A Theory for the Proton Transport of the Influenza Virus M2 Protein: Extensive Test against Conductance Data

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ABSTRACT A theory for calculating the proton flux through the influenza virus M2 channel is tested here against an extensive set of conductance data. The flux is determined by the rate constants for binding to the His37 tetrad from the two sides of the membrane and the corresponding unbinding rate constants. The rate constants are calculated by explicitly treating the structure and dynamics of the protein. Important features revealed by previous studies, such as a gating role for Val27 at the entrance to the channel pore, and channel activation by viral exterior pH, are incorporated in this theory. This study demonstrates that the conductance function of the M2 proton channel can now be quantitatively rationalized by the structure and dynamics of the protein.

INTRODUCTION

One of the coat proteins of the influenza A virus, M2, is a proton channel (1,2). This channel activity is essential for viral replication (3). M2 functions as a tetramer; each monomer has a single transmembrane helix. The pore for proton transport is formed at the center of the transmembrane helix bundle, with the N-terminals facing the viral exterior and the C-terminals facing the viral core. The channel is activated by the low pH of the endosome while the virion is encapsulated there. Proton transport through the M2 protein acidifies the viral core, facilitating the release of viral RNA to the host cell. The structure, dynamics, and function of the M2 proton channel have been the focus of intense studies (4–19). These studies have set the stage for correlating channel function with protein structure and dynamics. Toward that goal, a microscopic theory that accounts for the structure and dynamics of the M2 protein was developed to calculate the proton flux (20). Qualitatively, the theory was promising in that it captured the main characteristics of the M2 conductance activity. In this article, we show that the theory is able to quantitatively reproduce an extensive set of conductance data (7,12,16).

In our theory, the motions of permeant protons are modeled as diffusive (see Fig. 1). It should be noted that the mechanism for proton movement through water involves shuttling along a water chain: an excess proton transiently associates with a water molecule to form a hydronium ion; a proton then hops from the hydronium ion to the next water molecule to form another hydronium ion (21). This Grotthuss mechanism differs from the hydrodynamic diffusion of other ions, and explains why the apparent diffusion constant, $D_{\text{app}}$, is around fivefold higher than those of other univalent ions of similar size. As noted previously (20), this diffusion constant can explain the high rate constant, $10^{10}$ M$^{-1}$ s$^{-1}$, of proton binding to an imidazole in aqueous solution (23), assuming that the binding is diffusion-limited. To pass through the M2 channel pore, we suppose that a permeant proton first binds to and then unbinds from an internal site, i.e., the His37 tetrad. This obligatory binding site has strong experimental support (17,18). Furthermore, the motion of the permeant proton through the pore is gated by Val27 on the N-terminal side and by Trp41 on the C-terminal side. The gating role of Trp41 has long been recognized (15). More recently, based on molecular dynamics (MD) simulations, Val27 has been proposed as a secondary gate (9). Although the gating dynamics of Val27 is assumed to be local, the gating dynamics of Trp41 is assumed to be coupled to the backbone dynamics of the transmembrane domain (11).

The transport of a proton through the M2 channel starts from the bulk solution in the viral exterior. The proton enters the pore by diffusion, passes through the Val27 gate when it is open, and diffuses further down the pore to bind to the (doubly protonated (18)) His37 tetrad. The bound proton is then released to either the N-terminal pore or, provided the Trp41 gate is open, the C-terminal pore. In the latter case, the proton diffuses out to the bulk solution in the viral core and completes the transport process. This process can also be reversed, bringing one proton from the viral core to the exterior bulk solution. Let the diffusion-influenced rate constants for proton binding to the internal site from the viral exterior and interior be $k_{\text{e}}^-$ and $k_{\text{i}}^-$, respectively, and the rate constants for releasing a bound proton to the viral exterior and interior be $k_{\text{e}}^+$ and $k_{\text{i}}^+$, respectively. When the proton concentrations in the viral exterior and interior are $C_{\text{e}}$ and $C_{\text{i}}$, respectively, the net proton influx is

$$I = \frac{-k_{\text{e}}^- C_{\text{e}} + k_{\text{i}}^- C_{\text{i}}}{k_{\text{e}}^- + k_{\text{i}}^- + k_{\text{e}}^+ C_{\text{e}} + k_{\text{i}}^+ C_{\text{i}}}.$$ (1)
In terms of the exterior pH, one may write $C_e = 10^{-pHe}$; in a similar way, $C_i = 10^{-pHi}$.

Equation 1 is derived from a set of steady-state rate equations. In previous rate-equation based models of ion transport, the rate constants are simply taken as adjustable parameters (24–26). Our theory goes one step further: we calculate the rate constants of Eq. 1 by treating the diffusional motion of the permeant ion and the gating motions of the channel protein. Thus, we directly incorporate the structure and dynamics of the M2 channel into our theory. Note that in our treatment, the ion-transport system is now represented at the same level as in the Poisson-Nernst-Planck theory (25,27,28). However, our theory is distinct from the Poisson-Nernst-Planck theory in two respects. In the latter theory, one calculates the ion flux from the steady-state electrodiffusion gradient. The channel pore in our theory explicitly contains an obligatory binding site for the permeant ion, and we calculate the rate constants, $k_{e+}$ and $k_{i-}$, for the permeant ion binding to this internal site from the two sides of the membrane. More importantly, our channel is not static but dynamic, and we explicitly account for the effects of the gating motions of the channel on $k_{e+}$ and $k_{i+}$. Note that $k_{e+}$ or $k_{i+}$ is an effective rate constant involving the whole process of a proton starting in the bulk solution, diffusing into the dynamic channel and to the internal site, and finally binding to it. The last step is assumed to be fast (corresponding to an absorbing boundary condition), and hence, the overall binding rate constant is diffusion-controlled.

