Supporting Text

Here we supply additional material concerning the construction of the kinetic models and electrostatic calculations.

A Multi-Ion Model for Channel Permeation

Experimentally, intracellular ion concentrations cannot be controlled when recording from ooyctes in two-electrode or cell-attached modes. Typically, cytoplasmic sodium is 20 mM and the potassium concentration is 120 mM. Therefore, a realistic model of ion permeation under these conditions needs to account for the mixing of potassium and sodium ions in the channel. With this in mind, we wanted to extend the realistically accurate model of ion movement in Fig. $3A$ to include two ion species. As we will see, this generalization allows us to compute the permeability ratio from the reversal potential in exactly the same manner as the experiments.

The Model and Its Rate Constants. Consider the diagram shown in Fig. $3B$ in which each ion can be either potassium (blue) or sodium (red). The total number of states is 32 and the total number of rate constants is 88. Ions occupy the same spatial configurations as states a, b, c, d, and e in the model in Fig. $3A$, and the diagram in Fig. $3B$ reflects this by showing five subgroups. Moreover, transitions can only be made between subgroups connected with arrows. Transitions cannot be made from one state in a subgroup to another state in the same subgroup. Between subgroups a, b, and c, states are connected to a single state in the other subgroup. For instance, state 1 in a is connected to only one state of subgroup b, state 9, and subgroup 12 in b is connected to only one state of subgroup c, state 20. This also holds for transitions between subgroups d and e, but not for transitions out of d and e to a, b, or c. For instance, state 32 of e can accept either a potassium or sodium ion into the cavity allowing transitions to states 15 or 16 in subgroup b. With these rules in mind, the kinetic equations corresponding to this model can be determined from the state diagram as follows:

$$\dot{S}_1 = -(k_{1,9} + k_{a,d})S_1 + k_{9,1}S_9 + k_{d,a}c_i^K S_{25}$$
$$\dot{S}_2 = -(k_{2,10} + k_{a,d})S_2 + k_{10,2}S_{10} + k_{d,a}c_i^{Na} S_{25}$$
$$\dot{S}_3 = -(k_{3,11} + k_{a,d})S_3 + k_{11,3}S_{11} + k_{d,a}c_i^K S_{26} \qquad [\mathbf{1}]$$
$$\vdots$$
$$\dot{S}_{32} = k_{b,e}(S_{15} + S_{16}) + k_{28,32}S_{28} - (k_{e,b}(c_i^K + c_i^{Na}) + k_{32,28})S_{32},$$

We are interested in the steady-state occupancies, S_i where $i \in \{1, 2, 3, \ldots, 32\}$, which can be determined by numerically inverting the above matrix equation, $MS = \vec{0}$, given the concentrations and the rate constants (S is the vector of occupancies S_i). This is accomplished by first computing the LU decomposition of the rate constant matrix, M, followed by solving the equation $Uy = \vec{1}$ for y. The steady-state solution is then given by $S = y/|y|$, where normalizing y enforces single-site occupancy. A small machine precision quantity is added to the final diagonal element of U to better condition the matrix before performing back substitution. The individual ionic fluxes along each path can be determined by considering the flow between adjacent states along each of the pathways (1 or 2) as follows:

$$J_1^{\mathrm{K}} = p(k_{\mathrm{d,a}} c_i^{\mathrm{K}}(S_{25} + S_{26} + S_{27} + S_{28}) - k_{\mathrm{a,d}}(S_1 + S_3 + S_5 + S_7))$$
$$J_1^{\mathrm{Na}} = p(k_{\mathrm{d,a}} c_i^{\mathrm{Na}}(S_{25} + S_{26} + S_{27} + S_{28}) - k_{\mathrm{a,d}}(S_2 + S_4 + S_6 + S_8))$$
$$J_2^{\mathrm{K}} = p(k_{\mathrm{e,b}} c_i^{\mathrm{K}}(S_{29} + S_{30} + S_{31} + S_{32}) - k_{\mathrm{b,e}}(S_9 + S_{11} + S_{13} + S_{15}))$$
$$J_2^{\mathrm{Na}} = p(k_{\mathrm{e,b}} c_i^{\mathrm{Na}}(S_{29} + S_{30} + S_{31} + S_{32}) - k_{\mathrm{b,e}}(S_{10} + S_{12} + S_{14} + S_{16})),$$
[2]

