of a protein, our data suggest a model of striking simplicity for the M₂ ion channel protein, with the side chain of Trp⁴¹ blocking the pore of the M₂ channel when pH⁴ is high and with this side chain leaving the pore when pH⁴ is low. Thus, the Trp⁴¹ side chain acts as the gate that opens and closes the pore.

The prediction that the influenza A virus M₂ protein has a proton-selective ion channel activity (Refs. 1 and 2 and reviewed in Ref. 3) arose from a coupling of various observations on the life cycle of influenza virus. The M₂ protein is an integral membrane protein that is expressed at the plasma membrane of influenza virus-infected cells and is incorporated in small amounts into budding virions (4, 5). Studies on the mechanism of action of the anti-viral drug, amantadine (1-aminoadamantane hydrochloride), indicated that viral escape mutants resistant of action of the anti-viral drug, amantadine (1-aminoadamantane hydrochloride), indicated that viral escape mutants resistant to amantadine acting two steps in the life cycle, virus uncoating and virus maturation. The effect of amantadine on inhibition of uncoating is general to all strains of influenza A virus (7, 8) (reviewed in Refs. 3, 9–11). When a virion has entered the cell by receptor-mediated endocytosis and the virus particle is in the acidic environment of the endosomal lumen, the M₂ ion channel is activated and conducts protons across the viral membrane. The lowered internal virion pH is thought to weaken protein-protein interactions between the viral matrix protein (M₁) and the ribonucleoprotein (RNP) core (7, 12–15) (reviewed in Ref. 16). In the presence of amantadine, influenza virus uncoating is incomplete, because the M₁ protein is not released from the RNPs and the RNPs fail to enter the nucleus. Normally, influenza virus RNPs are transcribed and replicated in the nucleus (reviewed in Ref. 10). For some influenza virus subtypes, amantadine inhibits a “late” step in virus replication. The M₂ ion channel activity is activated during transport of the M₂ protein through the exocytic pathway; this ion channel activity raises the luminal pH of the trans Golgi network (TGN), equilibrating pH with that of the cytoplasm (1, 17–22). Thus, the intraluminal pH of the TGN is kept above the threshold at which the hemagglutinin (HA) conformational change to the low pH-induced form occurs, therefore preventing HA aggregation, which blocks virus release from cells.

Direct evidence that the M₂ protein has an ion channel activity was obtained by using electrophysiological techniques and oocytes of Xenopus laevis (23–29) or mammalian cells (30–33) that expressed the M₂ protein. It was found that the M₂ channel is blocked specifically by amantadine, is highly proton-selective, and is opened (activated) when the N-terminal ectodomain is exposed to a low pH environment (25, 30, 31, 34). Furthermore, when mutations in the M₂ protein TM domain that confer resistance to amantadine were introduced into the M₂ protein and the mutants expressed in oocytes, the ion channel activity was found to be insensitive to amantadine (25). In addition, when either peptides corresponding to the M₂ TM domain or purified M₂ protein were incorporated into planar bilayers, an amantadine-sensitive current was measured (34–37).

The M₂ ion channel protein is a homotetrameric integral membrane protein with each chain of the mature protein containing 96 amino acid residues (2, 4, 5, 38–43). The coding regions for the M₂ protein have been conserved in all known strains of avian, swine, equine, and human influenza A viruses, and the amino acid sequence of the M₂ protein TM domain has been conserved to a greater extent than the remainder of the protein (44). The TM domain consists of 19 residues, and a considerable body of experimental evidence indicates that the M₂ protein TM domain constitutes the proteinaceous core (the channel pore) that allows a flux of protons across the membrane. M₂ protein TM domain histidine 37 is essential for both ion selectivity and activation by a low pH environment at the M₂ N-terminal ectodomain that resides external to the virion (pH⁴) (25, 28).

