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Free-Energy Profiles for Ions in the Influenza M₂-TMD Channel

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Abstract

M₂ transmembrane domain channel (M₂-TMD) permeation properties are studied using molecular dynamics simulations of M₂-TMD (1NYJ) embedded in a lipid bilayer (DMPC) with 1 mol/kg NaCl or KCl saline solution. This study allows examination of spontaneous cation and anion entry into the selectivity filter. Three titration states of the M₂-TMD tetramer are modeled for which the four His37 residues, forming the selectivity filter, are net uncharged, +2 charged, or +3 charged. M₂-TMD structural properties from our simulations are compared with the properties of other models extracted from NMR and X-ray studies. During 10 ns simulations, chloride ions rarely occupy the positively-charged selectivity filter, whereas from umbrella sampling simulations, Cl[−] has a lower free-energy barrier in the selectivity-filter region than either Na⁺ or NH₄⁺, and NH₄⁺ has a lower free-energy barrier than Na⁺. For Na⁺ and Cl[−], the free-energy barriers are less than 5 kcal/mol, suggesting that the 1NYJ conformation would probably not be exquisitely proton selective. We also point out a rotameric configuration of Trp41 that could fully occlude the channel.

Keywords

Interhelical distances; Side chain rotamers; Potential of mean force; Influenza A virus; Membrane channel; Salt

Introduction

The influenza A virus is a genus of the orthomyxovirus family of viruses. It is coated by a cell membrane containing several copies of three integral membrane proteins: a receptor-cleavage protein known as neuraminidase, a fusion protein known as hemagglutinin, and a proton channel known as an M₂ channel.^{1,2}

After the endocytosis process of the virus in an epithelial cell of a host, the engulfed virus appears in an endosome. In the endosome, which has a pH range of 4.9–5.2,³ the M₂ channel assumes an active form that allows protons to pass through it to acidify the matrix of the virus prior to the fusion of the virion membrane with the endosomal membrane.^{4–8} This acidification facilitates the uncoating process that allows a release of the viral genome.

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Amantadine (1-Aminoadamantane hydrochloride) has an antiviral activity for the influenza A viruses at low concentrations ($\leq 5 \mu\text{M}$).^{5,7,9–16} It blocks the M_2 channel and disallows it from passing protons to the virion interior.^{5,7,8,12,15–21} Previous experimental data indicate that the M_2 channel is an essential part in virus replication and assembly.^{1,11,22–26}

The M_2 channel is comprised of four identical monomers,^{4,22,27–30} and each monomer consists of 97 amino acids.² Each monomer is divided into three domains: an extracellular N-terminal domain of 18 to 23 amino acid residues, an intracellular C-terminal domain of 54 amino acid residues, and an internal transmembrane domain (TMD) of 20 to 25 amino acid residues.^{1,2} The internal domains of the M_2 channel form a left-handed bundle of four α -helices,^{31,32} which are stabilized by disulfide bonds between the N-terminal domains at Cys17, Cys19, or both.^{4,27} Thus, the homotetrameric structure could be a pair of disulfide-linked dimers^{4,33} or four variably-linked monomers.²⁷ The α -helices of the M_2 -TMD channel are tilted in such a way that the N-terminal end of the channel is wider than the C-terminal end.²⁹ This tilted-helix bundle geometry enables the amantadine molecule to enter the channel and interact with the residues close to the N-terminal end and prevents it from passing through the C-terminal end of the channel.²⁹ The M_2 -TMD α -helices have an amphiphilic nature that also contributes to their role as a target of the amantadine interaction.^{4,17}

Under ideal conditions, the M_2 channel only transports protons.^{7,15,34,35} and this permeation increases as the external pH decreases⁵ below 8.5 until it reaches a saturation level at pH 4.^{7,15} The permeation of other ions through this channel has been suggested from experiments as well.^{5,6,8,14,35,36} It is not known whether experimental artifacts or variations in protein structure for different expression systems are responsible for these differences. Mutagenesis studies suggested that the Trp41 side chains function as a gate that opens and closes the M_2 -TMD pore for proton current at low and high pH, respectively,³⁶ whereas the His37 side chains function as a selectivity filter for protons.^{6,37}