where $p = 1.6 \times 10^{-7}$ is the conversion factor from ions/sec to picoAmps, and the total flux is $J_{\mathrm{Total}} = J_1^{\mathrm{K}} + J_1^{\mathrm{Na}} + J_2^{\mathrm{K}} + J_2^{\mathrm{Na}}$. The rate constants are related to the energetics of the system as follows. We write the energy of ions in each site in the channel as E_i^j where $i \in \{S0, S1, S2, S3, S4, C\}$ and $j \in \{\mathrm{K}, \mathrm{Na}\}$. We require that $E_C^{\mathrm{K}} = E_C^{\mathrm{Na}}$. Once a forward rate constant is given, the backward rate is determined through detailed balance given these energies. For instance, for all rates between a and b:

$$k_{1,9} = k_{9,1} e^{(E_{S2}^{\mathrm{K}} + E_{S4}^{\mathrm{K}} + E_{\mathrm{r}}) - (E_{S1}^{\mathrm{K}} + E_{S3}^{\mathrm{K}})}$$
$$k_{2,10} = k_{10,2} e^{(E_{S2}^{\mathrm{K}} + E_{S4}^{\mathrm{K}} + E_{\mathrm{r}}) - (E_{S1}^{\mathrm{K}} + E_{S3}^{\mathrm{K}})}$$
$$k_{3,11} = k_{11,3} e^{(E_{S2}^{\mathrm{Na}} + E_{S4}^{\mathrm{K}} + E_{\mathrm{r}}) - (E_{S1}^{\mathrm{Na}} + E_{S3}^{\mathrm{K}})}$$
$$k_{4,12} = k_{12,4} e^{(E_{S2}^{\mathrm{Na}} + E_{S4}^{\mathrm{K}} + E_{\mathrm{r}}) - (E_{S1}^{\mathrm{Na}} + E_{S3}^{\mathrm{K}})}$$
$$k_{5,13} = k_{13,5} e^{(E_{S2}^{\mathrm{K}} + E_{S4}^{\mathrm{Na}} + E_{\mathrm{r}}) - (E_{S1}^{\mathrm{K}} + E_{S3}^{\mathrm{Na}})}$$
$$k_{6,14} = k_{14,6} e^{(E_{S2}^{\mathrm{K}} + E_{S4}^{\mathrm{Na}} + E_{\mathrm{r}}) - (E_{S1}^{\mathrm{K}} + E_{S3}^{\mathrm{Na}})}$$
$$k_{7,15} = k_{15,7} e^{(E_{S2}^{\mathrm{Na}} + E_{S4}^{\mathrm{Na}} + E_{\mathrm{r}}) - (E_{S1}^{\mathrm{Na}} + E_{S3}^{\mathrm{Na}})}$$
$$k_{8,16} = k_{16,8} e^{(E_{S2}^{\mathrm{Na}} + E_{S4}^{\mathrm{Na}} + E_{\mathrm{r}}) - (E_{S1}^{\mathrm{Na}} + E_{S3}^{\mathrm{Na}})},$$
[3]

where the energy of the cavity ion E_C does not enter the equations because an ion occupies that site in both states; however, the repulsion energy E_{r} increases the energy of states in the a subgroup. Writing all backward rates in this manner ensures that detailed balance is maintained throughout the system. The molecular calculations influence the kinetic model through modification of rate constants involving entry or exit from the cavity position. We write the total energy of the cavity binding site in terms of the electrostatic component $E_{\mathrm{c,elec}}$ from the continuum calculations and a constant entropic component, $E_{\mathrm{c,S}}$, which we assume does not vary with mutations at the cavity sites. The cavity energy is then $E_C = E_{\mathrm{c,elec}} + E_{\mathrm{c,S}}$, and the rate constants depend on this value as follows:

$$
\begin{aligned}
k'_{\mathrm{a,d}} &= k_{\mathrm{a,d}} e^{(E_{\mathrm{c,elec}} + E_{\mathrm{c,S}})/2}; & k'_{\mathrm{d,a}} &= k_{\mathrm{d,a}} e^{-(E_{\mathrm{c,elec}} + E_{\mathrm{c,S}})/2} \\
k'_{\mathrm{b,e}} &= k_{\mathrm{b,e}} e^{(E_{\mathrm{c,elec}} + E_{\mathrm{c,S}})/2}; & k'_{\mathrm{e,b}} &= k_{\mathrm{e,b}} e^{-(E_{\mathrm{c,elec}} + E_{\mathrm{c,S}})/2} \\
k'_{9,17} &= k_{9,17} e^{(E_{\mathrm{c,elec}} + E_{\mathrm{c,S}})/2}; & k'_{17,9} &= k_{17,9} e^{-(E_{\mathrm{c,elec}} + E_{\mathrm{c,S}})/2} \\
k'_{10,18} &= k_{10,18} e^{(E_{\mathrm{c,elec}} + E_{\mathrm{c,S}})/2}; & k'_{18,10} &= k_{18,10} e^{-(E_{\mathrm{c,elec}} + E_{\mathrm{c,S}})/2} \\
k'_{11,19} &= k_{11,19} e^{(E_{\mathrm{c,elec}} + E_{\mathrm{c,S}})/2}; & k'_{19,11} &= k_{19,11} e^{-(E_{\mathrm{c,elec}} + E_{\mathrm{c,S}})/2} \\
k'_{12,20} &= k_{12,20} e^{(E_{\mathrm{c,elec}} + E_{\mathrm{c,S}})/2}; & k'_{20,12} &= k_{20,12} e^{-(E_{\mathrm{c,elec}} + E_{\mathrm{c,S}})/2} \\
k'_{13,21} &= k_{13,21} e^{(E_{\mathrm{c,elec}} + E_{\mathrm{c,S}})/2}; & k'_{21,13} &= k_{21,13} e^{-(E_{\mathrm{c,elec}} + E_{\mathrm{c,S}})/2} \\
k'_{14,22} &= k_{14,22} e^{(E_{\mathrm{c,elec}} + E_{\mathrm{c,S}})/2}; & k'_{22,14} &= k_{22,14} e^{-(E_{\mathrm{c,elec}} + E_{\mathrm{c,S}})/2} \\
k'_{15,23} &= k_{15,23} e^{(E_{\mathrm{c,elec}} + E_{\mathrm{c,S}})/2}; & k'_{23,15} &= k_{23,15} e^{-(E_{\mathrm{c,elec}} + E_{\mathrm{c,S}})/2} \\
k'_{16,24} &= k_{16,24} e^{(E_{\mathrm{c,elec}} + E_{\mathrm{c,S}})/2}; & k'_{24,16} &= k_{24,16} e^{-(E_{\mathrm{c,elec}} + E_{\mathrm{c,S}})/2},
\end{aligned}
$$
[4]

where the unprimed rates are the rate constants when the cavity binding energy is zero, the primed

rates are the values used to solve for the flux.

We impose additional physical constraints on the model through the rate constants. We assume that ion movement into and out of the cavity to the cytoplasm is equal for both ions, and that the repulsion energy, E_r, biases the transitions between b and e relative to a and d. Therefore, there is a single rate set $(k_{a,d}, k_{d,a})$ for all transitions between subgroups a and d and a single rate set $(k_{b,e}, k_{e,b})$ for all transitions between subgroups b and e. These two sets of rates are related through the repulsion energy as follows:

$$k_{b,e} = k_{a,d}e^{-E_r/2}; \quad k_{e,b} = k_{d,a}e^{+E_r/2}. \tag{5}$$

In a similar manner, the transitions between subgroups d and e are given in terms of the equivalent transitions between subgroups a and b:

$$\begin{aligned}
k_{25,29} &= k_{1,9}e^{-E_r/2}; & k_{29,25} &= k_{9,1}e^{+E_r/2} \\
k_{26,30} &= k_{3,11}e^{-E_r/2}; & k_{30,26} &= k_{11,3}e^{+E_r/2} \\
k_{27,31} &= k_{5,13}e^{-E_r/2}; & k_{31,27} &= k_{13,5}e^{+E_r/2} \\
k_{28,32} &= k_{7,15}e^{-E_r/2}; & k_{32,28} &= k_{15,7}e^{+E_r/2}.
\end{aligned} \tag{6}$$

