Chemical Rescue of Histidine Selectivity Filter Mutants of the M2 Ion Channel of Influenza A Virus

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The influenza virus M2 proton-selective ion channel activity facilitates virus uncoating, a process that occurs in the acidic environment of the endosome. The M2 channel causes acidification of the interior of the virus particle, which results in viral protein-protein dissociation. The M2 protein is a homotetramer that contains in its aqueous pore a histidine residue (His-37) that acts as a selectivity filter and a tryptophan residue (Trp-41) that acts as a channel gate. Substitution of His-37 modifies M2 ion channel properties drastically. However, the results of such experiments are difficult to interpret because substitution of His-37 could cause gross structural changes to the channel pore. We described here experiments in which partial or, in some cases, full rescue of specific M2 ion channel properties of His-37 substitution mutants was achieved by addition of imidazole to the bathing medium. Chemical rescue was demonstrated for three histidine substitution mutant ion channels (M2-H37G, M2-H37E, and M2-H37T) and for two double mutants in which the Trp-41 channel gate was also mutated (H37G/W41Y and H37G/W41A). Currents of the mutant protein on introduction of imidazole or an imidazole analog into the buffer solution. This chemical rescue demonstrated that the loss of catalytic function resulted from the lack of the histidine residue and not from large scale structural changes. Chemical rescue experiments also provided insight into the role of specific M2 ion channel properties of His-37 substitution mutants in the activity of mixed oligomers (11) showed the active state of the M2 ion channel protein to exist minimally as a homotetramer.

Despite the small size of the active M2 oligomer, several lines of evidence indicate that ion channel activity is intrinsic to the M2 protein. First, ion channel activity has been observed in three different expression systems, Xenopus oocytes (4, 6), mammalian cells (6, 12), and yeast (13, 14). Second, M2 channel activity has also been recorded in artificial lipid bilayers from a reconstituted peptide corresponding to the TM domain of the M2 protein (15) and from purified M2 protein (16, 17). Thus, due to its structural simplicity, the M2 ion channel is a potentially useful model for studying ion channels in general.

The ion selectivity of the M2 ion channel has been studied with voltage clamp (18, 19) and ion flux studies (17, 19) indicating that the channel is nearly perfectly selective for protons. The channel is inactive for extracellular pH values higher than pH 7.5 but becomes active when extracellular pH is lowered. This greater activity is due to two factors, greater abundance of protons and increased channel opening due to an interaction between protonated His-37 and the indole side chain of Trp-41, the putative “gate” of the ion channel (20, 21). Experiments in which His-37 of the TM domain was replaced by site-directed mutagenesis with Gly, Ala, Glu, Lys, and Arg (22) indicate that His-37 is essential for the proton selectivity of the channel and its activation by low pH. However, it is possible that substitution of even a single residue in the closely packed pore of this protein might bring about large structural changes in the protein, making a comparison of the mutant and wild-type (wt) channels difficult. For some enzymes that transport protons as part of their catalytic cycle, substitution of histidine in the active site with alanine or glycine resulted in loss of enzymatic activity, e.g. carbonic anhydrase II (23–25), copper amine oxidase (26), aldolase (27), (S)-mandelate dehydrogenase (28), bacterial luciferase (29), the reaction center of photosynthetic bacteria Rhodobacter sphaeroides (30), and protein kinases (31). However, one way to overcome the argument that the amino acid substitution caused gross structural changes in the protein was achieved by partial or complete chemical rescue of the mutant protein on introduction of imidazole or an imidazole analog into the buffer solution. This chemical rescue demonstrated that the loss of catalytic function resulted from the lack of the histidine residue and not from large scale structural changes. Chemical rescue experiments also provided insight...
into the function of the histidine residue in the wt enzyme, providing direct evidence that the imidazole side chain of histidine acts as an intermediate proton acceptor/donor, in which protons were accepted by the histidine molecule from one chemical moiety and subsequently donated to a second chemical moiety.

Chemical rescue of mutant ion channels has been reported in only a few instances. However, an important property of the M2 ion channel suggested that histidine substitution mutants might be susceptible to chemical rescue by imidazole. This property is the ability of the channel to be inhibited by amantadine, a molecule of approximately the same size as imidazole. We reasoned that it was likely that the diameter of the channel pore might be large enough to accommodate the imidazole molecule, and we applied imidazole buffer to three histidine substitution mutants. It was found that partial rescue was possible for each mutant M2 ion channel and that the imidazole-enhanced currents were able to be inhibited by Cu(II). These results confirm the role of the imidazole side chain of histidine in proton transport across the M2 channel pore and support the hypothesis that the imidazole side chain of histidine acts as an intermediate proton acceptor/donor to relay protons through the aqueous pore of the M2 ion channel while obstructing the flow of larger cations.