This study explores the behavior of ionic species in the M_2 -TMD channel based on solid state NMR observables³⁸ and initially thought to represent the closed state. Previous simulations^{29,33,39–46} with this NMR structure³⁸, generally using neat water for the bath, have frequently demonstrated that, when the selectivity filter is multiply-titrated, it relaxes to a water-containing structure. It was observed that this structure can pass protons but still has a high barrier to Na^+ .²¹ However, the relaxed water-filled structure observed in these simulations raises questions about the occupancy of the charged selectivity filter. Namely, do Cl^- occupy the charged selectivity filter and neutralize it? Is the free-energy barrier to Cl^- passage lower than that of cation passage when there is a fixed charge in the selectivity filter? With slight relaxations, does the structure retain its tightness against Na^+ transport? Are there significant structural differences between the 1NYJ model and other models extracted from NMR and X-ray experiments? We conducted molecular dynamics (MD) and umbrella sampling simulations with, for the first time to our knowledge, concentrated electrolyte solutions as the bath, to address those questions.

Methods

Model System and Parameters

The initial system consisted of an M_2 -TMD homotetramer, with the 1NYJ structure,³⁸ embedded in 96 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) molecules as a lipid bilayer, as well as 6400 water molecules (with the TIP3P model)⁴⁷, and 1 mol/kg NaCl or NH_4Cl saline solution. The 1NYJ structure represents the M_2 -TMD channel as a homotetramer with a left-handed bundle of four α -helices.³⁸ The M_2 -TMD α -helix was obtained from solid-state NMR spectroscopy in a hydrated DMPC bilayer, which consists of

22–46 residues with the amino acid sequence: NH_3^+ -Ser 22-Ser-Asp-Pro-Leu-Val-Val-Ala-Ala-Ser-Ile-Ile-Gly-Ile-Leu-His37-Leu-Ile-Leu-Trp41-Ile-Leu-Asp-Arg-Leu46- COO^- .38 The initial crystal-like structure was constructed, using the VMD software,48 in a box with initial dimensions of $62 \times 62 \times 80 \text{ \AA}$ in x , y , and z dimensions, respectively. This system was denoted $\text{M}_2\text{-TMD}_{\text{His}_4^{+0}}$.

Another two $\text{M}_2\text{-TMD}$ systems were modeled using the same traditional empirical-force-field approach as the $\text{M}_2\text{-TMD}_{\text{His}_4^{+0}}$. In the first system, two opposite histidine residues were protonated, whereas in the second system three histidine residues were protonated using a covalent bond in the force field. The former system was denoted $\text{M}_2\text{-TMD}_{\text{His}_4^{+2}}$, whereas the later was denoted $\text{M}_2\text{-TMD}_{\text{His}_4^{+3}}$. This approach allowed us to examine the limiting electrostatic effects of His37 titration.

The ammonium ion (NH_4^+) force field was not available in the empirical force field. Its parameters were obtained using the Extensible Computational Chemistry Environment (ECCE)49 after performing quantum mechanics calculations using NWChem50·51. The ammonium ion was built in ECCE and geometrically optimized at the RHF level of theory with the 6-311++G(3df,3pd) basis set, with Pople(3df,3pd) as a polarization function, and with Pople-style as a diffusion function. The bond lengths for the ammonium ion were derived from the resulting structure. The atomic partial charges were derived using electrostatic potential fitting. Energy constants were taken from the CHARMM, version 22, force field for primary amines. The ammonium ion parameters are listed (Table I). The calculated partial charges for ammonium atoms are somewhat stronger than the OPLS charges, H: 0.35 and N: -0.40,52 but we expect this to have only minor impacts on total mean force potential. To produce electroneutrality for the $\text{M}_2\text{-TMD}$ systems, appropriate numbers (4, 2, or 1) of Cl^- were removed from $\text{M}_2\text{-TMD}_{\text{His}_4^{+0}}$, $\text{M}_2\text{-TMD}_{\text{His}_4^{+2}}$, or $\text{M}_2\text{-TMD}_{\text{His}_4^{+3}}$, respectively.

System Preparation

The total simulation time for system preparation was 25 ns, with different periodic boundary conditions and harmonic restraints (Table II). This preparation allowed relaxation of the channel and disordering the lipid molecules, ions, and water molecules starting from the crystal-like structure.