Activation of most ligand- and voltage-gated ion channels is a process that requires detection of the activating signal and coupling to a “gate,” the portion of the protein that prevents...
conduction while in the unactivated state (45). For many ion channel proteins, the portion of the protein that detects the activating signal (46–50) or acts as the activation gate (51–54) may be as large as an entire TM domain consisting of several turns of an α-helix (55, 56). The gate of Shaker-type channels has been studied by comparing atomic structures of the closed state of the KcsA channel with the open state of the calcium-activated MthK channel (57, 58). The gate is postulated to be formed by the cytoplasmic residues of the “inner” transmembrane helix. In the closed state the cytoplasmic residues of this helix oppose one another closely at a conserved Ala residue, and opening results from a splaying apart of the cytoplasmic residues starting from a conserved Gly residue that serves as a “hinge.” This postulated splaying open of the helices in the open state is consistent with the increased accessibility of the internal residues to organic reagents that occurs when the Shaker family channels are activated (52, 59). The influenza virus M₂ protein is a model of minimalism because it has only a single multifunctional TM domain, which contains the pore of the channel (25), acts both to target the protein initially to the membrane of the rough endoplasmic reticulum (ER) and anchors the protein in the ER membrane (61), and, as we show here, contains the activation gate.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis, Protein Expression, and Measurement of Electrical Activity**—The cDNA encoding the influenza virus A/Udorn/72 M₂ protein was subjected to site-directed mutagenesis using four-primer PCR. The nucleotide sequence of the entire coding region of the altered cDNAs was confirmed by nucleotide sequencing.

**Culture and Microinjection of Oocytes**—Oocytes were removed from female *X. laevis* (Nasco, Fort Atkinson, WI), defolliculated by treatment with collagenase B (2 mg/ml; Roche Molecular Biochemicals), and incubated in ND96 (96 mM NaCl, 2 mM KCl, 3.6 mM CaCl₂, 1 mM MgCl₂, 2.5 mM pyruvic acid, 5 mg/ml gentamicin, 5 μM HEPES, pH 7.6, osmolality ~210 mOsm/kg) at 19 °C. Oocytes at stage V were microinjected with 50 nl of mRNA (1 ng/nl) on the day after defolliculation, incubated for 24 h at 19 °C before use (25).

**Measurement of Membrane Current and Intracellular Injections during Recording from Oocytes**—Whole-cell currents were measured using a two-electrode voltage clamp. Electrodes were filled with 3 mM KCl, and the oocytes were bathed in either Barth’s solution (88 mM NaCl, 1.3 mM KCl, 2.4 mM NaHCO₃, 0.3 mM NaNO₃, 0.71 mM CaCl₂, 0.82 mM MgSO₄, 15 mM HEPES, pH 7.5, osmolality ~210 mOsm/kg) or a modified solution containing 120 mM NaCl in 2 s. Oocyte holding potential was ~20 mV unless stated otherwise. Voltage clamping was achieved with a two-electrode voltage clamp apparatus and the PCLAMP® program. Intracellular injections of CuCl₂, hydroyxynbenzyl methanethiosulfonate, and their water controls were confirmed by inclusion of a small quantity of 6-carboxyfluorescein. The quantity of fluorescent dye present in the intracellular injections and also conducted outward H⁺ current upon return to an alkaline bathing solution (arrow). *B*, oocytes expressing the wt M₂ protein bathed in alkaline solution did not display outward currents when injected with HCl. Those expressing the M₂-W41C and -F (see arrow) mutant proteins did (ANOVA *p* < 0.01; injections confirmed with a fluorescent indicator, ANOVA *p* = 0.25; M₂-W41C not shown), showing that the mutant channel proteins were open at high pH values, whereas under these conditions the wt M₂ channel protein was closed.