**EXPERIMENTAL PROCEDURES**

**Mutant and mRNA Synthesis, Culture, and Microinjection of Oocytes**—Mutations were introduced by PCR in a high expression vector that has a portion of the Xenopus 5’ globin untranslated region (32). Oocytes were removed from female Xenopus laevis (Nasco, Fort Atkinson, WI), defolliculated, microinjected with 50 nl of mRNA, and cultured in ND96 (pH 8.5) at 19 °C before use (18). The amounts of mRNA injected were as follows: wt (+100 ng), M2–H37G (–44 ng), M2–H37S (–25.5 ng), M2–H37T (–39 ng), M2–H37G/W41A (–180 ng), and M2–H37G/W41Y (–308 ng). Oocytes expressing the histidine substitution mutant proteins were incubated in ND96 for at least 24 h before recording, whereas recordings from oocytes expressing the wt protein were made ~65 h after injection.

**Recording Solutions**—Oocytes were bathed in either normal Barth’s solution or modified Barth’s solution during recording. Normal Barth’s solution contained (in mM) 88 NaCl, 1 KCl, 2.4 NaHCO3, 0.3 NaNO3, 0.71 CaCl2, and 0.82 MgSO4. The osmolality of solution with imidazole buffer was 202 mosM/kg. The composition of solutions used in Na+ ion substitution experiments (Table III and Table IV) performed in the presence of 50 mM imidazole buffer was 88 mM NaCl, 1 KCl, 2.4 NaHCO3, 50 mM imidazole buffer (pH 5.5), 0.3 NaNO3, 0.71 CaCl2, and 0.82 MgSO4. In solutions buffered with MES and HEPES, the osmolality of solution with imidazole buffer was adjusted with mannitol.

**TABLE I**

| Genotype | Vrev (88 mM Na+), mV | Vrev (88 mM NMDG), mV |
|----------|---------------------|----------------------|
| H37G     | −7.6 ± 0.2          | −42 ± 1.7           |
|          | (n = 3)             | (n = 3)             |

*p < 0.0001.

**TABLE II**

| Genotype | Vrev (88 mM Na+), mV | Vrev (88 mM NMDG), mV | Vrev (89 mM K+), mV |
|----------|---------------------|----------------------|---------------------|
| WT       | 56 ± 2.0            | 55 ± 2.1             | 51 ± 1.8            |
| (n = 9)  |                     | (n = 9)              | (n = 5)             |
| H37G     | 8.6 ± 1.0           | 14 ± 2.0             | 11 ± 1.9            |
| (n = 11) | (n = 11)            | (n = 5)              |                     |
| W41A     | 44 ± 5.7            | 44 ± 6.0             | 40 ± 6.8            |
| (n = 4)  | (n = 4)             | (n = 4)              |                     |

*p < 0.05.  
*p < 0.0001. The p values refer to NMDG.

**TABLE III**

| Genotype | Vrev (mV) | ΔpH (pH units) | Vrev (mV) | ΔpH (pH units) |
|----------|-----------|----------------|-----------|----------------|
| H37G     | 15 ± 0.6  | 0.26 ± 0.02    | 10 ± 0.4* | 0.24 ± 0.07    |
| (n = 8)  | (n = 3)   | (n = 8)        | (n = 3)   |                |

*p < 0.0001.

pounds capable of chemical rescue, the composition of the solutions was (in mM) 88 NaCl, 1 KCl, 2.4 NaHCO3, 0.3 NaNO3, 0.71 CaCl2, 0.82 MgSO4, 15 MES (pH 5.5), osmolality ~202 mosM/kg. The order of presentation of test solutions, the time of recovery in normal solution, and the time during which oocytes were exposed to low pH solutions were important for obtaining recordings in which reversible changes in current flow and acidification occurred and are described in Supplemental Information 1.

**Measurement of Membrane Current and Intracellular pH of Oocytes**—Whole-cell currents were measured using a two-electrode voltage clamp amplifier (Dagan TEV 200A) at ~25 °C using electrodes filled with 3 M KCl. Oocyte holding potential was ~20 mV unless stated otherwise. Recording of intracellular pH was done with silanized microelectrodes filled with protonophore as described previously (19).

