Supporting Methods

Sample Preparation of M2 TM Domain Vesicles for Use in Bilayer and Multilayer Conductance Studies. Lipid vesicles were prepared by mixing phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, and cholesterol (Avanti Polar Lipids) at a molar ratio of 4:1:1:2 (total mass of 10 mg) with or without 0.05 mg of M2 TM domain in chloroform, drying to a thin layer under N_2, vortexing together with 1 ml of saline solution (150 mM KH_2PO_4), and sonicating four times for 1 min each at 2-min intervals. The vesicles were stored at 4°C for no longer than 1 week.

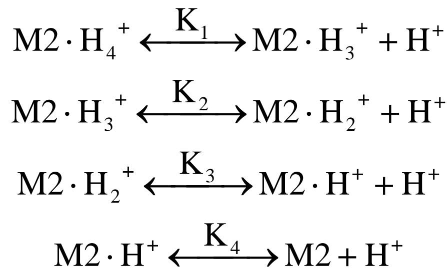
For uptake measurements, a 30-μl aliquot of freshly vortexed vesicle suspension was added to 3 ml of saline solution (0.2 mM tricine/200 mM Na_2SO_4) and stirred for 5 min at room temperature (25°C). A sensitive and selective pH electrode probe (Accu pHast) was used to detect the changes, which typically range over a few hundredths of a pH unit, small enough to be linear on a logarithmic scale. The ratio of $[K^+]$ between the inside and outside of the vesicles (≈100 after vesicle injection) was designed to produce a membrane potential, after activity corrections, of –103 mV for a perfectly K^+ selective membrane, whereas the pH gradient was negligible. After the pH had stabilized near pH 6.9, a baseline was recorded for 2 min. Three microliters of valinomycin (25 mg/ml in ethanol) was then added while stirring. Three minutes later, 7.5 μl of carbonyl cyanide *m*-chlorophenylhydrazone (200 mM in ethanol) was added while stirring. Independent experiments with ethanol with and without liposomes show that the ethanol causes a slight shift in pH, apparently due to the high pK_a of ethanol relative to water. After about 5 min, two back titrations were performed with 30 μl of HCl (1 mM) at 2-min intervals to provide a calibration for the titration of ≈3 ml of extravesicular buffer volume with 30 neq H^+.

Deconvolution Analysis of the NMR Data. For the overlapped ^{15}N chemical shifts that are substantially overlapped, signal deconvolution is necessary to extract the signal intensities. To deconvolute these overlapping resonances, three parameters were defined: peak position, peak height, and peak half-height linewidth. The chemical shift positions were fixed, because they do not vary significantly as a function of pH and the linewidth

for each resonance varies only slightly through the titration. For each simulated spectrum using a narrow range of linewidths and peak heights, a difference spectrum was calculated with the experimental spectrum and judged by standard deviation with the noise level from the experimental spectrum. Only those simulations generating difference spectra with a standard deviation <150% that of the noise level were included in the data analysis of the proportion of charged and uncharged histidine residues.

Modeling of Multistep Proton Dissociation of the M2 TM Domain. We propose that the M2 proton channel can release protons in a fractional style (Scheme 1).

Scheme 1



$$M2 \cdot H_4^+ \xleftrightarrow{K_1} M2 \cdot H_3^+ + H^+$$

$$M2 \cdot H_3^+ \xleftrightarrow{K_2} M2 \cdot H_2^+ + H^+$$

$$M2 \cdot H_2^+ \xleftrightarrow{K_3} M2 \cdot H^+ + H^+$$

$$M2 \cdot H^+ \xleftrightarrow{K_4} M2 + H^+$$

Thus, each equilibrium constant can be written as:

$$K_1 = \frac{[M2 \cdot H_3^+][H^+]}{[M2 \cdot H_4^+]} \qquad [\mathbf{1}]$$

$$K_2 = \frac{[M2 \cdot H_2^+][H^+]}{[M2 \cdot H_3^+]} \qquad [\mathbf{2}]$$

$$K_3 = \frac{[M2 \cdot H^+][H^+]}{[M2 \cdot H_2^+]} \qquad [\mathbf{3}]$$

$$K_4 = \frac{[M2][H^+]}{[M2 \cdot H^+]} \ . \qquad [\mathbf{4}]$$

Here, for simplicity, we use the concentration of different species instead of activities. Other useful equations (Eqs. **5**–**7**), shown below, can be derived from Eqs. **1**–**4**:

$$K_1K_2 = \frac{[M2 \cdot H_2{}^+][H^+]^2}{[M2 \cdot H_4{}^+]} \qquad [\mathbf{5}]$$

$$K_1K_2K_3 = \frac{[M2 \cdot H^+][H^+]^3}{[M2 \cdot H_4{}^+]} \qquad [\mathbf{6}]$$

$$K_1K_2K_3K_4 = \frac{[M2][H^+]^4}{[M2 \cdot H_4{}^+]} \ . \qquad [\mathbf{7}]$$

The ^{15}N cross-polarization magic-angle spinning spectra cannot differentiate between the various $M2{\bullet}H^+{}_{1\text{–}4}$ tetramers. However, the concentration ratio of neutral histidine [His] and charged histidine [$HisH^+$] can be obtained from the NMR spectra and expressed as a function of pH from the combination of Eqs. **1**–**7**.

$$\begin{aligned}
\frac{[His]}{[HisH^+]} &= \frac{[M2 \cdot H_3{}^+] + 2[M2 \cdot H_2{}^+] + 3[M2 \cdot H^+] + 4[M2]}{4[M2 \cdot H_4{}^+] + 3[M2 \cdot H_3{}^+] + 2[M2 \cdot H_2{}^+] + [M2 \cdot H^+]} \\
&= \frac{\frac{[M2 \cdot H_3{}^+]}{[M2 \cdot H_4{}^+]} + 2\frac{[M2 \cdot H_2{}^+]}{[M2 \cdot H_4{}^+]} + 3\frac{[M2 \cdot H^+]}{[M2 \cdot H_4{}^+]} + 4\frac{[M2]}{[M2 \cdot H_4{}^+]}}{4 + 3\frac{[M2 \cdot H_3{}^+]}{[M2 \cdot H_4{}^+]} + 2\frac{[M2 \cdot H_2{}^+]}{[M2 \cdot H_4{}^+]} + \frac{[M2 \cdot H^+]}{[M2 \cdot H_4{}^+]}} \qquad [\mathbf{8}] \\
&= \frac{\frac{K_1}{10^{-pH}} + 2\frac{K_1K_2}{10^{-2pH}} + 3\frac{K_1K_2K_3}{10^{-3pH}} + 4\frac{K_1K_2K_3K_4}{10^{-4pH}}}{4 + 3\frac{K_1}{10^{-pH}} + 2\frac{K_1K_2}{10^{-2pH}} + \frac{K_1K_2K_3}{10^{-3pH}}}
\end{aligned}$$

The number of protons (N_{proton}) released from a four-charged histidine complex is related to the concentration ratio of neutral histidine [His] and charged histidine [$HisH^+$],

$$N_{proton} = \frac{4}{1 + \frac{[HisH^+]}{[His]}} \quad . \qquad [\mathbf{9}]$$

Therefore, N_{proton} as a function of K_1, K_2, K_3, and K_4 is

$$N_{proton} = \frac{4}{1 + \frac{4 + 3\frac{K_1}{10^{-pH}} + 2\frac{K_1 K_2}{10^{-2pH}} + \frac{K_1 K_2 K_3}{10^{-3pH}}}{\frac{K_1}{10^{-pH}} + 2\frac{K_1 K_2}{10^{-2pH}} + 3\frac{K_1 K_2 K_3}{10^{-3pH}} + 4\frac{K_1 K_2 K_3 K_4}{10^{-4pH}}}} \quad . \qquad [\mathbf{10}]$$

The proton dissociation constants K_1, K_2, K_3, and K_4 were obtained by fitting the curve of N_{proton} vs. pH by using ORIGIN 5.0 software (Microcal, Amherst, MA).