**RESULTS**

To identify the region of the M₂ ion channel protein that serves as its activation gate and to assess the extent to which the M₂ ion channel gate was open, oocytes of *X. laevis* expressing the M₂ protein were used. Because the M₂ ion channel is both activated by elevated H⁺ concentration and conduct H⁺ (31), the absence of inward H⁺ currents in the presence of low H⁺ concentration does not permit the determination that the channel is closed. One effective way to show that the channel is closed is to show that in the presence of a deliberately induced high H⁺ concentration inside the cell there is no outward current while membrane voltage is held at a constant value with high driving force for H⁺. Thus, we assessed gating by measuring the presence of outward currents using two different acidification/alkalinization protocols while membrane potential was clamped to a constant value. Both these procedures showed the gate of the wt M₂ ion channel to be closed in high pH medium (Fig. 1, A and B). First, the oocytes were bathed in acidic (pH 5.9) medium, imposing an inward current sufficient to acidify the ooplasm by about one pH unit (31), and then the medium was switched rapidly to an alkaline medium (pH 8.5; Fig. 1A). The outward current expected if the gate were open in the alkaline medium was not observed (Fig. 1A). Secondly, HCl was injected into oocytes that were bathed in alkaline medium (Fig. 1B), and again outward currents were not observed. In earlier experiments we had observed that the currents of oocytes expressing the M₂-W41C mutant protein were larger than those for wt M₂ protein (62), leading us to suspect that the large iodole group of the highly conserved Trp⁴¹ residue might limit current amplitude. Thus, to test the possibility that Trp⁴¹ acts as the channel gate, mutant proteins containing residues at position 41 with less bulky side chains were examined for outward currents. When Ala, Cys, or Phe were substituted for tryptophan, outward currents were able to flow from acidified oocytes (Fig. 1A, arrow). Two findings supported the conclusion that this difference between mutant and wt M₂ proteins was due to gating by pHₗₒᵤₜ and not due to a general inability of the wt M₂ protein to conduct outward current (rectification). First, the membrane conductance of oocytes expressing the wt protein, measured upon reintroduction of bathing solution of pH 8.5 following bathing in low pH solution, returned to nearly the low value.
measured at pH 8.5 prior to acidification. Secondly, the wt M2 channel protein has been demonstrated to conduct outward current when pHout is low and the membrane voltage is sufficiently positive (26, 31). Outward currents were also observed for oocytes expressing M2-W41C (7 oocytes) and M2-W41F (8 oocytes) mutant proteins when HCl was injected intracellularly. In contrast to oocytes expressing the wt protein, the conductance of oocytes expressing each of these three mutant proteins, measured upon reintroduction of bathing solution of pH 8.5 following bathing in low pH solution, remained at the elevated value measured during the acidification in low pH solution. To better understand the gating of the M2 proton channel, we compared the data obtained from M2 protein with data obtained from a H+–transporting compound that does not have the ability to be gated, the protomophore FCCP. In contrast to the findings for wt M2 protein, current did flow from acidified oocytes treated with FCCP when pHout was increased (Fig. IA; see also Ref. 31). This result is similar to that obtained when the indole side chain of Trp41 of the M2 protein was replaced with Ala, Cys, or Phe. We also measured currents for acidified oocytes expressing the M2-W41Y mutant protein and found that outward H+ current did not flow after the transition from low to high pHout (see Fig. 4). We ascertained that the outward currents of oocytes expressing the M2 protein were specific to the M2 protein by applying the specific M2 ion channel inhibitor, amantadine (100 μM) (25, 29). The outward currents from oocytes expressing the mutant M2 proteins (Fig. 2A), but not the currents from oocytes treated with FCCP (31), were inhibited by amantadine. Thus, these data indicate the gating properties of the M2 ion channel protein are determined by the side chain of the residue at position 41 of the TM domain and can be influenced by a single hydroxyl moiety.

The amplitude of the charge carried by the outward current (Fig. 2B) and the extent of acidification of the oocyte while it was bathed in low pH medium were quantified for each of several values of acidification. The acidification was measured from the change in reversal voltage, Vrev, of the amantadine-sensitive current (31). It was found that the charge was proportional to the extent of the acidification that occurred while the oocyte was bathed in solution of low pH (Fig. 2C). This relationship was very useful because it allowed the comparison of the outward currents from different oocytes that have different levels of expression of the M2 protein.

Given that Trp41 functions as a gate for outward current, it was important to determine whether this residue also influences the openness of the channel at high values of pHout. Thus the relationship between conductance and pHout of the wt channel protein with that of the M2-W41A, -C, -F, and -Y mutant proteins was compared. If the gate of the M2 ion channel protein is removed by a mutation that does not remove its selectivity filter, then the relationship between conductance and pHout ought to show a greater degree of openness at high pHout for the residue 41 mutant channels than for the wt M2 channel. As shown in Fig. 3, the relationship between conductance and pHout was shifted to higher pH values for all the M2 mutant ion channels, indicating a greater degree of openness for the mutant channels at high pHout (see below for discussion of M2-W41Y mutant). The conductance measured at pH 8.2, normalized to the value at pH 4.5, was consistently higher for oocytes expressing the mutant proteins than for oocytes expressing the wt protein. We were unable to measure this small conductance with sufficient precision to tell whether there was a biologically significant difference among the mutant proteins in their minimum conductance. We also compared the Vrev of the currents of oocytes expressing these four mutant proteins with that of the wt protein. The measurement was made 10 s after switching from bathing medium of pH 8.5 to bathing medium of pH 5.9, at which time some acidification of the oocyte had already begun (31). The values for Vrev were in the range of 35–42 mV and did not differ between wt and the various M2-W41 mutants tested (ANOVA, p = 0.28, 28 oocytes), indicating that the ion selectivity of these four mutant proteins does not differ significantly from that of the wt protein. Thus, the observed shift to higher values...
of pH_{out} (Fig. 3) is consistent with the mutant M2 proteins lacking a region that blocks the pore at high pH_{out}.