**Immunofluorescence of Living Oocytes**—The relative expression levels of the mutant M2 proteins at the surface membrane of the oocytes were measured with respect to that of the wt ion type protein. This was done by incubating the oocytes after recording with a solution containing a monoclonal antibody directed against the N-terminal ectodomain of the influenza A M2 protein (monoclonal antibody 14C2) (33). Individual oocytes were washed with ND96 lacking pyruvate and gentamycin (4 °C, three times), incubated in ND96 containing 2% bovine serum albumin (4 °C, 1 h), incubated in primary antibody (1:500 in 2% bovine serum albumin, 4 °C, 1 h), washed with ND96 (4 °C, three times for 10 min), incubated in secondary antibody (goat anti-mouse IgG1 (γ) labeled with Alexa Fluor® 546 (catalog number 70030-200, Molecular Probes, Medford, OR), 10 or 20 μg/ml in 2% bovine serum albumin in ND96, 4 °C, and washed with ND96 (4 °C, five times for 10 min). Fluorescence was quantified using a PTI image master microfluorometer (London, Ontario, Canada) with a 20× 0.5 NA objective. Approach
Effect of imidazole buffer on the ion selectivity of the wt M2 ion channel and the M2-H37G mutant ion channel. $V_{rev}$ was measured using the amantadine-sensitive component of current. Note that $V_{rev}$ for the mutant ion channel changes much less when Na$^+$ is replaced in imidazole buffer than in MES buffer.

| Genotype | $V_{rev}$ (MES buffer, 88 mM Na$^+$) | $V_{rev}$ (MES buffer, 79.66 mM NMDG) | $V_{rev}$ (imidazole buffer, 88 mM Na$^+$) | $V_{rev}$ (imidazole buffer, 79.66 mM NMDG) |
|----------|------------------------------------|-------------------------------------|----------------------------------------|------------------------------------------|
| WT       | $55 \pm 5.9$                       | $60 \pm 5.3$                        | $60 \pm 2.0$                           | $69 \pm 1.3$                             |
|          | $(n = 3)$                           | $(n = 3)$                            | $(n = 3)$                              | $(n = 3)$                                |
| H37G     | $5.3 \pm 0.2$                      | $-11 \pm 0.8^a$                     | $41 \pm 1.8$                          | $45 \pm 1.4$                             |
|          | $(n = 3)$                           | $(n = 3)$                            | $(n = 3)$                              | $(n = 3)$                                |

*$p < 0.05.$

RESULTS

When oocytes expressing the wt M2 protein are bathed in solutions of low pH, but not solutions of high pH, an inward proton current flows across their membrane, and the cytoplasm becomes acidified (Fig. 1A). Previous work had shown that oocytes expressing histidine substitution mutant proteins have membrane currents, but these currents flowed at both high pH and low pH (22). We tested whether proton flow was included in the currents of oocytes expressing the histidine substitution mutant proteins by recording intracellular pH of the oocytes and found that acidification occurred when the oocytes were bathed in solutions of low pH (Fig. 1B), showing that protons can pass through the pore of the mutant ion channel in the absence of histidine (see Supplemental Information 2). However, results with mutant enzymes that normally contain histidine as part of a proton transport pathway indicated that proton transport rates are increased when imidazole buffer is used (23–25). We thus applied imidazole buffer to oocytes expressing histidine substitution mutant proteins, and we found that current amplitude and acidification were increased significantly ($p < 0.05, n = 4$) (Fig. 1B). Increases were not observed for oocytes expressing the wt protein (Fig. 1A). These observations suggested that the presence of imidazole buffer might cause the properties of the mutant ion channels to resemble more closely those of the wild-type ion channel (i.e., “rescue” the channel properties). We thus analyzed the key properties of the mutant ion channels with and without imidazole buffer. Our studies focused on the M2-H37G mutant ion channel because the currents of oocytes expressing this genotype were more reproducible from experiment-to-experiment than those of oocytes expressing the other mutant ion channels.

Ion Selectivity of Histidine Substitution Mutants in MES Buffer

The ion selectivity for the M2-H37G, M2-H37S, and M2-H37T mutant ion channels was studied by measuring their current-voltage relationships in media of various pH, buffer, and ionic composition (Fig. 2 and Tables I and II). These mutant ion channels displayed much less proton selectivity than the wt ion channel when studied at high pH (pH 7.5 or pH 8.5) and low pH (pH 5.5) in MES buffer.

Measurements at High pH—Oocytes expressing these mutant ion channels had standing currents when bathed at pH 7.5 or pH 8.5 (Fig. 2A). The standing current for the M2-H37G mutant ion channel became outward when Na$^+$ in the bathing medium was replaced with NMDG (holding voltage $-20$ mV). This suggested that the mutant ion channel conducted Na$^+$, unlike the wt channel that does not conduct Na$^+$ at all. To test if Na$^+$ carried the inward current of the M2-H37G mutant ion channel at pH 7.5, reversal voltage ($V_{rev}$) Table I) was measured and found to be less negative in media containing Na$^+$ ($-7.6 \pm 0.2$ mV, $n = 3$ mean $\pm$ S.E.) than in media containing NMDG ($-42 \pm 1.7$ mV, $n = 3$), consistent with the conclusion that Na$^+$ is conducted through the mutant channel.

