The homotetrameric M₂ integral membrane protein of influenza virus forms a proton-selective ion channel. An essential histidine residue (His-37) in the M₂ transmembrane domain is believed to play an important role in the conduction mechanism of this channel. Also, this residue is believed to form hydrogen-bonded interactions with the ammonium group of the anti-viral compound, amantadine. A molecular model of this channel suggests that the imidazole side chains of His-37 from symmetry-related monomers of the homotetrameric pore converge to form a coordination site for transition metals. Therefore, membrane currents of oocytes of Xenopus laevis expressing the M₂ protein were recorded when the solution bathing the oocytes contained various transition metals. Membrane currents were strongly and reversibly inhibited by Cu²⁺ with biphasic reaction kinetics. The biphasic inhibition curves may be explained by a two-site model involving a fast-binding peripheral site with low specificity for divalent metal ions, as well as a high affinity site (Kₘ₁ = 2 μM) that lies deep within the pore and shows rather slow-binding kinetics (Kₘ₂ = 18.6 ± 0.9 μM⁻¹ s⁻¹). The pH dependence of the interaction with the high affinity Cu²⁺-binding site parallels the pH dependence of inhibition by amantadine, which has previously been ascribed to protonation of His-37. The voltage dependence of the inhibition at the high affinity site indicates that the binding site lies within the transmembrane region of the pore. Furthermore, the inhibition by Cu²⁺ could be prevented by prior application of the reversible blocker of M₂ channel activity, BL-1743, providing further support for the location of the site within the pore region of M₂. Finally, substitutions of His-37 by alanine or glycine eliminated the high affinity site and resulted in membrane currents that were only partially inhibited at millimolar concentrations of Cu²⁺. Binding of Cu²⁺ to the high affinity site resulted in an approximately equal inhibition of both inward and outward currents. The wild-type protein showed very high specificity for Cu²⁺ and was only partially inhibited by 1 mM Ni²⁺, Pt²⁺, and Zn²⁺. These data are discussed in terms of the functional role of His-37 in the mechanism of proton translocation through the channel.

**Cu(II) Inhibition of the Proton Translocation Machinery of the Influenza A Virus M₂ Protein**

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The M₂ protein of influenza A virus is thought to function as an ion channel that permits protons to enter virus particles during uncoating of virions in endosomes. In addition, influenza virus-infected cells the M₂ protein causes the equilibration of pH between the acidic lumen of the trans-Golgi network and the cytoplasm (reviewed in Refs. 1 and 2). The M₂ protein contains a 24-residue N-terminal extracellular domain, a single internal hydrophobic domain of 19 residues which acts as a transmembrane domain and forms the pore of the channel, and a 54-residue cytoplasmic tail (3). Chemical cross-linking studies showed the M₂ protein to be minimally a homotetramer (4–6), and statistical analysis of the ion channel activity of mixed oligomers indicated that the minimal active oligomer is a homotetramer (7). Despite the small size of the active M₂ oligomer, several pieces of evidence indicate that the ion channel activity is intrinsic to the M₂ protein. First, ion channel activity has also been observed in two expression systems in addition to oocytes, mammalian cells (8) and yeast (9). Second, the activity has also been reconstituted in artificial lipid bilayers (10) from purified M₂ protein. The currents associated with the M₂ ion channel are inhibited by amantadine, its methyl derivative rimantadine, and the spirene-containing compound BL-1743 (Structure 1). Mutant viruses resistant to amantadine or BL-1743 have been isolated, and most have been found to map to the extracellular half of the transmembrane domain of the M₂ protein (11–13). These mutant M₂ proteins have ion channel activity that is insensitive to the compound used to generate the resistant mutant virus (8, 12, 14–16). Inhibition by BL-1743 also has the useful property that, unlike inhibition by amantadine, it is reversible on the time scale of physiological experiments (15).

The ion selectivity of the M₂ ion channel has been examined with ion substitution and reversal voltage measurements. The channel possesses at least 10⁵-fold selectivity for H⁺ (8, 17). This finding has been confirmed with intracellular pH measurements, but the former experiments indicate that other monovalent cations such as Na⁺ also permeate (17).