Although these results confirmed our suspicion that the bulky indole side chain of Trp41 is the gate of the channel, it is possible nevertheless that other amino acids in the inner TM-spanning region of the protein, but not the wider amantadine-accessible outer region of the TM domain (29, 63), also participate in the gating process. Therefore, we performed cysteine-scanning mutagenesis for the residues that form the cytoplasmic-proximal α-helical turns of the TM domain (42, 62). We used the presence of outward currents into alkaline solutions as an indication that the gate was open. It was found that replacement by cysteine at only one position, Trp41, produced a protein that allowed significant outward currents to flow (Fig. 4).

To determine whether mutation to cysteine at residue 41 causes large scale changes in the conformation of the protein, a cysteine-specific reagent was injected into oocytes expressing the M2-W41C mutant protein, and the gating properties were examined to see whether they were altered in a manner consistent with the known biology. Ideally, one would prefer to restore the function of the wt M2 channel, but the reagent necessary to produce a Trp-like side chain, 7-indole methanethiosulfonate, was not commercially available. We thus resorted to restoring the function of the M2-W41C mutant channel from the M2-W41F control mutant protein, because the M2-W41Y mutant channel resembled the wt M2 channel by not permitting outward currents to flow from acidified oocytes into solutions of high pH_{out}. The reagent chosen to do this, hydroxybenzyl methanethiosulfonate, was expected to produce an altered channel that mimicked the M2-W41Y mutant channel by forming a Tyr-like side chain consisting of a phenol adduct with cysteine. It was considered likely that this adduct would alter the function of the M2-W41C mutant channel protein, causing it to mimic the M2-W41Y mutant channel protein if the conformation of the M2-W41C mutant protein permits the reagent to

**FIG. 3.** Relationship between membrane conductance and pH_{out} for oocytes expressing the wt M2 ion channel protein and the M2-W41A, -C, -F, and -Y mutant proteins. Conductance was normalized to the value obtained at pH 4.5. Note the shift to higher pH values for the mutants (ANOVA p < 0.01).

**FIG. 4.** Cysteine scanning mutagenesis of the M2 ion channel protein in the TM region from the His^{77} selectivity filter to the cytoplasmic end. For each residue, outward H^{+} current was measured and its amplitude normalized to the amount of prior oocyte acidification (as measured by the change in V_{rev}, as described in Fig. 2C). The numerals below each residue are the number of cells from which recordings were made. Mutation to cysteine of only residue 41 resulted in the presence of detectable outward currents (p < 0.01, two-way ANOVA Student-Newmann-Keuls). Asterisks indicate mutations for which deviation from wt is significant.

**FIG. 5.** Hydroxybenzyl methanethiosulfonate (structure shown) reduced the amplitude of outward currents of the M2-W41C mutant protein that were measured after return of pH of the bathing medium from a low to a high value (see Fig. 1A). Reaction with the protein would be expected to produce a tyrosine-like adduct. Because the outward currents of the M2-W41Y mutant protein are smaller than those of the M2-W41C mutant protein, this modification would be expected to reduce outward current amplitude. The reagent was injected into oocytes that expressed either the M2-W41C or control M2-W41F mutant proteins, and the outward currents were measured (chart below). The amplitude of the outward current (normalized as described in Fig. 2C) was compared with the amplitude of the current of oocytes that were not injected with the reagent. The normalized outward current amplitude was reduced significantly (*) (ANOVA p < 0.05) for the oocytes expressing the M2-W41C mutant protein but not for the oocytes expressing the M2-W41F control mutant protein.
react with its cysteine side chain. Amantadine-sensitive currents were recorded 30 min after injection of 50 nl of 40 mM reagent during a 2-min period in pH 6.2; during the 30-min period, oocytes were bathed in medium of pH 7.5. Intracellular injection of this reagent significantly decreased the amplitude of outward currents from acidified oocytes (see Fig. 5, 30% reduction; ANOVA p < 0.05), consistent with the data obtained for the M2-W41Y mutant protein. The currents of control oocytes expressing M2-W41F mutant protein were not affected by intracellular injection of hydroxybenzyl methanethiosulfonate (ANOVA p > 0.48, n = 6; injections confirmed by co-injection of fluorescent tracer). This result confirms the notion that the nature of the side chain at M2 residue 41 determines the gating properties of the M2 ion channel.