Measurements at Low pH—Oocytes expressing the wt M2 ion channel and the M2-H37G mutant ion channel had steady inward currents at pH 5.5 ($-20$ mV holding voltage, Fig. 2A). The inward current amplitude of oocytes expressing the M2-H37G mutant ion channel decreased when Na$^+$ in the medium
was replaced by NMDG, suggesting that the mutant ion channel has Na\(^+\) conductance at low pH. However, the amplitude of the decrease achieved by this replacement was not as large when studied at pH 5.5 (0.55 \pm 0.05 \mu A, \(n = 6\)) as it was at pH 7.5 (0.99 \pm 0.2 \mu A, \(n = 3\); \(p < 0.05\)). Consistent with the interpretation that the channel has Na\(^+\) conductance at pH 5.5, Na\(^+\) replacement made the \(V_{\text{rev}}\) considerably more negative for oocytes expressing the M2-H37G mutant ion channel (Table II). In contrast, neither inward current amplitude nor the \(V_{\text{rev}}\) for oocytes expressing the M2-H37T channel was affected by replacement of Na\(^+\) in the bathing medium with NMDG (6, 18, 19). To test for the possibility that the mutant ion channels also conduct K\(^+\) in the bathing medium with NMDG (6, 18), we measured the current-voltage relationship for the wt ion channel (upper) and the M2-H37G mutant ion channel (lower). Note that substantial currents remain in the presence of amantadine for the mutant channel (4) and that the slope conductance of the channel is not significantly affected by the Na\(^+\)-free solution with imidazole buffer (pH 5.5; Table IV). This was done while the cells were bathed in medium buffered with either MES or imidazole (pH 5.5) as it was at pH 7.5. The \(V_{\text{rev}}\) did not change significantly (data not shown). Thus, the M2-H37G mutant protein forms a poorly selective cation channel.

 Ion Selectivity of Histidine Substitution Mutant Ion Channels in Imidazole Buffer

To test the dependence of ion selectivity of the M2-H37G mutant ion channel on the concentration of imidazole buffer in the bathing medium, we measured the current-voltage relationship (Fig. 2B) and \(V_{\text{rev}}\) for oocytes that were bathed in solutions with each of several concentrations of imidazole buffer between 1.5 and 50 mM at pH 5.5 (Fig. 3). For these measurements we studied the amantadine-sensitive component of current. Neither the amplitude of the inward current (Fig. 2B) nor the \(V_{\text{rev}}\) of oocytes expressing the wt channel varied significantly with imidazole concentration (Fig. 3A), nor was \(V_{\text{rev}}\) affected by replacement of Na\(^+\) in the bathing medium with NMDG (6, 18). However, the \(V_{\text{rev}}\) of oocytes expressing the M2-H37G mutant ion channel became more positive with increased imidazole concentration (Fig. 2B and Fig. 3B), consistent with increased proton conductance resulting from higher imidazole concentration. The current-voltage relationship for the M2-H37G mutant ion channel was increased by replacing MES buffer with imidazole buffer (Fig. 2B); this increase in slope occurred for both inward and outward current. In contrast, the current-voltage relationship for the wt ion channel was changed very little by imidazole buffer (Fig. 2B). To test ion selectivity further, we measured the effect on \(V_{\text{rev}}\) of replacement of Na\(^+\) in the bathing medium with NMDG (50 mM buffer, pH 5.5; Table IV). This was done while the cells were bathed in medium buffered with either MES or imidazole (pH 5.5) as it was at pH 7.5.
Specific Activity of Histidine Substitution Mutant Ion Channels in Imidazole Buffer

A notable characteristic of the wt M2 ion channel is its low single channel conductance or specific activity (17, 34). We therefore compared the relative activity of the histidine substitution mutant ion channels expressed in individual oocytes with that of the wt ion channel by first measuring the oocyte current in imidazole buffer (50 mM) and then quantifying the relative surface expression of M2 protein expressed in each oocyte with immunofluorescence using an antibody specific for the N-terminal ectodomain of the protein (33). This epitope would not be expected to be altered by mutation of the transmembrane domain histidine residue (monoclonal antibody 14C2) (see “Experimental Procedures” and Fig. 4). The currents of the oocytes expressing the three mutant ion channels were much higher than those expressing the wt ion channel, but the relative levels of surface expression were lower for the mutant ion channel. Relative to the wt ion channel, the ratio of current to immunofluorescence for the mutant ion channels was (mean ± S.E.) M2-H37G, 6.8 ± 1.9, n = 3; M2-H37S, 12 ± 2.3, n = 3; and M2-H37T, 4.1 ± 1.0, n = 3. This finding is consistent with the mutant ion channels having a higher specific activity than the wt ion channel.
occurred within the range pH 6.5 to pH 8.5 for the mutant ion channel and the maximal slope occurred within the range pH 4.5 to pH 6.5 for the wt ion channel. Thus, imidazole buffer partially modified gating of the mutant ion channel.