Both the lipid deuterium order parameters and the lipid surface area are sensitive to the changes in the bilayer structures due to many factors such as the lipid hydration level, number of lipid molecules, temperature, statistical ensemble, surface tension, and saline-solution concentration.53–60 Surface tension is the force applied per unit length to a filament required to stretch a liquid film.61·62 Mathematically, the surface tension per interface (γ) can be calculated from the pressure tensors, averaged over the central cell containing a lipid bilayer system normal to the z direction, as follows:56

$$\gamma = \frac{1}{2} \left(L_z \times \left(P_{zz} - \frac{1}{2}(P_{xx} + P_{yy}) \right) \right), \quad (1)$$

where L_z is the length of the unit cell, P_{zz} is the component of the pressure tensor normal to the bi-layer surface, P_{xx} and P_{yy} are the tangential components of the pressure tensor, and

the angle brackets denote an average over time. For simulating a pure lipid bilayer in pure water, the appropriate surface tension has been evaluated to be within the range of 30–45 dyn/(cm·interface).^{56·58·63·64} However, with a more complex lipid bilayer system, such as one that includes salt and polypeptide, the surface tension is unknown.

We carried out a test for the suitable surface tension using a gramicidin A (gA) channel system because of a well estimated surface area for the gA channel⁶⁵, whereas it is unknown for the M₂-TMD channel. Briefly, the test was done by fixing the gA channel in the original structure, 1JNO structure,⁶⁶ to avoid channel-surface perturbation effect on the lipid bilayer properties,⁶⁷ applying two planar harmonic restraints on the lipid phosphorus atoms ($k_f = 10 \text{ kcal}/(\text{mol} \cdot \text{\AA}^2)$) at $z = \pm 17.5 \text{ \AA}$ to agree with the experimental head–head or phosphate–phosphate thickness of 35.3 $\text{\AA}$,^{68·69} and using 2.5 unit increments of the surface tension in the range of 0–75 dyn/(cm·interface). Based on accuracy and precision in the lipid molecular surface area consistent with the experimental DMPC molecular surface area,⁶⁹ the optimal surface tension was found to be 40 dyn/(cm·interface).

The Equilibration 3 stage was then carried out with $\gamma = 40 \text{ dyn}/(\text{cm} \cdot \text{interface})$, a fixed channel in the 1NYJ structure, and two planar harmonic restraints on the lipid phosphorus atoms ($k_f = 10 \text{ kcal}/(\text{mol} \cdot \text{\AA}^2)$) at $z = \pm 17.5 \text{ \AA}$ (Table II). This stage was run for 20 ns, enough time to obtain stable lateral dimensions for the system under study.⁷⁰ The purpose of this stage is to prepare a realistic lipid environment for the polypeptide channel.

During the subsequent equilibrations, the restraints on the channel and the lipid phosphorus atoms were progressively reduced without significant changes in the system structure. The simulations in the NPAT ensemble (Equilibrations 4–10) utilized a tetragonal box having a lateral dimension of 59.849 $\text{\AA}$, the average value from the second 10 ns of the Equilibration 3 stage. Both the Equilibration 10 stage and the routine 10 ns production run (done in parallel with the umbrella sampling simulations) were performed with the channel center-of-mass (COM) as the only restraint.

Molecular Dynamics Simulations

Simulations were performed with NAMD software,⁷¹ version 2.6, using version 31 of the CHARMM force field.^{72–77} Trajectory files were written every 1 ps for both the 10 ns test-ion-free production run and the umbrella sampling production runs using a time step of 2 fs and the NPAT ensemble. A smoothing function was applied to both the electrostatic and the van der Waals forces at a distance of 8 $\text{\AA}$ with a switching cutoff distance of 12 $\text{\AA}$, a pair list distance of 14 $\text{\AA}$, and all bonds with hydrogen being rigid using the ShakeH algorithm. The non-bonded interactions parameters (switching, spherical cutoff, and pair list distances) were carefully chosen to achieve realistic macromolecular simulations.⁷⁸ The Particle Mesh Ewald (PME) algorithm, implemented to add a reciprocal space long-range electrostatic energy to the limited non-bonded component, was used with an interpolation function of order 5, and PME box grid size of 128, 128, 256 in x , y , and z direction, respectively. Langevin dynamics were used to maintain the temperature at 303.15 K with a damping coefficient of 5 ps^{-1} , whereas the pressure in the z direction was maintained at 1 atm using the Nosé–Hoover Langevin piston method with an oscillation of 1 ps and a damping time of 0.1 ps.^{79·80}