The membrane potential biases the energetics of ions at particular sites in the channel. We have adopted the convention used by Kutluay $et\ al.$ (1) for the fraction of the membrane potential between ion binding sites. The modified rates for monovalent cations are:

$$\begin{aligned}
k'_{1-8,9-16} &= k_{1-8,9-16}e^{\delta_2\psi}; & k'_{9-16,1-8} &= k_{9-16,1-8}e^{-\delta_2\psi} \\
k'_{9-16,17-24} &= k_{9-16,17-24}e^{(\delta_2+2\delta_3)\psi/2}; & k'_{17-24,9-16} &= k_{17-24,9-16}exp^{-(\delta_2+2\delta_3)\psi/2} \\
k'_{d,a} &= k_{d,a}e^{\delta_1\psi/2}; & k'_{a,d} &= k_{a,d}e^{-\delta_1\psi/2} \\
k'_{e,b} &= k_{e,b}e^{\delta_1\psi/2}; & k'_{b,e} &= k_{b,e}e^{-\delta_1\psi/2} \\
k'_{25-28,29-32} &= k_{25-28,29-32}e^{\delta_2\psi}; & k'_{29-32,25-28} &= k_{29-32,25-28}e^{-\delta_2\psi},
\end{aligned} \tag{7}$$

where on the left side are all outward rates and on the right are all inward rates, and the primed variables are used to solve for the flux. In the above equation we have used a short hand in which $k_{1-8,9-16}$ represents the eight rate constants $k_{1,9}, k_{2,10}, \ldots, k_{8,16}$.

Determining Permeability Ratios From the Model. Computationally, we solve for the total channel flux, J_{Total}, first under conditions I. $c_o^K = 0.090$ M, $c_o^{Na} = 0.0$ M, $c_i^K = 0.120$ M, $c_i^{Na} = 0.020$ M and then under conditions II. $c_o^K = 0.0$ M, $c_o^{Na} = 0.090$ M, $c_i^K = 0.120$ M, $c_i^{Na} = 0.020$ M. For each condition, the equations are solved for membrane potentials from -150 mV to +150 mV, and the reversal potential is determined numerically with the interpolation protocol in Matlab. The permeability ratio is then determined from the change in reversal potentials according to the following standard formula:

$$\frac{P_{Na}}{P_K} = \frac{c_o^K}{c_o^{Na}}e^{\Delta E_{rev}F/RT} \tag{8}$$

3

Fitting the Model to Experimental Data. The kinetic rate constants were determined by fitting the model to the experimentally determined permeability ratios and the single channel conductance values. All forward rates and ion energies were treated as free parameters for a total of 31 free parameters in this model. We then used a Nelder-Mead search algorithm to search through this parameter space and minimize the following error function (2):

$$
\begin{aligned}
\text{error function} = \ & \sum_{i=1}^{9} (P_i - P_i^0)^2 + \tfrac{1}{3600} \sum_{i=1}^{3} (g_i - g_i^0)^2 + H(E_r)E_r + \ldots \\
& + \tfrac{1}{20} \sum_{i=S0}^{S4} H(E_i^K - (E_i^{Na} - 2))(E_i^K - (E_i^{Na} - 2)) + \ldots \\
& + \tfrac{1}{4.5E9}(H(k_{da} - k^0)(k_{da} - k^0) + \sum_{i=K}^{Na} H(k_{ed}^i - k^0)(k_{ed}^i - k^0)),
\end{aligned}
\qquad [\mathbf{9}]
$$

where the superscript values P_i^0 and g_i^0 are the experimental values, $H(x)$ is the Heaviside step function which is 0 for arguments < 0 and 1 for arguments > 0. P_i^0 in the first term is the i*th* permeability ratio of the nine experimentally measured values depicted as diamonds in Fig. 4*A*. For any given set of parameters, the model is solved nine times as discussed in the last subsection and each theoretical P_i is saved to compute the first term of the error. Next, the model is solved three times with the electrostatic binding energies, $E_{c,elec}$, corresponding to S177W in potassium ($c_o^K = 0.150$ M, $c_o^{Na} = 0.0$ M, $c_i^K = 0.120$ M, $c_i^{Na} = 0.020$ M, at -100 mV), S177W-N184D in potassium ($c_o^K = 0.150$ M, $c_o^{Na} = 0.0$ M, $c_i^K = 0.120$ M, $c_i^{Na} = 0.020$ M, at -100 mV) and S177W in sodium ($c_o^{Na} = 0.150$ M, $c_o^K = 0.0$ M, $c_i^{Na} = 0.020$ M, $c_i^K = 0.120$ M, -200 mV). These outer ionic concentrations correspond to the pipette solution in the cell-attached configuration used to determine the single-channel conductances, g_i^0, in Fig. 7. The final theoretical values from the model correspond well with the experimental ones: $g_1^0 = 35.4, g_1 = 27.1$ (S177W in potassium), $g_2^0 = 54.6, g_2 = 53.6$ (S177W-N184D in potassium) $g_3^0 = 11.3, g_3 = 12.4$ (S177W, in sodium) (all in pS). The third term in Eq. **9** simply requires that the repulsion energy be positive. The fourth term is a sum over the filter site energies enforcing the notion that the sites should be at least slightly selective for potassium. This is a weak restraint. The last two terms penalize the inward rates of ions from the cytoplasm or the extracellular space if they exceed $k^0 = 8 \times 10^8 \, \text{M}^{-1} \cdot \text{s}^{-1}$. Based on theoretical considerations of potassium and sodium's diffusion coefficients, it is hard to rationalize rates that are in great excess to this value. The prefactors in front of the second, third, fourth, and fifth error terms in Eq. **9** ensure that all terms have nearly equal weight during the fitting processes. Finally, there is nothing in the search algorithm to restrict negative rate constants, if this happens, the error function is assigned a large positive value, the solution of the model equations is skipped, and new parameters are selected to continue the search.