Equation 1 predicts that the proton influx exhibits a sharp increase when pH_e is lowered to the third pK_a of the His$^{37}$ tetrad, which is 6.3 ± 0.3 (18). Because of the asymmetry in geometry and in gating dynamics between the N-terminal pore and the C-terminal pore (see Fig. 1), $k_{e+}$ and $k_{i+}$ have different values; correspondingly, $k_{e-}$ and $k_{i-}$ are also different. As a result, the influx and efflux are predicted to be asymmetric when the pH gradient across the membrane is reversed. All of these are qualitative features observed in M2 conductance measurements (12,16). Here, we extend and refine our theory for proton transport and show that it is capable of quantitatively reproducing a significant amount of experimental conductance data.

The unbinding rate constants, $k_{e-}$ and $k_{i-}$, are related to the corresponding binding rate constants, $k_{e+}$ and $k_{i+}$, through the dissociation constants, $K_{de}$ and $K_{di}$. If there is no change in chemical potential when a proton is transferred from the bulk solution on either side of the membrane to the internal site, then both $K_{de}$ and $K_{di}$ are equivalent to the third pK_a of the His$^{37}$ tetrad, which hereafter will be denoted as pK$_{a0}$. In this case, we have

$$K_{de} = \frac{k_{e-}}{k_{e+}} = 10^{-pK_{a0}}, \quad (2a)$$

$$K_{di} = \frac{k_{i-}}{k_{i+}} = 10^{-pK_{a0}}. \quad (2b)$$

If a transmembrane voltage is applied, then there are differences in chemical potential, to be denoted as $\Delta U_e$ and $\Delta U_i$, respectively, between the internal site and the two bulk solutions. We now have

$$K_{de} = \frac{k_{e-}}{k_{e+}} = 10^{-pK_{a0} e^{\Delta U_e/k_B T}}, \quad (3a)$$

$$K_{di} = \frac{k_{i-}}{k_{i+}} = 10^{-pK_{a0} e^{\Delta U_i/k_B T}}, \quad (3b)$$

where $k_B$ is Boltzmann’s constant and $T$ is the absolute temperature. These relations allow us to focus on the binding rate constants $k_{e+}$ and $k_{i+}$.

**Binding to the internal site in the static pore**

We treat the binding to the internal site as diffusion-controlled. Therefore, the internal site, as a disk spanning the cross section
of the pore, serves as an absorbing boundary to protons coming to the pore from either side of the membrane. Though the theory allows the binding to be treated as partial absorption, it has been argued previously that proton binding to the His37 tetrad is diffusion-controlled and an absorbing boundary condition is appropriate for modeling the M2 proton channel (20). For now, the pore is assumed to be static, and correspondingly, the rate constants are denoted as $k_{se}$ and $k_{si}$. Let $x$ denote the coordinate along the pore axis, with value 0 at the entrance from the viral exterior and value $L$ at the exit to the viral core. The pore is assumed to be axially symmetric, with radius $a_c$ at $x = 0$ and radius $a_i$ at $x = L$. The internal site is located at $x = L_c$. We denote the potential of mean force along the pore axis as $U(x)$, and the cross-sectional area as $\sigma(x)$. The reference of $U(x)$ is chosen such that $U(0) = 0$. The difference in potential of mean force between the two ends of the pore, $U(L) - U(0)$, is denoted as $\Delta U$. Note that $\Delta U = \Delta U_e - \Delta U_i$. This basic model, except for the boundary condition at the internal site, is similar to what underlies the Poisson-Nernst-Planck theory, and is hence subject to all the caveats noted previously for the Poisson-Nernst-Planck theory (27).

The rate constants are given by (A. M. Berezhkovskii, A. Szabo, and H.-X. Zhou, unpublished)

$$\frac{1}{k_{se}} = \frac{1}{4Da_c} + \int_0^{L_e} dx \left[ D\sigma(x) e^{-U(x)/kT} \right]^{-1},$$

$$\frac{1}{k_{si}} = \frac{1}{4Da_i} + \int_{L_e}^L dx \left[ D\sigma(x) e^{-(U(x)-\Delta U)/kT} \right]^{-1}.$$  

These expressions bear resemblance to some of the results in the Poisson-Nernst-Planck theory (25). In fact, for the static pore considered at the moment, it can be shown that when the internal binding site is represented as a deep energy well, our theory gives the same result for the ion flux as does the Poisson-Nernst-Planck theory. We model the N-terminal pore as a cylinder, with radius $a$. The C-terminal pore is assumed to be a topless cone, with radius $a$ at the internal binding site and radius $a_c$ at the pore exit. When $U(x) = 0$, we find that

$$\frac{1}{k_{se}} = \frac{1}{4Da} + \frac{L_e}{\pi Da},$$

$$\frac{1}{k_{si}} = \frac{1}{4Da} + \frac{L_i}{\pi Da},$$

where $L_e = L - L_c$. These results are found in an earlier work (20).