To add support to the notion that the indole side chain of M2-Trp41 is capable of preventing current flow through the conducting pore of the M2 protein, we investigated whether this side chain prevented a reagent applied intracellularly from accessing the cytoplasmic portion of the channel pore. Advantage was taken of our finding (64) that Cu2+ but not Cu1+ applied extracellularly, is capable of inhibiting the M2 ion channel. The mechanism of inhibition is by coordination with the His37 selectivity filter, located closer to the lipid interior of the membrane than M2-Trp41. The accessibility of intracellularly applied Cu2+ to the His37 residue in the wt M2 and M2-W41A mutant proteins was tested. The mutant M2-W41A was chosen for study because alanine has the smallest side chain among the residue 41 mutant proteins that were constructed, and indeed M2-W41A has larger currents than the wt M2 protein. Oocytes expressing the wt M2 channel and the M2-W41A mutant were bathed in low pH solution to activate the M2 ion channel, and the conductance of the oocytes was

![Fig. 6. Accessibility of the pore of the wt M2 ion channel protein and M2-W41A mutant protein to intracellularly injected Cu2+](image)

![Fig. 7. Model for activation of the M2 ion channel showing only the TM domain from residues 24 to 44 (green); the selectivity filter His37 (red), and the gate Trp41 (blue) are highlighted.](image)

For clarity these residues are shown for only two of the four subunits; the residues facing the viewer and the subunit closest to the viewer are omitted. Upper panel shows scheme for the closed wt M2 channel. The channel is closed when pHout is high because His37 is not charged and Trp41 obstructs the pore near its cytoplasmic end. Middle panel shows the wt M2 channel in the open state. With low pHout His37 is charged, allowing rotation of Trp41 to a conformation parallel to the pore's axis, permitting H+ to flow. Lower panel shows the M2-W41F mutant protein. In this case the smaller side chain of the Phe mutant permits passage through the pore regardless of pHout.
measured every minute (Fig. 6). After the first conductance measurement, Cu\(^{2+}\) was injected intracellularly, and the effect of Cu\(^{2+}\) injection on oocyte conductance was measured. For control oocytes expressing either the wt M2 protein or the M2-W41A mutant protein that were not injected with Cu\(^{2+}\), conductance increased with time while they were bathed in solutions of low pH because the cytoplasm of the oocytes became acidified and the concentration of conducting ions near the membrane increased. The currents for the M2-W41A mutant channel increased more than for the wt M2 channel, because the mutant channel allowed more protons to pass. For oocytes expressing the wt M2 protein, the conductance continued to increase even after the injection of Cu\(^{2+}\) (injections confirmed by co-injection of fluorescent tracer). However, for oocytes expressing the M2-W41A mutant protein, the conductance decreased after intracellular injection of Cu\(^{2+}\) (two-way ANOVA \(p < 0.01\)). These results indicate that accessibility of the His37 selectivity filter to intracellularly injected Cu\(^{2+}\) is limited by the bulky indole side chain of M2-Trp41.

**DISCUSSION**

Taken together, these data suggest a model for the activation of the M2 ion channel (Fig. 7). In the presence of high pH\(_{\text{out}}\) the channel pore is obstructed by the indole side chain of Trp41. When pH\(_{\text{out}}\) is lowered, the His37 H* selectivity filter becomes protonated; as a result, the indole of Trp41 rotates to permit H* to flow. This movement may be accomplished by cation-pi interactions (65, 66). After returning to high pH\(_{\text{out}}\) outward current will not flow because the deprotonation of the His37 selectivity filter in high pH medium causes the indole of Trp41 to return to its pore-blocking position. If Trp41 is mutated to have a smaller side chain, pore blockage cannot occur. Thus, His37 acts as the detector of low pH\(_{\text{out}}\), and Trp41 acts as the gate. These data are consistent with the notion that the Trp41 region of the M2 ion channel undergoes pH-dependent conformational changes as deduced from cysteine-scanning mutagenesis experiments. It was observed that residues 40–43 showed a pH-dependent propensity to form inter-subunit disulfide bonds on oxidation (67). Our data do not allow us to determine the contribution made to the gating process by the positively charged nitrogen atom of the imidazole side chain of His37. Furthermore, we cannot distinguish which of the four His residues of each tetramer participate in transport of H* from those that participate in interactions with Trp. Our observation that the M2-W41Y mutant channel is more similar to the wt M2 channel in its gating behavior than the M2-W41F mutant channel is consistent with our proposed mechanism of His-Trp interactions mediating channel gating. For the small ribonucleo-""
Influenza M₂ Ion Channel Activation Gate

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