Recently, we proposed a model for the three-dimensional structure of the transmembrane region of M₂, based on a mathematical analysis of the functional properties of a series of mutants (18). The predicted structure consists of four α-helices arranged with approximate 4-fold symmetry about a central channel, which spans the transmembrane region of the protein. This model is in very good agreement with spectroscopic data (19–21) and with an independently proposed structure based on molecular dynamics calculations (22). Our model provides a rationale for the proton selectivity of the channel; the tetramer defines a continuous, water-filled pore, which is interrupted at only one position, His-37 (Fig. 1A) which may act as a proton shuttle similar to that of carbonic anhydrase (23, 24). A con-
A considerable body of evidence shows that His-37 is indeed essential for the activity of M₂, and mutation of this residue leads to a channel that conducts ions in a pH-independent manner (25).

Amantadine inhibits M₂ with a dissociation constant of approximately 0.2 μM and a relatively slow rate of association (16). This drug binds to the channel with a half-time on the order of 10–20 min when the drug is present at low micromolar concentrations (the second order rate constant ranges from 150 to 1000 s⁻¹ M⁻¹ for M₂ from different amantadine-sensitive subtypes of virus). Interestingly, another positively charged, hydrophobic drug, BL-1743, is known to bind M₂ at a similar rate, although its rate of dissociation varies widely; the half-time for dissociation of BL-1743 from M₂ is approximately 3 min (15), while amantadine dissociates at a rate too slow to be experimentally measured. The relatively slow rates at which these drugs associate with the channel may relate to the mechanism by which they enter the pore. Both drugs are relatively bulky and may experience some steric hindrance as they enter the channel. In addition, the partial or full dehydration of positively charged groups as the drugs enter the channel may be energetically difficult, leading to a slow rate of penetration. We suggest that these drugs interact with the lining of the pore, which is composed primarily of hydrophobic groups that form a widening near the center of the bilayer similar to that found in the K⁺ channel (26).

To test further the role of His-37 in the function and inhibition of M₂, we have examined the ability of Cu(II) to inhibit the channel. Examination of the model (Fig. 1) indicated that this ion could interact with the four histidine imidazole groups similarly to the role proposed for histidine as the chelating ligand for Cu(II) binding to prion protein (27). We demonstrate that Cu(II) indeed binds to a high affinity site within the protein in a slow, time-dependent process.

**EXPERIMENTAL PROCEDURES**

Site-specific mutagenesis of M₂ cDNA was performed as described previously (15, 19). In vitro synthesis of mRNA was performed using the mMessage mMachine T7 Transcription Kit (Ambion, Austin, TX).

**Microinjection and Culture of Oocytes**—Ovarian lobules from individually identified Xenopus laevis females (Nasco, Fort Atkinson, WI) were surgically removed and treated with collagenase B (2 mg/ml; Boehringer Mannheim) in Ca²⁺-free OR-2 solution (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES-NaOH, pH 7.5) at 24 °C for 30–45 min to liberate oocytes from follicle cells. Defolliculated oocytes were washed in OR-2 and maintained in ND-96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 2.5 mM sodium pyruvate, 5 mM...
**RESULTS**

**Inhibition of Currents by Cu**

The inhibition of currents by Cu²⁺ is a potent, time-dependent inhibitor of the channel activity of oocytes that express the M₂ protein (Fig. 2 and Table I). In the absence of M₂ protein, *Xenopus* oocytes have very small currents in the range of pH 4.0–pH 9.0. Oocytes that express M₂ protein have currents that are dependent on pH of the bathing medium (14, 25). For pH 7.5 the current is double the background current found in control oocytes. However, at pH 6.2 the current is about 8-fold higher than the current at pH 7.5 (14, 25). Bathing oocytes that express wild-type M₂ protein in a solution that contains 100 μM amantadine causes the currents to decrease to background levels within 2–3 min (14, 16). Prolonged exposure of oocytes to Cu²⁺ can be toxic, so we took a number of precautions to ensure that the observed inhibition was specific to the M₂ ion channel. Before applying media containing transition metals, we first confirmed that the whole-cell current of each oocyte was activated by low pH (see Fig. 2). We also tested each cell for the inhibition of current by amantadine by applying the drug after washing out the transition metal. The data reported here are from oocytes whose currents were increased 4–6-fold by decreasing pH out from pH 7.5 to pH 6.2 and whose currents were reduced to 100–200 nA by amantadine (100 μM for 2 min). These values for activation by low pH and inhibition by amantadine are within the range for M₂-expressing oocytes in the absence of transition metals and thus provide assurance that the inhibition reported is specific to the M₂ ion channel and is not confounded with nonspecific leakage currents.