**Effect of a transmembrane voltage**

A transmembrane voltage, $V$, corresponds to a change of $\Delta U = eV$ in the potential of mean force, where $e$ is the protonic charge. We assume that the change is linearly accrued along the pore, resulting in a potential of mean force (29, 30)

$$U(x) = \frac{eV}{L}x.$$  

The differences in potential between the internal site and the two bulk solutions are

$$\Delta U_e = \frac{eV}{L} \frac{L_e}{L},$$

$$\Delta U_i = -eV \frac{L_i}{L}.$$  

Equation 4 now becomes

$$\frac{1}{k_{se}} = \frac{1}{4Da} + \frac{e^a - 1}{\alpha \pi Da^2},$$

$$\frac{1}{k_{si}} = \frac{1}{4Da} + \frac{1}{\pi D} \int_0^L dx \left( \frac{e^{-Vx/Lk_BT}}{[a_i + (a - a_i)x/L]^2} \right).$$

Equation 8a, which introduces a symbol $\alpha$ to denote $\Delta U_e/k_BT$, is found in an earlier work (20). No closed-form expression for the integral in Eq. 8b is available; hence, we evaluate it here by numerical integration.

**Val27 gating**

As noted above, our channel is not static but dynamic. We model the four Val27 residues near the pore entrance as a gate at $x = 0$ that stochastically opens and closes. Let the open-closed transition rates be $\omega_o$ and $\omega_c$,

$$\text{open} \xrightleftharpoons[\omega_c]{\omega_o} \text{closed},$$

and let $\omega = \omega_o + \omega_c$. Following earlier work (31), the rate constant for binding from the viral exterior is given by

$$\frac{1}{k_{se}} = \frac{1}{k_{se}^o} + \frac{\omega}{\omega_o} \left[ \frac{1}{\omega T_k} + \frac{1}{\omega T_k'} \right].$$

We now explain the two new quantities appearing in the brackets. $T_k(s)$ is the Laplace transform,

$$\hat{k}_{eb}(s) = \int_0^\infty e^{-st} k_{eb}(t),$$

of the time-dependent rate coefficient, $k_{eb}(t)$, for protons in the exterior bulk solution being absorbed at the pore entrance. The steady-state value of $k_{eb}(t)$ is $4Da$, the inverse of the first term in Eq. 5a. An accurate approximate expression for $\hat{k}_{eb}(s)$ is (32)
\[ s k_{eb}(s) / 4D a = 1 + (\pi/4)(s a^2/D)^{1/2} - \frac{(1 - \pi/4)(s a^2/D)^{1/2}}{(1 - \pi/4)\pi/2(\pi^2/8 - 1) + (s a^2/D)^{1/2}}. \]  

The other quantity, \( \hat{k}_{ap}(s) \), is the Laplace transform of the total flux across the N-terminal pore entrance, under the condition that the initial distribution of the proton is uniformly zero in the N-terminal pore and its distribution function at the pore entrance is maintained at 1. The boundary condition at the other end of the N-terminal pore, i.e., at the internal binding site, is absorbing. For the linear potential of Eq. \( 6 \), the result is (see Appendix A)

\[ s \hat{k}_{ap}(s)/(\pi Da^2/L_e) = \gamma \coth \gamma - \alpha/2, \]  

where

\[ \gamma = (s L_e/d^2/D + \alpha^2/4)^{1/2}. \]  

When \( U(x) = 0 \), a condition realized by setting \( \alpha = 0 \), Eq. 12a reduces to the result given previously (20).

If the gating dynamics is slow on the timescales of the diffusional motion over the pore entrance cross section and down the pore, i.e., \( \omega \ll D/a^2 \) and \( D/L_e^2 \), it can be shown that Eq. 9 reduces to (33)

\[ k_{c+} = \frac{\omega_o}{\omega_c} k_{se+}. \]  

**Trp\(^{41} \) gating**

In our model, the gating dynamics of Trp\(^{41} \) is coupled to the backbone dynamics of the transmembrane domain (11). Essentially, as far as binding to the internal site from the interior bulk solution is concerned, the transmembrane domain interconverts between two conformations. The open conformation allows for binding to the internal site, with the rate constant given by \( k_{a+i} \) of Eq. 8b. In the other, the closed conformation, binding is prohibited. The ion flux for a channel that has an internal binding site and interconverts between two conformations has been derived (24). The result formally reduces to Eq. 1 when the conformational interconversion is much faster than proton binding/unbinding (see Appendix B), a condition that has been argued to apply to the M2 proton channel (20). The effective rate constant for proton binding to the internal site from the interior bulk solution is then given by

\[ k_{i+} = p_{open} k_{ai+}, \]  

where \( p_{open} \) is the equilibrium fraction of the open conformation.

**Activation by low pH\(_e\)**

The M2 proton channel is activated by low pH\(_e\). Here, we assume that activation amounts to converting the closed conformation to the open conformation. We model activation explicitly by assuming that \( p_{open} \) depends on pH\(_e\); that is, \( p_{open} \) increases gradually as pH\(_e\) is lowered. In contrast to pH\(_e\), pH\(_i\) does not play any role in our modeling of channel activation, because, as we proposed previously (11), access to the His\(^{37} \) tetrad is blocked by the Trp\(^{41} \) gate until the C-terminal portion is already in the open conformation.