The final set of rate constants used to compute these conductance values as well as Fig. 4*A* are:

$$
\begin{aligned}
k_{1-8,9-16} &= [0.17,\ 8.1,\ 0.32,\ 0.66,\ 0.72,\ 1.1 \times 10^{-5},\ 0.034,\ 48] \\
k_{9-16,1-8} &= [0.17,\ 8.3,\ 0.059,\ 0.12,\ 0.18,\ 2.8 \times 10^{-6},\ 0.0015,\ 2.2] \\
k_{9-16,17-24} &= [7.4 \times 10^{3},\ 2.3 \times 10^{3},\ 0.14,\ 3.8 \times 10^{2},\ 0.001,\ 1.5,\ 6.2,\ 4.6] \\
k_{17-24,9-16} &= [43,\ 4 \times 10^{2},\ 4.1 \times 10^{-5},\ 3.23.3 \times 10^{-5},\ 1.4,\ 0.01,\ 0.22] \\
k_{25-28,29-32} &= [0.17,\ 0.32,\ 0.71,\ 0.033] \\
k_{29-32,25-28} &= [0.18,\ 0.06,\ 0.19,\ 0.0016] \\
k_{c,d}^{K} &= 100.0 \\
k_{c,d}^{Na} &= 11 \\
k_{d,c}^{K} &= 57 \\
k_{d,c}^{Na} &= 16 \\
k_{a,d} &= 6.5 \\
k_{d,a} &= 0.021 \\
E_{c,S} &= 15, \\
E_{r} &\simeq 0.0,
\end{aligned}
\qquad \textbf{[10]}
$$

where all rates are multiplied by 1×10^{9}, units are in $\mathrm{M}^{-1} \cdot \mathrm{s}^{-1}$ for concentration-dependent rates, and s^{-1} for all others. All values are given at zero membrane potential for the wt channel, $E_{c,\mathrm{elec}} = -9.8\,k_{\mathrm{B}}T$.

A Model for Ion Permeation with Two Sites and Four States, and Its Relation to Molecular Calculations

We wanted to test the robustness of our theoretical results by attempting to fit our experimental permeability data with a minimal model of ion permeation, which has far fewer parameters than the more accurate model presented. This kinetic model follows from the two site diagram in Fig. 8A in which there is a single cavity site, c, and a single filter site, f. There are then four possible ion configuration within the channel labeled a, b, c, and d.

At steady state, the kinetic equations corresponding to the four state model follow from the state diagram in Fig. 8A:

$$
\begin{bmatrix}
-k_{ab}c_i - k_{bd}c_o & k_{ba} & k_{db} & 0 \\
k_{ab}c_i & -k_{ba} - k_{bd}c_o - k_{bc} & k_{cb} & k_{db} \\
k_{bd}c_o & k_{bc} & -k_{db} - k_{ab}c_i - k_{cb} & k_{ba} \\
0 & k_{bd}c_o & k_{ab}c_i & -k_{ba} - k_{db}
\end{bmatrix}
\begin{bmatrix}
a \\ b \\ c \\ d
\end{bmatrix}
=
\begin{bmatrix}
0 \\ 0 \\ 0 \\ 0
\end{bmatrix}. \quad \textbf{[11]}
$$