Addition of Cu²⁺ to the bathing solution (pH out = 6.2) gives rise to a time-dependent decrease in the amantadine-sensitive channel activity, approaching full inhibition at long times (Fig. 2 and Table I). The time course of inhibition differs from that for amantadine or other hydrophobic drugs, which generally show simple, pseudo-order first order decay kinetics under conditions where the drugs are in large excess. Instead, the data for inhibition by Cu²⁺ is biphasic, and the data can be fit by a sum of two exponentials (Fig. 2 and Table I), representing a fast and a slow process with both relaxation times linearly proportional to the concentration of Cu²⁺ between 100 and 1000 μM. Indeed, we show below that the initial rapid rate (93 ± 5 M⁻¹ s⁻¹) is associated with a nonspecific, partial block of the channel that is not dependent on His-37, whereas the slower process (18.6 ± 9 M⁻¹ s⁻¹) requires the presence of histidine at position 37. The second order rate constant associated with this latter process is nearly 2 orders of magnitude slower than the value observed for inhibition of the M₂ channel activity (A/UDorn subtype) by amantadine.

**Fig. 2. Time course of inhibition of the wild-type M₂ ion channel protein by Cu²⁺.** The symbols in this and the following figures show the recorded currents (mean ± S.E. for 4 cells), and the lines show the currents fit with a double exponential function. See Equation 1.

**Table I**

| Parameter | 0.1 mM (●) | 0.5 mM (■) | 1.0 mM (▲) |
|-----------|-----------|-----------|-----------|
| Iₘₜ | 0.11 ± 0.01 | 0.14 ± 0.01 | 0.14 ± 0.01 |
| αₖ | 0.48 ± 0.02 | 0.46 ± 0.02 | 0.25 ± 0.0 |
| Kₐₘ (Global) | 93 ± 5 M⁻¹ s⁻¹ | 93 ± 5 M⁻¹ s⁻¹ | 93 ± 5 M⁻¹ s⁻¹ |
| Kₖₘ (Global) | 18.6 ± 9 M⁻¹ s⁻¹ | 18.6 ± 9 M⁻¹ s⁻¹ | 18.6 ± 9 M⁻¹ s⁻¹ |

The abbreviation used is: MES, 4-morpholineethanesulfonic acid.
the ratio of slow off to fast on rates (concentration experiment). The lines are fits to the double exponential function as shown in Equation 2.

\[
I(t) = I_c + a_1(I_c - I_s)[1 - \exp(-k_{on1}t)] + (1 - a_1)(I_c - I_s)[1 - \exp(-k_{on2}t)] \quad \text{(Eq. 2)}
\]

See Table II for parameters that were fitted to these data.

**Table II**

Parameters fitted to the time course after exposure to Cu²⁺ (see Fig. 3)

| Parameter               | 0.1 mM (○) | 0.5 mM (■) | 1.0 mM (▲) |
|-------------------------|------------|------------|------------|
| \( I_{on} \)            | 0.52 ± 0.25| 0.42 ± 0.17| 0.54 ± 0.54|
| \( I_{off} \)           | 0.059 ± 0.005| 0.049 ± 0.005| 0.054 ± 0.005|
| \( \alpha_f \)          | 7.6 × 10⁻⁶ | 4.6 ± 0.02  | 0.72       |
| \( k_{on1} \) (Global)  | 89 ± 0.0002 s⁻¹ | 89 ± 0.0002 s⁻¹ | 89 ± 0.0002 s⁻¹ |
| \( k_{on2} \) (Global)  | 1.84 ± 1 × 10⁻⁶ s⁻¹ | 1.84 ± 1 × 10⁻⁶ s⁻¹ | 1.84 ± 1 × 10⁻⁶ s⁻¹ |

**Fig. 3.** Time course of washout after Cu²⁺ exposures of Fig. 1. Note the apparently paradoxical faster recovery rate for the 1 mM concentration experiment. The lines are fits to the double exponential function as shown in Equation 2.

**Fig. 4.** pH dependence of onset of inhibition and recovery from inhibition by 0.25 mM Cu²⁺. ○, pH 6.2; ■, pH 5.7; ▲, pH 5.2. The arrow indicates the time at which washout began, and the data points are connected by the lines.