**Inhibition of Histidine Substitution Ion Channels by Amantadine and Cu(II)**

The wt M2 channel is completely inhibited by amantadine, and this inhibition is essentially irreversible (4, 5). In contrast, the activity of the histidine substitution mutant ion channels was only partially inhibited by amantadine. For example, the currents of oocytes expressing the M2-H37G mutant ion channel, studied in MES buffer, were inhibited only 62 ± 2.8% (mean ± S.E., n = 4) when the inhibitor (100 μM) was applied in bathing media of either high or low pH (see Fig. 2B and Table VI). The amantadine inhibition of the mutant ion channels was reversible within the time scale of these experiments (~85–100% reversibility in 5 min). Thus, amantadine inhibition of the histidine substitution mutant ion channels was incomplete and reversible, in contrast to the complete and irreversible inhibition found for the wt ion channel. Cu(II) causes a rapid, incomplete inhibition of the M2-H37G mutant ion channel, in contrast to the slow, complete inhibition observed for the wt ion channel (35).

**Inhibition by Amantadine and Cu(II) in the Presence of Imidazole Buffer**

These two inhibitors have been shown to inhibit the wt ion channel, and we thus tested the influence of imidazole buffer on their ability to inhibit the M2-H37G mutant ion channel. Imidazole buffer increased inhibition of the M2-H37G mutant ion channel by amantadine (100 μM). Inhibition measured at pH 5.5 was greater in imidazole buffer (82 ± 0.5% mean ± S.E., n = 4) than in MES buffer (62 ± 2.8%), and the inhibition of the mutant channel increased with higher values of pH of the bathing medium with imidazole buffer (Fig. 5B). In the presence of either MES or imidazole buffer, the currents and acidification caused by the wt ion channel were fully inhibited by amantadine (100 μM). Possible explanations for these results are as follows: 1) imidazole buffer acted on the M2-H37G mutant ion channel to increase proton current and decrease the current for other cations; and 2) amantadine inhibited only the proton current. The specificity for the proton current could explain the increased inhibition of the mutant ion channel by amantadine.

Inhibition of the wt ion channel by Cu(II) is important because it is known that inhibition occurs by coordination with His-37 (35), and if imidazole buffer is able to occupy a site(s) in the mutant channel pore, Cu(II) would be expected to inhibit the mutant ion channel. We therefore tested the ability of Cu(II) to cause inhibition of inward current and acidification of oocytes expressing the M2-H37G mutant ion channel when bathed in imidazole buffer. The inward current and acidification of the M2-H37G mutant channel were partially inhibited by Cu(II) (220 μM) in 15 mM imidazole-buffered solution (Fig. 6B; inhibition of the inward current was 38 ± 0.2% mean ± S.E., n = 4; p < 0.05). At this value of pH, less than 1% of the total imidazole in the bulk solution was complexed with Cu(II), and thus the inhibition observed could not be attributed to a decrease in the concentration of free imidazole buffer. Although the percentage inhibition of the M2-H37G mutant by Cu(II) was lower in imidazole buffer than in MES buffer, the absolute amount of inhibition was larger in imidazole buffer (0.6 ± 0.06 μA) than in MES buffer (0.4 ± 0.1 μA, p < 0.05, n = 3), demonstrating that the imidazole-sensitive portion of the current is inhibited by Cu(II). Most surprisingly, the wt ion channel was not inhibited by Cu(II) dissolved in imidazole buffer (Fig. 6B; pH 5.5, free concentration of Cu(II) 220 μM); these conditions cause inhibition in MES buffer (35) (see Supplemental Information 4). Thus, the M2-H37G mutant ion channel is inhibited by Cu(II) when studied in imidazole buffer, suggesting that the site of inhibition by Cu(II) is in the pore of the mutant ion channel.

**Intracellular Injection of Imidazole Was without Significant Effect Upon the M2-H37G Mutant Ion Channel**

If bulk flow of protonated imidazole is responsible for the increased current in imidazole buffer, then injection of imidazole into the cytoplasm would be expected to increase outward currents. Thus, imidazole was injected intracellularly into oocytes expressing the M2-H37G mutant ion channel. The injection of imidazole to a final concentration of 4–14 mM (confirmed with fluorescent tracer) resulted in an insignificant (~5%) increase of current amplitude for cells measured in MES buffer (pH 5.5, 50 mM). This is in contrast to the 5-fold increase (500 ± 89%, n = 5) measured when 15 mM imidazole buffer was introduced into the bathing medium.