**Model parameters**

In our theory, we derive rate constants for proton binding from an explicit treatment of the structure and dynamics of the M2 channel. The results involve only a few well-characterized parameters. These include the proton diffusion constant \( D \); the dissociation constant of the permeating proton, as measured by the third pKa, pKa\(_{i0} \), of the His\(^{37} \) tetrad; four parameters determined by the structure of the M2 transmembrane domain (the full length of the pore \( L \), the radius \( a \) and length \( L_e \) of the N-terminal pore, and the radius \( a_i \) of the C-terminal pore entrance in the open conformation); and three parameters determined by the dynamics of the M2 protein (transition rates \( \omega_o \) and \( \omega_c \) of the Val\(^{27} \) secondary gate and the equilibrium fraction \( p_{open} \) of the C-terminal open conformation). The value of \( D \) has been measured to be 10^3 A^2/ns (22). The value of pKa\(_{i0} \) has also been measured (18); here, pKa\(_{i0} \) was fixed at 6. The structure of the M2 transmembrane domain (5–7) provides a solid basis for the geometric parameters of the pore; we set \( L \), \( L_e \), and \( a \) to be 35, 21, and 3 Å, respectively. Our choice of 5 Å for the value of \( a_i \) was guided by a model of the open conformation developed previously based on MD simulations (11). With these parameters, the binding rate constants \( k_{se+} \) and \( k_{ai+} \) for a static pore have values of 0.7 x 10^9 and 1.7 x 10^9 M^-1 s^-1, respectively, at \( V = 0 \) (see Eq. 5); values of 1.3 x 10^9 and 1.0 x 10^9 M^-1 s^-1, respectively, at \( V = -60 \) mV; and values of 0.3 x 10^9 and 2.7 x 10^9 M^-1 s^-1, respectively, at \( V = 60 \) mV (see Eq. 8).

There is less certainty on the three gating parameters, \( \omega_o \) and \( \omega_c \), and \( p_{open} \). We restricted these parameters to the following ranges: \( \omega_o \) between 0.1 and 1 ns^-1; \( \omega_c \) between 1 and 10 ns^-1; and \( p_{open} \) between 0.1 and 10%. These ranges were guided by MD simulations (9,11) and solid-state NMR experiments (8). The precise values of the three parameters were adjusted for each set of experimental data and are listed in the figure legends below. With these choices of parameters, the Val\(^{27} \) gating dynamics is close to the slow-gating limit: \( k_{c+} \) approaches the result given by Eq. 13. For example, at \( \omega_o = 0.1 \) ns^-1 and \( \omega_c = 1 \) ns^-1, Eq. 9 predicts 10.3-, 9.7-, and 8.6-fold decreases from \( k_{se+} \) for \( k_{c+} \), at \( V = -60, 0, \) and 60 mV, respectively.
In comparison, Eq. 13 predicts an 11-fold decrease for all three voltages.

RESULTS

Here, we quantitatively test our theory against an extensive set of experimental data on M2 proton conductance. The experimental data of Chizhmakov et al. (12,16) were obtained from the whole-cell patch-clamp technique, which measured the total current conducted by multiple M2 proton channels, but the M2 copy numbers in the cells were unknown. (Only the currents due to M2 were used for the comparison here; that is, the rimantadine-resistant currents were subtracted from all the data.) In comparing against these experimental data, we scaled the calculated results for a single channel; the scaling factor was adjusted for optimal agreement. Chizhmakov et al. reported data for the M2 channels from two strains of influenza virus. Structural determinations and MD simulations have so far been restricted to the M2 of the Weybridge strain. Since our theory accounts for the structure and dynamics of the protein, in testing against the experiments of Chizhmakov et al. only the Weybridge conductance data were used. We further test our theory against the recent experimental data of Busath and co-workers (7). The latter data were obtained from M2 channels reconstituted into liposomes and report single-channel proton flux deduced from the known M2 copy number.

Current-voltage relations at fixed $p_{He}$ and $p_{Hi}$

Chizhmakov et al. (12,16) measured current-voltage relations for a large number of interior and exterior pH values. All of these can be reproduced quite well by our theory. Fig. 2 shows the comparison of the experimental data with the calculation results. Consistent with our modeling of activation by $p_{He}$, the values of $p_{open}$ used for producing the results that match the experimental data increases with decreasing $p_{He}$ (see Fig. 2 legend). As Fig. 3 shows, the dependence of $p_{open}$ on $p_{He}$ can be fitted to the function

$$p_{open} = p_{open0} e^{-\left(p_{Hi}/p_{He}\right)^5},$$

(15)

where $p_{open0} = 12.7\%$ and $p_{H0} = 6.2$. It is interesting to note that the value of $p_{H0}$, which characterizes the transition of $p_{open}$ from 0 at $p_{He} \gg 1$ to the value $p_{open0}$ at $p_{He} = 0$, is close to the third $pK_a$ value of the His$^{37}$ tetrad.