**Fig. 5.** Voltage dependence of onset of inhibition and recovery from inhibition by 0.25 mM Cu²⁺. ○, -40 mV; ■, -20 mV; ▲, 0 mV; ▼, +20 mV; ●, +40 mV. The arrow indicates the time at which washout began. Lines are fits to double exponential functions as yielding the below tabulated fast and slow forward \((k_{onf} \text{ and } k_{ons})\) and backward \((k_{off} \text{ and } k_{ons})\) rate constants. See Table III for parameters that were fitted to these data.

**Voltage Dependence of Cu²⁺ Binding**—If the Cu²⁺-binding sites lie within the transmembrane region, then the rate of association of the positively charged copper ion may be accelerated at negative applied potentials. To test this possibility we measured the time course of inhibition by 250 μM Cu²⁺ at various holding voltages. We found that the rate of onset of inhibition was greater for more negative holding voltages (Fig. 5 and Table III), consistent with the presence of at least one Cu²⁺-binding site that is located at least partially inside the electric field of the membrane.

**Directionality of Cu²⁺ Inhibition**—Cationic, open-pore blocker molecules usually attenuate only the current originating from the side of the membrane to which they are applied. An example of this is the block of the outward, but not inward, K⁺ current of the squid axon by internally applied tetraethylammonium⁺ cation (28). We tested the directionality of the inhibition by Cu²⁺ by measuring the current-voltage relationship of the oocyte (pH_out = 6.2) with slowly varying ramps of current before and after various times after the application of Cu²⁺. For these experiments, the leakage current in the presence of amantadine was subtracted in order to obtain an accurate estimate of the reversal potential, and the membrane voltage was restricted to +50 mV to avoid activating endoge-
nous channels. We found (in six cells) that inward and outward currents were attenuated equally (see Fig. 6) and that the reversal voltage remained constant with increasing percentage inhibition by Cu\(^{2+}\). Thus, inhibition by Cu\(^{2+}\), like inhibition by amantadine (see Fig. 6, inset), is bi-directional.

Binding of BL-1743 Prevents Binding of Cu\(^{2+}\)—If Cu\(^{2+}\) binds to an internal site, it should be possible to block binding by prior application of a compound that either competes for the same site or occupies the outer regions of the pore. The compound BL-1743 has been shown to be a reversible inhibitor of the currents of the M\(_2\) ion channel (15). Mutations conferring resistance to BL-1743 map to the pore region immediately above His-37 (as viewed in Fig. 1), suggesting that BL-1743 penetrates deeply into the pore.

We tested the ability of BL-1743 to prevent inhibition by Cu\(^{2+}\). The test depended on the fact that recovery from inhibition by BL-1743 (Fig. 7 and Ref. 15) is considerably faster than that from 0.5 mM Cu\(^{2+}\) (4 min versus 4 h, respectively). To determine whether BL-1743 prevents inhibition by Cu\(^{2+}\), we performed the following steps: (i) inhibited the channel completely with BL-1743; (ii) while maintaining the concentration of BL-1743 constant, added 0.5 mM Cu\(^{2+}\) to the solution for 5 min (such that the Cu\(^{2+}\) would have completely inhibited the currents had it been applied alone); (iii) washed out free Cu\(^{2+}\) from the recording chamber briefly (2 min) while BL-1743 was still maintained in the solution; (iv) washed out BL-1743 and measured the time course of recovery. Our earlier results demonstrated that the time course of recovery from inhibition by Cu\(^{2+}\) alone is much slower than that from BL-1743. Thus, if Cu\(^{2+}\) had gained access to an internal binding site in the presence of BL-1743, then during washout of BL-1743 the recovery would have been slow and incomplete. However, we found that the time course of the recovery during washout of BL-1743 did not differ from the time course that would have been measured had Cu\(^{2+}\) not been applied (Fig. 7). Thus, BL-1743 prevented the binding of Cu\(^{2+}\) to a presumably internal site.

Mutation of His-37 Eliminates the High Affinity Cu\(^{2+}\)-binding Site—The above results strongly suggest that the high affinity Cu\(^{2+}\)-binding site lies within the transmembrane pore of M\(_2\). To confirm these observations, we examined the ability of Cu\(^{2+}\) to inhibit a number of variants of M\(_2\) in which potential chelating groups were altered. The amino acid sequence of the transmembrane region of M\(_2\) (residues 24–43) is: Asp-Pro-Leu-Val-Val-Ala-Ala-Ser-Ile-Ile-Gly-Ile-Leu-His-Leu-Ile-Leu-Trp-Ile-Leu. The only titratable residue in the transmembrane