**The Chemical Nature of Rescuing Buffers**

To determine whether only the naturally occurring imidazole side chain of histidine was capable of increasing current and conductance of the M2-H37G mutant channel, we applied a number of other buffer molecules while measuring inward current and conductance, and we compared these variables with their values measured in MES buffer (Fig. 7, 15 mM buffer concentration, pH 5.5). MOPSO buffer (pKa 6.9, identical to that of imidazole) did not increase either current or conductance of the mutant ion channel. The currents and conductance with 1-methylimidazole were significantly higher than those
Chemical rescue of the proton transfer function of the inactive carbonic anhydrase II H64A mutant protein by 4-methylimidazole achieved about 40% of the wt activity (36, 37). The crystal structure of the enzymatically rescued protein showed that the 4-methylimidazole moiety was placed in place through π-stacking interactions with Trp-5 of the protein; if Trp-5 was replaced with Ala then enzymatic rescue was not achieved (25). An essential, conserved feature of the TM domain of the M2 ion channel protein is the HXXW motif. We tested whether the increase in current of the M2-H37G mutant ion channel that occurred when MES buffer was replaced with imidazole buffer depended upon interactions with Trp-41. This was done by introducing a second mutation in which Trp-41 was replaced with either tyrosine or alanine. Before doing these experiments, however, we performed tests of the ion selectivity of the M2-W41A mutant ion channel by measuring its \( V_{rev} \) in various solutions at pH 5.5 (Table II). We found that \( V_{rev} \) was very similar to that of the wt ion channel and that \( V_{rev} \) was not altered by ion substitutions, indicating that this mutant ion channel has proton selectivity similar to that of the wt ion channel. The double mutant ion channels were expressed in oocytes, and their current, conductance, and \( V_{rev} \) were studied in MES and imidazole buffers (Table V). When imidazole buffer replaced MES buffer in the bathing medium (pH 5.5), inward current amplitude increased, and \( V_{rev} \) became more positive for both of these double mutant ion channels (Table V). In addition, conductance increased for both double mutants (ratio of conductance in imidazole buffer to MES buffer was 2.4 ± 0.1, \( n = 4 \), for M2-H37G/W41A and 4.4 ± 0.4, \( n = 3 \), for M2-H37G/W41Y). The specific activity of these double mutant proteins was determined by measuring currents and surface expression relative to those for the wt ion channel protein (Fig. 4). Relative to the wt ion channel, the ratio of current to immunofluorescence for the double mutant ion channels was (mean ± S.E.): M2-H37G/W41A, 4.0 ± 0.43, \( n = 3 \), and M2-H37G/W41Y, 9.6 ± 5.1, \( n = 4 \). Neither double mutant ion channel was significantly inhibited by amantadine (as low as 1% inhibition for the M2-H37G/W41Y mutant ion channel; Table VI). Thus, partial rescue of ion selectivity and proton transport can be achieved independent of Trp-41.

**DISCUSSION**

This study confirms that the TM domain histidine residue of the M2 ion channel protein of influenza A virus is essential for its ability to conduct protons with high selectivity and suggests that histidine acts as an intermediate proton acceptor/donor group. When the His-37 was replaced by Gly, Ser, or Thr, the mutant ion channels were capable of transporting protons, but the proton selectivity of each mutant ion channel, measured in MES buffer, was diminished. The use of imidazole buffer improved proton selectivity, increased proton transport, and improved gating of the mutant ion channels. The finding that partial rescue of three histidine substitution mutant ion channel proteins can be achieved with imidazole added to the bathing medium suggests that the mutation of four closely positioned residues in the TM pore did not cause gross structural alterations that would invalidate the comparison of mutant and wt proteins. Rescue is specific to imidazole, the side chain of histidine, supporting the conclusion that histidine plays a
key role in proton transport and selectivity. The ability of three different histidine substitution mutant ion channels to be rescued by added imidazole, together with the ability of Cu(II) to inhibit the currents in the presence of imidazole, suggests that rescue was probably achieved by imidazole acting as an intermediate proton acceptor/donor group in which the imidazole molecule accepted protons from the buffered solution on the outside of the ion channel pore and donated them to the solution on the other side of the pore. These results are consistent with histidine acting in the wt ion channel as an intermediate proton acceptor/donor group.

Properties of Histidine Substitution Mutant Ion Channels

**Studied in MES Buffer**—The histidine substitution mutant ion channels M2-H37G, M2-H37S, and M2-H37T were found to be deficient in ion selectivity, were not activated by low pH, and were not fully inhibited by amantadine. In addition, Cu(II) inhibition of the M2-H37G mutant channel was incomplete and rapidly reversible. However, the present study demonstrated that the M2-H37G mutant ion channel conducts protons and that the proton flux can be partially inhibited by amantadine. The ion selectivity of the M2-H37G mutant ion channel measured in MES buffer, inferred from its $V_{rev}$, showed that it possesses substantial Na$^+$ conductance at pH 8.5, that its relative Na$^+$ conductance decreased with pH, that it possesses substantial Na$^+$ and K$^+$ conductance at pH 5.5, and had undetectably low Cl$^-$ conductance. A possible explanation for the observation that oocytes expressing this mutant ion channel undergo nearly equal amounts of acidification as oocytes expressing the wt ion channel, but with smaller amplitude of inward membrane current than oocytes expressing the wt ion channel (Fig. 1), is that under these conditions considerable outward K$^+$ current flows through the mutant channel, balancing the inward proton and Na$^+$ current. In summary, although a histidine substitution mutant ion channel is capable of proton transport, the ion selectivity of the channel is greatly compromised by substitution of histidine.