$p_{He}$-dependent proton flux at fixed $p_{Hi}$ and voltage

In Fig. 4 A, we show the dependence of the inward proton flux on $p_{He}$ when $p_{Hi}$ is fixed at 7.4. Two different voltages, $-60$ mV and $+60$ mV, are applied. Both sets of experimental data are well reproduced by the calculations. Chizhmakov et al. (12) presented their data as the chord...
conductance, \( g = I/(V - V_{H}) \), where \( V_{H} \) is the equilibrium potential, given by \( 58.4(pHi - pHe) \text{ mV} \). In our calculations, \( p_{open} \) was \( pHe \)-dependent, as given by Eq. 15, to model activation by \( pHe \). If \( p_{open} \) were chosen to be independent of \( pHe \), the chord conductance would show a turnover at the low \( pHe \) end. (In fact, even with a \( pHe \)-dependent \( p_{open} \), a slight turnover is discernible in the theoretical curve for \( V = 60 \text{ mV} \).) At fixed \( pHi \) and \( V \), whether \( p_{open} \) is independent of \( pHe \) or has a \( pHe \) dependence like Eq. 15, the inward proton flux monotonically increases when \( pHi \) is lowered. However, the magnitude of \( V - V_{H} \) also increases when \( pHi \) is lowered. If \( p_{open} \) is independent of \( pHe \), the proton flux eventually saturates and lags behind the linearly increasing \( |V - V_{H}| \), resulting in a turnover at low \( pHe \). That the experimental data do not show a turnover corroborates the assumption that \( p_{open} \) increases upon lowering \( pHe \), which is our way of modeling activation by \( pHe \).

**Fig. 4 B** presents the dependence of the outward proton flux on \( pHe \) when \( pHi \) is fixed at 6.0 and the voltage is at 60 mV. Again, the experimental data (12) are well reproduced by the calculations. Note that here the dependence of the chord conductance on \( pHe \) has a shape that is very different from that in **Fig. 4 A**. Our theory exquisitely captures both of the two different shapes.

### Single-channel proton flux

With M2 channels reconstituted into liposomes, it has become possible to deduce single-channel proton flux (7,14,19). A defining feature of M2 is its low proton flux, of the order of 100 protons/channel/s. Our theory predicts such low M2 proton flux. For example, for the case shown in **Fig. 2 A** with \( pHe = 5.8 \) and \( pHi = 7.3 \), the flux is 87 protons/channel/s at \( V = -70 \text{ mV} \).

Busath and co-workers (7) reported single-channel M2 proton fluxes for \( pHe = 6.5, 6, \) and 5.5 while holding \( pHi \) at 8 and \( V \) at \(-114 \text{ mV} \). **Fig. 5** compares our predictions with the experimental data. The theory is in quantitative agreement with the experiment for all three \( pHe \) values.

The results reported above are for a single set of geometrical parameters (\( L = 35 \text{ Å}, L_e = 21 \text{ Å}, a = 3 \text{ Å}, \) and \( a_i = 5 \text{ Å} \)). Very similar results are produced by our theory when these parameters are varied in physically reasonable

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**FIGURE 3** Fitting of the \( pHe \) dependence of \( p_{open} \) to Eq. 15. Symbols are \( p_{open} \) values listed in the Fig. 2 legend; the curve is the fit to Eq. 15.

**FIGURE 4** Dependence of proton flux on \( pHe \). (A) Inward flux, with \( pHi \) fixed at 7.4 and \( V = \pm 60 \text{ mV} \). (B) Outward flux, with \( pHi \) fixed at 6.0 and \( V = 60 \text{ mV} \). Symbols are the same as for the experimental data of Chizhakov et al. (12). \( \xi_0 \) represents the chord conductance at \( pHi = 4.0 \) and \( V = -60 \text{ mV} \) in A and the chord conductance at \( pHi = 6.0 \) and \( V = 60 \text{ mV} \) in B. Curves are theoretical calculations. The values of the three gating parameters are \( \omega_o = 0.2 \text{ ns}^{-1} \) and \( \omega_c = 1 \text{ ns}^{-1} \) for A and \( \omega_o = 0.1 \text{ ns}^{-1} \) and \( \omega_c = 10 \text{ ns}^{-1} \) for B; and \( p_{open} \) as given by Eq. 15.

**FIGURE 5** Comparison of calculated and experimental results for single-channel proton flux. The continuous curve displays our theoretical calculation with \( \omega_o = 0.1 \text{ ns}^{-1} \) and \( \omega_c = 2 \text{ ns}^{-1} \); symbols show the data of Busath and co-workers (7).
ranges (e.g., $L \sim 25$–$35$ Å, $L_c/L \sim 0.6$, $a = 2.5$–$3$ Å, and $a/\alpha = 1.5$–$2$). As for the dynamic parameters ($\omega_o$, $\omega_c$, and $p_{open}$), the results are qualitatively similar as long as $\omega_o$ and $\omega_c$ are in the nanosecond range and $\omega_o/\omega_c$ and $p_{open}$ are $\leq 10\%$.

**DISCUSSION**

We have shown that our microscopic theory, which incorporates the structural and dynamical properties of the M2 protein, is capable of quantitatively reproducing an extensive set of conductance data. Some of the same data have been fitted to a phenomenological model (yielding an expression for the proton flux analogous to Eq. 1), in which rate constants are introduced as fitting parameters (26). In contrast, we calculate the rate constants using theories of diffusion-influenced reactions. Our theory thus allows the proton-conductance function to be directly correlated with the structure and dynamics of the protein.