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**Table III**

Parameters fitted to the time course of onset of inhibition and recovery from inhibition by 0.25 mM Cu\(^{2+}\) for membrane voltages between −40 and +40 mV (see Fig. 5)

| Parameter   | (○) −40 mV | (●) −20 mV | (▲) 0 mV | (▼) +20 mV | (●) +40 mV |
|-------------|-------------|-------------|-----------|------------|------------|
| \(k_{fp}\) (M s\(^{-1}\)) | 112.3       | 94.9        | 37.6      | 201.0      | 43.0       |
| \(k_{fr}\) (M s\(^{-1}\)) | 16.7        | 11.3        | 14.2      | 9.37       | 11.9       |
| \(10^2 k_{fr}\) (s\(^{-1}\)) | 51.8        | 0.388       | 0.71      | 2.8        | Unmeas.\(^a\) |
| \(10^2 k_{fr}\) (s\(^{-1}\)) | 0.31        | 0.386       | 0.084     | 0.076      | Unmeas.\(^a\) |

\(^a\)Unmeasured.

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**Fig. 6.** Cu\(^{2+}\) inhibits both inward and outward amantadine-sensitive currents. The current-voltage relationship of an oocyte expressing the wild-type M\(_2\) ion channel was measured at the times shown after application of 0.1 mM Cu\(^{2+}\) to the bathing medium. The inset shows the inhibition after addition of 10 \(\mu\)M amantadine to the bathing medium. Note that both inward and outward currents were inhibited by both compounds.
region is His-37, and this residue has been shown to be important for amantadine inhibition. Thus, we compared the ability of Cu$^{2+}$ to inhibit the mutant proteins M$_2$-H37A and M$_2$-H37G with its ability to inhibit the wild-type M$_2$ protein. In addition, we examined the role of the Asp-24, which lies near the N terminus of the $\alpha$-helix, and Ser-31, which lines a portion of the predicted pore. Finally, wild-type M$_2$ protein has two cysteine residues at positions 17 and 19 in the extracellular domain. It appeared unlikely that they would be responsible for binding Cu$^{2+}$ because these residues participate in disulfide bonding to stabilize the homotetramer (4–6). Nevertheless, we tested for this possibility by using a previously characterized “cysteineless” mutant in which each of the three cysteine residues of the M$_2$ protein, found at positions 17, 19, and 50, were mutated to serine.

We examined the time course of inhibition and recovery from inhibition of the currents of the M$_2$-H37A and M$_2$-H37G mutants by 0.1 and 1.0 mM Cu$^{2+}$. The limiting fractional inhibition was less than for the wild-type protein, and the rates of inhibition and recovery for both of these mutant proteins were faster than for the wild-type M$_2$ protein (Fig. 5 and Table III). Moreover, we found that the inhibition of neither mutant protein was strongly dependent on pH (see Fig. 9 for M$_2$-H37G), in contrast to the greater pH dependence found for the wild-type protein (Fig. 4). These results suggest that replacement of His-37 with a residue incapable of coordinating Cu$^{2+}$ leaves the ion channel with a low affinity binding site that is located near the outside of the electric field.

We also tested the possibility that the cysteine residues located at positions 17 and 19 (29), the charged aspartate residue at position 24, or the polar serine residue at position 31 might provide a low affinity binding site for Cu$^{2+}$ that may be partly responsible for the rapid recovery from inhibition by 1 mM Cu$^{2+}$ (see Fig. 3, Table II, and “Discussion”).

To determine the possible location of the low affinity Cu$^{2+}$-binding site in M$_2$-H37A and M$_2$-H37G, we measured the voltage dependence and pH dependence of their inhibition. The voltage dependence for both proteins (see Fig. 8 and Table IV for M$_2$-H37G) is similar and much less significant than for the wild-type protein (Fig. 5 and Table III). Moreover, we found that the inhibition of neither mutant protein was strongly dependent on pH (see Fig. 9 for M$_2$-H37G), in contrast to the greater pH dependence found for the wild-type protein (Fig. 4). These results suggest that replacement of His-37 with a residue incapable of coordinating Cu$^{2+}$ leaves the ion channel with a low affinity binding site that is located near the outside of the electric field.