**Evaluation of Chemical Rescue**—According to four criteria for ion channel function, imidazole buffer achieves substantial chemical rescue of histidine substitution mutant channels. First, ion selectivity is enhanced, in part by preventing Na$^+$ ions from being transported (Fig. 3 and Tables III and IV). Second, the transport of protons is increased (Fig. 1). Third, the gating of the channel is partially restored (Fig. 5A). Fourth, sensitivity to amantadine (Fig. 5B) and Cu(II) is increased. However, even in the presence of a high concentration of imidazole buffer, the properties of the mutant channels are not identical to those of the wt channel, and thus the rescue of the histidine substitution mutant ion channels by imidazole buffer is incomplete.

Chemical rescue of ion selectivity of the M2-H37G mutant ion channel by imidazole buffer was probably brought about by a combination of increased proton conductance and decreased conductance for Na$^+$ and K$^+$. In the presence of imidazole buffer the rate of acidification was greater than in MES buffer (Fig. 1), and the change in $V_{rev}$ due to replacing Na$^+$ in the bathing medium by NMDG was lower than in MES buffer (Tables III and IV). It is unlikely that the latter difference can be explained solely by an increase in proton conductance, and the most likely explanation is that decreased Na$^+$ conductance also resulted from bathing in imidazole buffer.

Gating of the M2-H37G mutant ion channel was improved modestly by imidazole buffer (Fig. 5), but this mutant channel did not achieve the same extent of closing at high pH as the wt channel. This might have been due to the inability of the imidazole buffer molecules to interact with the indole side chain of Trp-41 of the channel. In the wt M2 ion channel, gating depends on the interactions between His-37 and Trp-41 (20, 21). Current does not flow through the wt M2 ion channel when bathed in solutions of high pH (21, 34). The present study found that partial rescue of ion selectivity (Table V) and proton transport are possible for the double mutants M2-H37G/W41A and M2-H37G/W41Y in the presence of imidazole buffer. As partial rescue could be obtained in the absence of Trp-41, it is unlikely that the rescuing imidazole molecules interacted with the Trp-41 gate in the M2-H37G mutant ion channel in order to effect rescue. Thus, rescue of gating was incomplete. This independence from Trp is in contrast to observations of the catalytic function of mutant human carbonic anhydrase II (HCA II-H64A), for which partial enzymatic rescue by imidazole is only possible in the presence of the native Trp-5 residue (36, 37). The present findings suggest that the positioning of the rescuing imidazole molecule(s) in the mutant ion channels does not depend upon interaction with Trp-41.

**Specificity of Chemical Rescue**—Chemical rescue of the M2 ion channel is very specific to imidazole buffer. Buffers that are neutral when protonated (MES and MOPS) are not able to achieve rescue of proton transport of the M2-H37G mutant ion channel. Of the several buffers tested, the only other one that achieved rescue was structurally related to imidazole (1-methylimidazole), and rescue by this compound was less than that by imidazole itself (Fig. 7).

In a study of proton transfer rates by chemical rescue of the bacterial reaction center, the second order rate constant was found to depend upon the pK$\alpha$ of the rescuing buffer over a 9 log unit range (38). This dependence had a slope of about 10-fold per pH unit, a finding that was interpreted to indicate the presence of an intermediate proton acceptor/donor group located between the source of protons at the surface of the reaction center and their destination at Glu-212 of the reaction center. Unfortunately, an analysis of this type cannot be applied to the M2 ion channel because chemical rescue is specific to imidazole.