The physical (i.e., the proton diffusion constant), chemical (i.e., the dissociation constant of the permeant proton from the internal binding site), and geometrical parameters of our theory are well determined. There is somewhat less certainty on three dynamic parameters ($\omega_o$, $\omega_c$, and $p_{open}$) describing the gating of the channel, though even these parameters are tightly bound, based on previous MD simulations (9,11) and solid-state NMR experiments (8). Specifically, the simulations (9) showed that the Val27 gate at the entrance to the N-terminal pore is closed most of the time, and open-closed transitions occur on the nanosecond timescale. The conformation presented by the four Val27 residues is also observed in recent M2 structures (6,7), again indicating $\omega_o/\omega_c \ll 1$. A $p_{open}$ value of $\leq 10\%$, meaning that the C-terminal open conformation is a minor population, is supported by MD simulations (11) and the observation that peak positions of NMR spectra are unshifted when pH is lowered to a range where the channel becomes activated (8), though broadening of the peaks does suggest an increase in the population of the open conformation. Both the open-closed transitions of the Val27 gate and the interconversion of the C-terminal open and closed conformations are modeled as fast on the millisecond timescale. This aspect is supported by the observation that at both high and low pH, the NMR spectra of individual residues have single peaks, meaning that conformational averaging occurs fast on the NMR timescale (i.e., 1 ms). Furthermore, the time course of conductance induced by voltage change suggests “submillisecond ‘gating’ conformational changes” (16). It is important to note that with the tightly bound parameters, the theory produces the level of proton flux observed experimentally (7,14,19).

The results shown here seem to firmly establish the gating role of Val27. Such a role was first proposed based on MD simulations (9); Val27 broke the water wire through the pore during most of the simulations. Our recent study (20) found that inclusion of Val27 gating brings the predicted proton flux to the same range as measured. Now we find that inclusion of Val27 gating allows our theory to quantitatively reproduce conductance measurements.

We account for channel activation by viral exterior pH, explicitly by modeling $p_{open}$ as an increasing function as pH is lowered. Support is provided by the lack of a turnover of the chord conductance at low pH. As pH is lowered, an incoming proton in the N-terminal pore will likely interact with the His37 tetrad, thereby perhaps relieving the His37-Trp41 interactions and opening the Trp41 gate. However, the detailed mechanism of how a change in pH is propagated to a change in the population of the open conformation is still a matter of speculation (6,7,34), and the derivation of a mathematical relation between $p_{open}$ and pH like Eq. 15 remains to be investigated.

As noted above, a defining feature of M2 is its low proton flux, of the order of $10^7$ protons/channel/s. In comparison, the transport rates of potassium and sodium channels are $>10^9$ ions/s. In our theory, the low level of M2 proton flux arises first from the permeant proton’s obligatory binding to and unbinding from the internal site, i.e., the His37 tetrad. Moreover, the Trp41 primary gate and the Val27 secondary gate slow down the binding and unbinding. The role of Val27, as discussed above, has been suggested by MD simulations and is supported by M2 structures (6,7). The roles of His37 and Trp41 in fact are suggested by experimental studies (4,15,17,18). That the theory, with physically reasonable parameters, quantitatively reproduces an extensive set of experimental conductance data provides support for the roles of these key residues in the M2 functional mechanism.

Chen et al. (35) carried out MD simulations (with a multi-state empirical valence bond model accounting for proton-charged delocalization) to calculate the proton potential of mean force and the position-dependent diffusion constant. When the His37 tetrad was in the +1 charge state, a barrier was present around Val27, which is perhaps consistent with its putative role as a secondary gate. For the +3 charge state of the His37 tetrad, the potential of mean force was relatively flat, except for a barrier between His37 and Trp41. It is of interest that regardless of the protonation state of the His37 tetrad, the mean value of their position-dependent proton diffusion constant was $\sim 10^3$ Å$^2$/ns, which is the value used in our calculations. Chen et al. then modeled the proton transport as one-dimensional continuous electrodiffusion and applied the Poisson-Nernst-Planck theory to calculate the M2 proton conductance. They predicted a proton conductance of 53 pS for the +3 charge state of the His37 tetrad, which is equivalent to a proton flux of $3.3 \times 10^7$ protons/channel/s at $V = -100$ mV. This is 10-fold too high compared to an earlier measurement of Busath and co-workers (36) and five orders of magnitude too high compared to recent experimental data for single-channel proton conductance (7,14,19).
Our theory can be further tested. In particular, a change in solvent from H$_2$O to D$_2$O will affect the parameters in our theory in predictable ways. For example, the diffusion constant of the permeant ion, now a deuteron, will be reduced (22). Our predicted kinetic isotope effect can be tested by M2 currents measured in D$_2$O (13).

The basic framework of our theory allows for more realistic modeling of the structure and dynamics of the protein, through hybrid molecular dynamics and Brownian dynamics simulations. By Brownian dynamics simulations of the diffusive motion of the permeant proton and molecular dynamics simulations of the protein dynamics, we can calculate the binding rate constants with a more realistic representation of the protein (37). In the theory presented here, we have accounted for the potential of mean force arising from a transmembrane voltage, but neglected the potential of mean force that may arise from the interactions of the permeant proton with the protein and solvent environment. That the theory is able to reproduce well experimental results suggests that the latter type of potential of mean force either plays a negligible role in proton conductance or is mitigated by some other factor. The implementation of our theory by hybrid molecular dynamics and Brownian dynamics simulations will allow this and other detailed questions to be addressed.