![Fig. 7. Prior exposure to BL-1743 prevents inhibition by Cu$^{2+}$. The oocyte currents were measured at pH 6.2 to determine the maximal current. The current was then completely inhibited by BL-1743 (0.1 mM). While inhibited, 0.5 mM Cu$^{2+}$ was applied to the oocyte for 5 min, a long enough time to achieve full inhibition from which recovery would have been very slow had the Cu$^{2+}$ been applied alone (see Fig. 2). After 2 min washout of Cu$^{2+}$, the BL-1743 was finally washed out, and the time course of the fraction of the recovery was measured (●). This recovery was compared with the recovery from 0.1 mM BL-1743 alone (□) and the recovery from 0.5 mM Cu$^{2+}$ alone (▲). Note that the time course of recovery of current of oocytes treated with BL-1743 before Cu$^{2+}$ (●) was not distinguishable from that of oocytes inhibited by BL-1743 alone (□); data from Ref. 15.]

![Fig. 8. Inhibition of the current of the M$_2$-H37G mutant protein by 0.1 mM Cu$^{2+}$ is rapid, incomplete, and reversible. Inhibition is also relatively independent of membrane voltage (●, –40 mV; ▲, –20 mV; ○, 0 mV; ▼, +20 mV). See Table IV for parameters that were fitted to these data. The lines show single exponential fits with the following rate constants: inset, the time course of inhibition by 0.1 mM Cu$^{2+}$ of the M$_2$-H37A mutant protein (average of four cells) is compared with that of the wild-type protein (data from Fig. 1 and Fig. 2); note the rapid onset and offset of inhibition; black bars show times of application of Cu$^{2+}$. See Table IV for parameters that were fitted to these data.]

| Table IV |
| Parameters fitted to the inhibition of the current of M$_2$-H37G mutant protein by 0.1 mM Cu$^{2+}$ for voltages between –40 and +20 mV |
| $k_i$ (m$^{-1}$ s$^{-1}$) | 561 | 660 | 590 | 475 |
| $k_o$ (s$^{-1}$) | 0.0396 | 0.0320 | 0.031329 | 0.0086490 |

Metal Ion Specificity—To characterize further the ion specificity of the low and high affinity binding sites in M$_2$, we evaluated a series of transition metal ions, including Cd$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, Pt$^{2+}$, and Zn$^{2+}$. As was expected for the...
replacement of a relatively soft for a hard metal ion, the
replacement of Cu$^{2+}$ with Mg$^{2+}$ led to essentially no inhibition,
indicating that Mg$^{2+}$ does not interact with either site. Simi-
larly, Mn$^{2+}$, which has ligand preferences similar to Mg$^{2+}$, but
also shows some “soft” character, inhibited the channel by less
than 10% at 1 mM concentration. We next examined Cu$^{+}$
and Zn$^{2+}$, which have ligand preferences similar to Cu$^{2+}$ but have
a preference for octahedral or tetrahedral complexes and would
be less likely to assume the distorted square pyramidal com-
plex hypothesized for Cu$^{2+}$. These metal ions showed partial
inhibition of the channel (Table V), and recovery from inhibition
by these metal ions was nearly complete within 2–5 min.
We therefore tentatively assign their effects to interactions
with the low affinity site. In a similar manner, we examined Ni$^{2+}$
and Pt$^{2+}$, which have a preference for forming square planar complexes; again these ions gave rise to only partial, rapidly reversible inhibition of the channel (Table V). All of
these data suggest that the high affinity metal ion-binding site
is quite specific for Cu$^{2+}$, whereas the remaining, low affinity
site is less specific and able to interact with a variety of metal ions.
To confirm this suggestion, we tested the ability of 1 mM
Cd$^{2+}$, Ni$^{2+}$, and Zn$^{2+}$ to inhibit the currents of the M$_2$H37A
and M$_2$H37G mutant proteins. Indeed, all three metal ions inhibited these mutants in a manner similar to that observed
for the wild-type protein (data not shown).

**DISCUSSION**

The experiments described here demonstrate that Cu$^{2+}$ and
other transition metals are capable of inhibiting the M$_2$ ion
channel of influenza A virus. Inhibition and recovery from
inhibition can be explained by a model with two binding sites,
each capable of inhibiting the current upon binding a ligand.
One site, of low affinity and low specificity for Cu$^{2+}$, is located
near the outside of the electric field of the membrane and only
partially impedes ionic current through the channel. The sec-
ond site has higher affinity for Cu$^{2+}$, is located inside the
applied electric field, and more completely blocks current flow.
The high affinity site is probably formed by the association of
imidazole side chains of His-37 from the transmembrane heli-
ces of the M$_2$ tetramer.