As with the more detailed model, we have placed constraints upon the rates such that rates involving equivalent ion movement are equal: $k_{ac} = k_{bd}$, $k_{db} = k_{ca}$, $k_{ab} = k_{cd}$, and $k_{ba} = k_{dc}$. Using Mathematica, we analytically solved this system of equations to determine the occupancies $x = [a, b, c, d]$ in terms of the inner and outer ionic concentrations:

$$a = ((k_{db})^2 k_{bc} + (k_{ba})^2 (k_{db} + k_{cb}) + k_{ba}k_{db}(k_{ab}c_i + k_{bd}c_o + k_{db} + k_{bc} + k_{cb}))/\alpha$$
$$b = ((k_{ab}c_i)^2 k_{db} + k_{bd}c_o(k_{ba} + k_{db})k_{cb} + k_{ab}(k_{ba}(k_{db} + k_{cb}) + k_{db}(k_{bd}c_o + k_{db} + k_{cb})))/\alpha$$
$$c = ((k_{ba})^2 k_{bd}c_o + (k_{ab}c_i + k_{bd}c_o)k_{db}k_{bc} + k_{ba}(k_{ab}c_i(k_{bd}c_o + k_{bc}) + k_{bd}c_o(k_{bd}c_o + k_{db} + k_{bc})))/\alpha$$
$$d = ((k_{ab}c_i)^2(k_{bd}c_o + k_{bc}) + (k_{bd}c_o)^2 k_{cb} + k_{ab}c_i k_{bd}c_o(k_{ba} + k_{bd}c_o + k_{db} + k_{bc} + k_{cb}))/\alpha,$$

[12]

where α is defined as

$$
\begin{aligned}
\alpha \equiv \ & (k_{ab}k_{bd}c_o)^2(k_{bd}c_o + k_{db} + k_{bc}) + (k_{ba})^2(k_{bd}c_o + k_{db} + k_{cb}) + \cdots \\
& + k_{ab}c_i(k_{bd}c_o + k_{db})(k_{bd}c_o + k_{db} + k_{bc} + k_{cb}) + \cdots \\
& + (k_{bd}c_o + k_{db})(k_{db}k_{bc} + k_{bd}c_o k_{cb}) + \cdots \\
& + k_{ba}((k_{bd}c_o + k_{db})(k_{bd}c_o + k_{db} + k_{bc} + k_{cb}) + k_{ab}c_i(2k_{bd}c_o + 2k_{db} + k_{bc} + k_{cb})).
\end{aligned}
$$

From these probabilities, the net flux of ions through the channel is as follows:

$$J = \tfrac{1}{2}\left(\underbrace{[k_{ab}c_i(a+c) + k_{db}(c+d)]}_{\text{outward flux}} - \underbrace{[k_{bd}c_o(a+b) + k_{ba}(b+d)]}_{\text{inward flux}}\right) = P(c_i - c_o),$$

[13]

where P is the corresponding ion permeability, outward flux is defined to be positive, and the factor of 1/2 takes into account the double counting of the flux. The channel permeability as a function of the rate constants and ion concentrations follows from this last equation:

$$P = \frac{-k_{ab}k_{db}k_{bc}(k_{ab}c_i + k_{ba} + k_{bd}c_o + k_{db})}{\alpha}.$$

[14]

As with the more detailed model, the rate constants are related to the energetics of each channel state:

$$
\begin{aligned}
E_a &= 0.0 \\
E_b &= E_c \\
E_c &= E_f \\
E_d &= E_c + E_f.
\end{aligned}
$$

[15]

We define the ion configuration energies as: E_f: energy of the ion in the filter and E_c: energy of the cavity ion, just as in the model with five states. Again from the principle of detailed balance, these energy changes are related to the rate constants into and out of the cavity:

$$k_{ab}/k_{ba} = e^{E_a - E_b}$$
$$k_{bc}/k_{cb} = e^{E_b - E_c}$$
$$k_{bd}/k_{db} = e^{E_b - E_d} \qquad\qquad [\textbf{16}]$$
$$k_{ab}/k_{ba} = e^{E_c - E_d}$$
$$k_{bd}/k_{db} = e^{E_a - E_c}.$$

The electrostatic calculations affect the model through the change in rates involving transitions into and out of the cavity site:

$$k'_{ab} = k_{ab}\, e^{-1/2(E_{c,elec} + E_S)}; \quad k'_{ba} = k_{ba}\, e^{1/2(E_{c,elec} + E_S)}$$
$$k'_{bc} = k_{bc}\, e^{1/2(E_{c,elec} + E_S)}; \quad\; k'_{cb} = k_{cb}\, e^{-1/2(E_{c,elec} + E_S)}, \qquad\qquad [\textbf{17}]$$

where again we use the nomenclature that primed rates are the ones used in Eq. **14** to solve for the permeability and the unprimed rates are the values when the cavity binding energy is zero. The rates k_{bd} and k_{db} are constant and do not depend on binding energy of the cavity ion.