APPENDIX A

Derivation of Eq. 12a

Here, we derive Eq. 12a for $\tilde{\kappa}_{ab}(s)$, the Laplace transform of the total flux across the N-terminal pore entrance. The motion of ions in the pore is modeled as one-dimensional diffusion along the channel axis. We denote the product of the ion distribution function and the cross-sectional area as $p(x, t)$. It satisfies the diffusion equation (A. M. Berezhkovskii, A. Szabo, and H.-X. Zhou, unpublished).

$$\frac{\partial p(x, t)}{\partial t} = -D \frac{\partial^2 p(x, t)}{\partial x^2}, \quad (A1a)$$

$$J(x, t) = -De^{-U(x)/kT} \sigma(x) \frac{\partial}{\partial x} [e^{-U(x)/kT} \sigma(x)]^{-1} p(x, t). \quad (A1b)$$

In Eq. 11, $U(x)$ is the potential of mean force resulting from averaging over the cross section at $x$, with a reference chosen such that $U(0) = 0$, and $J(x, t)$ is the total flux through the cross section at $x$.

The initial condition for $p(x, t)$ is

$$p(x, 0) = 0. \quad (A2)$$

At the pore entrance, the boundary condition is

$$p(0, t) = \sigma(0). \quad (A3a)$$

We model binding to the internal site as diffusion-controlled, so that an absorbing boundary condition applies:

$$p(L_e, t) = 0. \quad (A3b)$$

The quantity we are looking for is

$$k_{b}(t) = J(0, t). \quad (A3c)$$

In the case of a cylindrical pore with radius $a$ and a linear potential $U(x)/kaT = ax/L_e$, Eq. A1 becomes

$$\frac{\partial p(x, t)}{\partial t} = D \frac{\partial^2 p(x, t)}{\partial x^2} + (Da/L_e) \frac{\partial p(x, t)}{\partial x}. \quad (A4a)$$

The Laplace transform of this equation (with the use of the initial condition of Eq. A2) is

$$\frac{\partial^2 \widehat{p}(x, s)}{\partial x^2} + (\alpha/L_e) \frac{\partial \widehat{p}(x, s)}{\partial x} - (s/D) \widehat{p}(x, s) = 0. \quad (A4b)$$

The solution of the last equation can be written as

$$\widehat{p}(x, s) = A_1 e^{-a^2/2 + \gamma s/L_e} + A_2 e^{-(a^2/2 - \gamma s)/L_e}, \quad (A5)$$

where $\gamma$ is given by Eq. 12b and $A_1$ and $A_2$ are coefficients to be determined by the boundary conditions of Eq. A3. The results of the coefficients are

$$A_1 = -\frac{a^2}{(e^{2\gamma} - 1)}, \quad (A6a)$$

$$A_2 = \frac{a^2 e^{2\gamma}}{(e^{2\gamma} - 1)}. \quad (A6b)$$

Calculating the flux at $x = 0$, we arrive at Eq. 12a.

We now briefly discuss two limiting cases. The first is when the potential is absent, and it is obtained by setting $\alpha$ to 0. Equation 12a then becomes

$$s\tilde{k}_{ab}(s) = \pi a^2 (sD)^{1/2} \coth((sL_e^2/D)^{1/2}). \quad (A7)$$

Using this expression in Eq. 9, we obtain the result for the rate constant for binding from the viral exterior under Val27 gating in the absence of a potential. This result was presented previously (20). Another limit is when $\omega \ll D/\alpha^2$ and $D/L_e^2$. Applying the condition $s \ll D/\alpha^2$ in Eq. 11, we find $s\tilde{k}_{ab}(s) \approx 4Da$, which is the inverse of the first term of Eq. 8a for $k_{ab}$. Applying the condition $s \ll D/L_e^2$ in Eq. 12a, we find $s\tilde{k}_{ab}(s) \approx 4Da^2/\alpha L_e(e^\alpha - 1)$, which is the inverse of the second term of Eq. 8a for $k_{ab}$. Taken together, we find that when $\omega \ll D/\alpha^2$ and $D/L_e^2$,

$$\frac{1}{\omega \tilde{k}_{ab}(\omega)} + \frac{1}{\omega \tilde{k}_{ab}(\omega)} \approx 4Da + \frac{\alpha}{\pi Da^2} = \frac{1}{k_{bc}}. \quad (A8)$$

Using this result in Eq. 9, we obtain the limiting expression for $k_{bc}$ given by Eq. 13.

APPENDIX B

Derivation of Eq. 14

Here, we examine the case where the channel protein can interconvert between two conformations. The proton transport in each channel conformation is as described in the main text, but now the channel can switch its conformation while the permeant proton is still in the pore. If the conformational interconversion is fast on the timescale of the diffusional motion of the permeant ion through the pore, then the fluctuating channel could be modeled as having a static structure, with the potential of mean force given by the lifetime-weighted average over the two conformations. The diffusional time, $D/L_e^2$, is of the order of 1 ns; it is very likely that the conformational interconversion is slower. In that situation, in calculating the ion flux, we may use rate equations to describe the conformational interconversion.
We denote the two conformations as A and B and use a subscript 0 or 1 when the internal binding site is either unoccupied or occupied. There are four possible states: \( A_0, A_1, B_0, \) and \( B_1 \). Their interconversion can be described by the following scheme (24):

\[
\begin{array}{c}
A_0 \\
\downarrow k_{AB0} \\
A_1 \\
\downarrow k_{AB1} \\
B_0 \\
\downarrow k_{BA0} \\
B_1 \\
\end{array}
\]