The strong voltage dependence of the level of inhibition of the
current of the wild-type channel by Cu$^{2+}$ (Fig. 5 and Table III)
suggests that the imidazole of His-37 is tightly coupled to the
high affinity binding site for Cu$^{2+}$. This is consistent with
models showing His-37 to be located well inside the presumed
transmembrane domain (18, 22), which begins with Pro-25.
Also, inhibition by low [Cu$^{2+}$] is dependent on pH$_{out}$. The
dependence of Cu$^{2+}$ inhibition on pH$_{out}$ occurs within a range
of pH that modulates the currents of the wild-type ion channel.
This modulation of the ionic current has been shown to depend
on the presence of His-37. Since His-37 is the only residue in the
transmembrane domain that is titratable in the range of
pH values studied, it is likely that this residue is also respon-
sible for the pH dependence of inhibition rate. A reasonable
explanation for the slower onset of inhibition at low pH (Fig. 4)
is that competition of H$^+$ and Cu$^{2+}$ for a binding site on the
imidazole of His-37 favors H$^+$ at low pH. Finally, the presence
of the reversible inhibitor BL-1743 prevents inhibition by low
[Cu$^{2+}$] (Fig. 7). BL-1743 has been shown to inhibit the M$_2$ ion
channel, and several mutants that are not affected by the
compound have been identified (15). Most of these mutants
map to residues of the transmembrane domain located between
Pro-25 and His-37, suggesting that the compound occupies the
outer region of the pore. It is probable that the compound
prevented inhibition by restricting access of Cu$^{2+}$ to His-37.

These results point to the predictive power of the current
model for M$_2$ and also add considerably to the mechanistic
understanding of this channel. We have recently proposed that
His-37 lies within the conduction pathway of the channel,
where it serves as a relay to selectively shuttle protons down
corresponding concentration gradient into the virus. In this
mechanism, the channel conducts more efficiently at low pH primarily
because of an increase in the concentration of the protonated
imididine histidine. His-37 allows efficient shuttling in vivo because it
has a pK$_a$ between pH 5 and 7 where the proton is not too
strongly bound. The finding that Cu$^{2+}$ binds to a high affinity
site associated with His-37 is consistent with this because the
normal ligation would require the histidine to be in its unpro-
tonated, basic form.

Although our experiments indicate that the inner, high af-
finitiy binding site is probably His-37, we were not able to
identify a single low affinity, exterior site. We mutated each
residue external to His-37 whose side chain might be thought
to bind divalent metal ions (Cys-17, Cys-19, Asp-24, and Ser-
31), and we found that inhibition by Cu$^{2+}$ was essentially the
same as that found for the wild-type M$_2$ protein.

As many ligand- and voltage-gated ion channels are modified
by transition metals, it would be of interest to compare the
effects of transition metals on the M$_2$ ion channel with the

**TABLE V**

Inhibition of wild-type M$_2$ ion channel activity by transition metals

| Metal | Concentration | Steady state fractional inhibition ($\text{Mean} \pm \text{S.E.}^a$) | Time | n  |
|-------|---------------|------------------------------------------------|------|----|
| Cu$^{2+}$ | 500 µM | 0.95 ± 0.01 | 600 | 4  |
| Cu$^{+}$ | 50 µM | 0.71 ± 0.05 | 60 | 4  |
| Zn$^{2+}$ | 1 mM | 0.28 ± 0.02 | 300 | 4  |
| Ni$^{2+}$ | 1 mM | 0.13 ± 0.01 | 300 | 4  |
| Pt$^{2+}$ | 1 mM | 0.35 ± 0.00 | 120 | 4  |
| Mg$^{2+}$ | 2 mM | 0.04 ± 0.01 | 300 | 4  |
| Mn$^{2+}$ | 1 mM | 0.07 ± 0.03 | 300 | 4  |

$^a$ Currents measured at $-120\text{ mV}$. 