We then fit this model to the experimental permeability ratios using the Nelder-Mead algorithm in a manner similar to that described above. The final set of parameters used in the search algorithm were $\{k^K_{ab}, k^K_{db}, k^K_{bc}, k^{Na}_{db}, k^{Na}_{bc}, E^K_f, E^{Na}_f, E_S\}$. This model has eight free parameters compared to 31 free parameters in the more exact model. Nonetheless, the results are presented in Fig. 8*B*, where we see that even this reduced model is capable of reproducing the experimentally observed data. As with the more detailed model, these results are reproduced by changing the electrostatic energy of the cavity binding site in a completely nonselective manner. In Fig. 8*C*, we see that a dramatic example of pathway switching from path 2 to path 1 at large negative cavity binding energies comes out of this model. The K$^+$ rate constants for the calculations in Fig. 8*B* are:

$$k_{ab} = 3.7 \text{ M}^{-1}, k_{ba} = 24.0, k_{bd} = 3.4 \text{ M}^{-1}, k_{db} = 0.07, k_{bc} = 43.0, k_{cb} = 0.14 \left(\times 10^9 \text{ s}^{-1} \right);$$

and the Na$^+$ rate constants are:

$$k_{ab} = 3.7 \text{ M}^{-1}, k_{ba} = 24.0, k_{bd} = 0.29 \text{ M}^{-1}, k_{db} = 7.7, k_{bc} = 1.8, k_{cb} = 7.4 \left(\times 10^9 \text{ s}^{-1} \right).$$

These values are given at the wt cavity energy, $E_{c,elec} = -9.8 \, k_B T$. The entropic component of the cavity site is $E_S = +11.6 \, k_B T$, the filter energies are $E^K_f = -3.9 \, k_B T$ and $E^{Na}_f = +3.3 \, k_B T$. In this model, the K$^+$ ion is stabilized in the filter by $7 \, k_B T$ relative to Na$^+$.

Determination of the Protonation State of N184D

Electrostatic Poisson-Boltzmann calculations were used to determine the pK$_a$ state of residues at position 184. We adopt the strategy detailed by Antosiewicz *et al.*(3) that compares the free energy of side-chain protonation/deprotonation in bulk media, ΔG_{bulk}, with the same electrostatic free energy calculation in the presence of the protein, $\Delta G_{protein}$. Then the shift in the pK$_a$ value of a site is calculated as follows:

$$pK_{\text{a,intrinsic}} = pK_{\text{a,initial}} - \frac{(\Delta G_{\text{protein}} - \Delta G_{\text{bulk}})}{ln(10)\,k_{\text{B}}T},$$ [18]

where $pK_{\text{a,initial}}$ is the known pK_a of the residue in free solution. We mimic protonation at N184D by simply adding a unit charge to oxygen OD2 in the charge file. All geometries are assumed fixed. The initial pK_a of Asp is taken as 4.0, and the intrinsic pK_a values for the successive deprotonation of Asp residues at position 184 are: 6.8 (3 protonated), 7.5 (2 protonated), 9.1 (1 protonated), 9.0 (0 protonated). The ionization state of the remaining three residues is shown in parentheses. That is, if two residues are negatively charged, the intrinsic pK_a of the remaining N184 residues is 9.1 that has a 1 in 40 chance of becoming deprotonated at pH 7.5. The local geometry of the fourth residue compared to the third residue gives rise to the drop in pK_a from 9.1 to 9.0. Without further combinatorial analysis, we assume that exactly two residues are charged during our calculations. This is in agreement with experimental studies of charged residues in the channel pore (4); however, Root and MacKinnon (4) observed that the two protonation events were independent of each other in CNG channels with a shifted pK_a of Glu from 4.3 (initial) to 7.6 (observed). The continuum calculations presented here predict pK_a shifts of similar magnitude, but the independence is not evident possibly because of incorrect assumptions about residue geometry, or the existence of carboxyl-carboxylate pairs formed between two N184D residues (4).

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