The vertical steps describe ion binding to and unbinding from the internal site; the rate constants \( k_{AB+}, k_{AB-}, \) etc. are the same as defined in the main text, but here they are specific to each conformation. The horizontal steps describe the conformational interconversion. The rate constants must satisfy detailed balance conditions

\[
\frac{k_{AB1}/k_{BA1}}{k_{AB0}/k_{BA0}} = \frac{k_{AB+}/k_{AB-}}{k_{AB-}/k_{AB+}} = \frac{k_{BA+}/k_{BA-}}{k_{BA-}/k_{BA+}}.
\]

(B1)

The ion flux can be calculated from the steady-state probabilities, \( q_{A0}, q_{A1}, q_{B0}, \) and \( q_{B1} \), of the four states:

\[
I = -k_{A+}C_eq_{A0} + k_{A-}q_{A1} - k_{B+}C_eq_{B0} + k_{B-}q_{B1}.
\]

(B2)

The time dependence of the probability of each state is determined by a rate equation. For example, for state \( A_0 \), the rate equation is

\[
\frac{dq_{A0}}{dt} = -(k_{A+}C_e + k_{A-}C_i + k_{AB0})q_{A0} + (k_{A-} + k_{A+})q_{A1} + k_{BA0}q_{B0}.
\]

(B3)

For the steady-state problem that we are interested in, each of these time derivatives is zero. In addition, the four steady-state probabilities sum to 1. Solving the algebraic equations for the steady-state probabilities, we find the ion flux to be

\[
I = \left( \frac{-C_e + C_i + k_{A+}C_i}{k_{A+} + k_{A-}} + G \right)\left( \frac{k_{A+}C_i}{k_{A+} + k_{A-}} + H \right)
\]

(B4)

where

\[
k_{A+} = p_{A0}k_{A+} + p_{B0}k_{B+}, \quad (B5a)
\]

\[
k_{A-} = p_{A0}k_{A-} + p_{B1}k_{B-}, \quad (B5b)
\]

\[
k_{B+} = p_{A1}k_{B+} - p_{B0}k_{B-}, \quad (B5c)
\]

\[
k_{B-} = p_{A1}k_{B-} - p_{B1}k_{B+} \quad (B5d)
\]

\[
F = \frac{k_{A+}/k_{A-}}{k_{A+}/k_{A-}} = k_{B+}/k_{B-}, \quad (B5e)
\]

\[
G = \frac{p_{A0}(k_{A+}k_{B+}C_e + F_k_{A+}C_i + k_{B-}C_i)/k_{BA0}}{p_{A1}(k_{A+}k_{B-}C_i + F_k_{A-}k_{B+}C_i)/k_{BA1}}, \quad (B5f)
\]

and

\[
H = \frac{p_{A0}(k_{A+}k_{B+} + p_{A1}k_{A+}k_{B-} + p_{B0}k_{A-}k_{B+})}{p_{B0}(k_{A+}k_{B-} + p_{A1}k_{A-}k_{B+} + p_{B1}k_{A+}k_{B-})} \quad (B5g)
\]

In Eq. B5, we have introduced the equilibrium fractions \( p_{A0} \) and \( p_{B0} \) of conformations A and B, respectively, in the unbound state, given by

\[
p_{A0} = \frac{k_{BA0}}{k_{BA0} + k_{AB0}}, \quad (B6a)
\]

\[
p_{B0} = \frac{k_{BA0}}{k_{BA0} + k_{AB0}} = 1 - p_{A0}. \quad (B6b)
\]

In a similar way, the equilibrium fractions \( p_{A1} \) and \( p_{B1} \) of conformations A and B, respectively, in the bound state are given by

\[
p_{A1} = \frac{k_{BA1}}{k_{BA1} + k_{AB1}} \quad (B6c)
\]

\[
p_{B1} = \frac{k_{BA1}}{k_{BA1} + k_{AB1}} = 1 - p_{A1}. \quad (B6d)
\]

The additional quantities \( k_{A\pm} \) in Eq. B5g are given by

\[
k_{A+} = k_{A+}C_e + k_{A+}C_i; \quad (B7a)
\]

\[
k_{A-} = k_{A-} + k_{A+}; \quad (B7b)
\]

and analogously for \( k_{B\pm} \).

When the conformational interconversion is fast on the timescale of ion binding and unbinding, i.e., when the first-order rate constants for the horizontal steps are much greater than the (pseudo) first-order rate constants for the vertical steps, \( G \) and \( H \to 0 \), and Eq. B4 reduces to Eq. 1. The effective binding and unbinding rate constants are given by Eq. B5a–d. Specializing to our modeling of the C-terminal gating in the M2 proton channel, A and B represent the open and closed conformations, respectively. In the closed conformation, binding to the internal site from the viral interior is prohibited, i.e., \( k_{B1} = 0 \). Equation B5 then reduces to Eq. 14 once \( p_{A0} \) is identified with \( p_{\text{open}} \) the equilibrium fraction of the open conformation, and \( k_{A\pm} \) is identified with \( k_{\text{on}} \), the rate constant for binding from the viral interior when the channel stays in the open conformation.

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