**FIG. 9.** Inhibition of the current of the M$_2$H37G mutant pro-
tein by 0.1 mM Cu$^{2+}$ is relatively independent of pH. The inhibi-
tion was measured for three values of pH: pH 6.2 (●), pH 5.7 (○), and
pH 5.2 (△).
effects on other ion channels. Among the best-studied of these channels is the Na⁺ channel; however, the inhibition of the Na⁺ channel by transition metals differs greatly from the inhibition of the M₂ ion channel by Cu²⁺. 1) Cu²⁺ inhibits both inward and outward currents of the M₂ ion channel, but transition metals inhibit the inward current of the cardiac Na⁺ channel. 2) The Cu²⁺-binding site in M₂ is highly specific for Cu²⁺, whereas Cd²⁺ and Mn²⁺ inhibit the cardiac Na⁺ channel but do not inhibit the M₂ ion channel. 3) The high affinity binding site differs; the imidazole of His-37 of the transmembrane domain of the M₂ ion channel is probably the high affinity binding site for Cu²⁺, but Cys-401 of the pore region of the cardiac Na⁺ channel is thought to be the high affinity binding site for Cd²⁺ and Zn²⁺ (30, 31). Such differences in metal binding indicate that these ion channel proteins have stable, but different, structures that present coordinating ligands of different affinities to permeating metal ions.

The results of our experiments have important implications for future work. First, Cu²⁺ has an unpaired electron and thus could serve as a probe for structural studies of the M₂ molecule using either electron paramagnetic resonance or ENDOR methods. Second, the inhibition of the M₂ ion channel by low [Cu²⁺] shares several important properties with the inhibition by amantadine; both are slowed by low pH, both inhibit currents in both inward and outward directions, and both depend on the presence of His-37. If Cu²⁺ and amantadine interact with the imidazole of His-37 in a similar fashion, then information gained using Cu²⁺ may be helpful in the design of inhibitors that are more useful than amantadine.

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APPENDIX

This Appendix derives kinetic models for two-state binding of Cu²⁺ to the M₂ ion channel protein. We envision an outer site located in the pore of the channel close to the outside of the membrane electric field and an inner site located near the inside and well within the transmembrane electric field. Binding of Cu²⁺ to the outer site is assumed to be capable of inhibiting the current to a fraction of its initial value, whereas binding to the inner site inhibits completely. States are defined by their location and Cu²⁺ occupancy: Sa is the state defined by unoccupied inner and outer sites, Sb by occupancy of only the outer site, Sc by only the inner site, and Sd by both sites occupied. Different non-equivalent kinetic schemes can be devised to connect the states. We used two as follows: the first (Scheme 1) prohibiting Sa to Sc but allowing a direct, reversible Sa to Sd transition; and the second (Scheme 2) allowing Sa to Sc and prohibiting Sa to Sd. Note that no distinction is made in this mechanism between the two sides of the membrane so it is possible that the Sd to Sa transition involves Cu²⁺ entering the cytoplasm of the oocyte.

The forward and reverse reaction rate constants for each transition are those defined in the following equations of Schemes 1 and 2.

\[
\frac{dS_a}{dt} = -(k_{1f}[Cu^{2+}] + k_{2f}[Cu^{2+}]^2)Sa + k_{3b}Sb + k_{4b}Sd
\]

\[
\frac{dS_b}{dt} = -(k_{4f} + k_{1b})Sb + k_{1f}[Cu^{2+}]Sa + k_{2b}Sc
\]

\[
\frac{dS_c}{dt} = -(k_{1f}[Cu^{2+}] + k_{4b})Sc + k_{3b}Sb + k_{3b}Sc
\]

\[
\frac{dS_d}{dt} = -(k_{4b} + k_{3b})Sd + k_{4f}[Cu^{2+}]Sa + k_{4f}[Cu^{2+}]Sc
\]

These equations, with the initial condition that Sa = 1 and all other states are empty, were used (by the program MLAB; Civilized Software, Inc., Bethesda, which has a built-in differential equation solving routine (33)) to generate current-time records for the three different Cu²⁺ concentrations (100, 500, and 1000 μM). Copper concentrations were taken to be step constant.

Standard errors, as reported by the MLAB program, are for qualitative comparison only because they depend on the un-supportable assumption that the curve-fitting error function varies linearly with the fitting parameters. The number of parameters in the models discourages quantitative interpretation of these results.

To compare the site affinities for Cu²⁺, the reactions Sa + Cu²⁺ → Sb and Sa + Cu²⁺ → Sc are the relevant equilibria. The “operational” site dissociation constants K_{outer} and K_{inner} corresponding to the above reactions can be calculated from the rate constant ratios. For Scheme 1, K_{outer} = k_{1f}/k_{2f} = 0.4 mM and K_{inner} = K_{outer} k_{2b}/k_{3b} = 1.9 μM. For Scheme 2, K_{outer} = k_{4b}/k_{1f} = 0.25 mM; K_{inner} k_{2b}/k_{3b} = 1.6 μM.
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