**Mechanism for Partial Chemical Rescue of Histidine Substitution Mutant Ion Channels**—The finding that it is possible for three histidine substitution ion channels to be rescued by imidazole supports a mechanism in which His-37 acts as an intermediate proton acceptor/donor group in these rescued mutant ion channels. This conclusion is further supported by several additional findings. First, imidazole buffer causes an increase in proton transport, as determined by rate of acidification (Fig. 1), and proton selectivity, as determined by relative independence of $V_{rev}$ from [Na$^+$] in the bathing medium (Tables III and IV). Second, the extent of rescue increases with the concentration of imidazole (Fig. 3). Third, low concentrations of Cu(II) insufficient to reduce bulk free imidazole concentration (see below) inhibit proton transport in the presence of imidaz-

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**Table VI**

Chemical Rescue of M2 Ion Channel Mutants

| Genotype | % of MES currents inhibited by amantadine | % of imidazole currents inhibited by amantadine |
|----------|------------------------------------------|-----------------------------------------------|
| wt       | 97 ± 0.2                                 | 98 ± 1.7                                      |
| H37G     | 62 ± 2.8                                 | 65 ± 1.8                                      |
| W41A     | 75 ± 2.9                                 | 75 ± 3.2                                      |
| H37G/W41A| 29 ± 13                                  | 58 ± 1.0                                      |
| H37G/W41Y| <1%                                      | 17 ± 0.5                                      |

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Comparison of the amantadine inhibition for oocytes expressing the wt M2, M2-H37G, M2-W41A, M2-H37G/W41A, and M2-H37G/W41Y mutant proteins. Note that amantadine does not significantly inhibit the M2-H37G/W41A and M2-H37G/W41Y double mutant ion channels. Currents and $V_{rev}$ were for the pH-sensitive component of current.
Inhibition of currents in the presence of imidazole buffer by Cu(II) is inconsistent with a mechanism for rescue in which bulk imidazole is conducted through the pore of the channel. Inhibition can be observed with 220 mM free Cu(II) in the presence of 15 mM imidazole, and under these conditions only about 1% of the imidazole is coordinated with Cu(II). This makes it unlikely that a decrease in the free imidazole concentration in the bulk solution causes the inhibition observed. A much more likely explanation is that Cu(II) coordinates with imidazole molecules that are located in the mutant ion channel pore, inhibiting their ability to bind or conduct protons. This explanation is strengthened by two observations made with the M2-H37G mutant ion channel. First, in the presence of imidazole buffer the slope of the current-voltage relationship for both outward and inward currents was equally enhanced. As the free imidazole concentration in the cytoplasm is negligible, it is unlikely that protonated imidazole molecules carry an outward proton current. Second, intracellular injection of imidazole is without effect. Thus, it is likely that imidazole molecules located in the pore are responsible for the observed chemical rescue.

We postulate that imidazole molecules act as intermediate acceptor/donors of protons conducted through the pore of the mutant ion channels (Fig. 8). Each of the three mutant ion channel proteins, when studied in MES buffer, is capable of conducting $\text{H}^+$, $\text{K}^+$, and $\text{Na}^+$ through their pore (Fig. 8B). These ion channels lack specificity because they lack pore-occupying imidazole side chains of His-37. When studied in imidazole buffer, imidazole occupies the channel pore (Fig. 8C) and alters channel function in two ways. First, imidazole partly occludes the pore, thereby impeding flow of $\text{K}^+$ and $\text{Na}^+$. Second, imidazole molecules act as intermediate proton acceptor/donor molecules, enhancing $\text{H}^+$ transport. Cu(II) is postulated to coordinate with pore-occupying imidazole molecules in this model, inhibiting their ability to bind protons, disabling them from acting as intermediate proton acceptor/donor molecules (Fig. 8D).

Implications for Proton Transport by the Wild-type M2 Ion Channel—The mechanism by which protons are transported through the pore of the wt M2 ion channel is not known. However, measurement of the kinetic isotope effect shows that it is unlikely that passage of hydronium ions is the principal mechanism (18). The remaining two possibilities are as follows: 1) His-37 acts as an intermediate proton acceptor/donor group, \textit{i.e.} each proton binds to His-37 and is subsequently released;
and 2) a “proton wire” forms through which protons are transported (41, 42). The finding that the single channel conductance of the channel is very low (17, 18) favors the former interpretation, although it is still possible that a very short lived proton wire could provide for a low average conductance, albeit with a very high conductance during the time of opening (41, 42). Our results point to imidazole functioning as an intermediate proton acceptor/donor in the rescued mutant ion channels, and it is consistent that the naturally occurring imidazole side chain of His-37 functions in a similar fashion in the wild ion channel. However, although the present results support the intermediate proton acceptor/donor mechanism, they do not exclude the short lived proton wire mechanism, and further studies will be needed to resolve this question.

In summary, these experiments confirm the essential role of His-37 in the selective transport of protons through the pore of the M2 ion channel protein and support the conclusion that mutation of His-37 in all four subunits of the tetrameric channel does not necessarily cause a gross distortion of the channel architecture. The results suggest that His-37 acts as an intermediate proton acceptor/donor group in the function of the wild ion channel. Three histidine substitution mutant ion channel proteins (M2-H37G, M2-H37S, and M2-H37P), studied in MES buffer, have very high specific activities and function as large, un gated cation pores. It is remarkable that the addition of imidazole buffer to the bathing medium is capable of partial gating of these mutant ion channels.

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