Analysis scripts were composed using VMD and Tcl81 commands. The deuterium order parameters of the deuterium-labeled segment were calculated from:^{53·55·57·59}

$$S_{CD}^{(i)} = \frac{1}{2} \langle (3 \cos^2 \theta_i - 1) \rangle, \quad (2)$$

where θ_i is the angle between the i th C–H vector and the z axis (bilayer normal), and the angle brackets denote an average over both acyl chains from all lipid molecules, all C–H vectors associated with the i th carbon, and time. These parameters have a range from -0.5 to 1.0 and reflect the lipid tails orientation (i.e., a -0.5 value represents fully ordered lipid tails, whereas a 1.0 value represents fully disordered lipid tails).

The root-mean-square displacement (RMSD) reflects the degree of similarity between three-dimensional protein structures. It was computed by measuring the RMSD between equivalent atoms after optimal superposition of the two structures (the protein structure in trajectory frame i and the initial trajectory frame).

The conformational stability of the side chains can be represented by their torsion angles (dihedral angles) such as χ_1 and χ_2 angles. For histidine side chains, the χ_1 angle is the counterclockwise rotation of the $C_\beta - C_\gamma$ bond around the $C_\alpha - C_\beta$ bond in $N - C_\alpha - C_\beta - C_\gamma$ chain of atoms, whereas χ_2 angle is the counterclockwise rotation of the $C_\gamma - N_{\delta_1}$ bond around the $C_\beta - C_\gamma$ bond in $C_\alpha - C_\beta - C_\gamma - N_{\delta_1}$ chain of atoms.⁸² For tryptophan side chains, the χ_1 angle is the counterclockwise rotation of the $C_\beta - C_\gamma$ bond around the $C_\alpha - C_\beta$ bond in $N - C_\alpha - C_\beta - C_\gamma$ chain of atoms, whereas χ_2 angle is the counterclockwise rotation of the $C_\gamma - C_{\delta_1}$ bond around the $C_\beta - C_\gamma$ bond in $C_\alpha - C_\beta - C_\gamma - C_{\delta_1}$ chain of atoms.⁸² These definitions give a positive torsion angle, whereas a negative torsion angle requires rotation in the opposite direction. The rotameric state was given to each side chain based on observing their χ_1 and χ_2 fluctuations around their averages, most of the simulation time scale (8–10 ns). A rotameric state combination is represented with a notation (His37, Trp41).

The tilt angle was computed by calculating the angle between the helix principle axis and the bilayer normal, and then averaged over all helices and trajectories.

The interhelical distance was computed by calculating the distance between two neighboring helices using their COM, and then averaged over all trajectories.^{21,83,84} This distance was also calculated at another two different points, at the C-terminal end of the principle axis and at the N-terminal end of the principle axis taken at equal distances from the helix COM.

Another interesting distance was computed after averaging over all trajectories is the

$\text{Trp}_{C_\gamma}^{41} - \text{His}_{N_{\delta_1}}^{37}$ distance, which is the distance between C_γ in Trp41 from helix _{i} and N_{δ_1} in His37 from helix _{$i+1$} .³⁸ The notation $H_{i-(i+1)}$ was used to denote distances between residues on helix _{i} and helix _{$i+1$} , where the latter is the clockwise adjacent helix as viewed from the C-terminus side.

Umbrella Sampling Simulations

All the umbrella sampling simulations were performed using the final frame of the Equilibration 10 stage of the system under study as an initial structure. In these simulations, the channel COM was restrained to within $0.1 \text{ \AA}$ of the origin ($k_f^{\text{COM}} = 1000 \text{ kcal}/(\text{mol} \cdot \text{\AA}^2)$) and the test ion, selected from the bath ions, was restrained to a plane normal to the z axis ($k_f = 30 \text{ kcal}/(\text{mol} \cdot \text{\AA}^2)$), with the position of the plane being varied along the transport coordinate (“reaction coordinate”). All k_f symbols designated force constants (i.e., harmonic spring constants).

We don't view the restraining force on the channel COM as a biasing force for the umbrella sampling, but rather as a structural restraint force designed to compensate for missing components in the system, that is, the rest of the experimental system (remainder of planar bilayer or plasmalemma, etc), and thus to establish a reference point for the coordinate system. Here we use the term "transport coordinate" because no bond-forming or -breaking takes place during ion transport. The transport coordinate is then defined to be independent of the ion channel position, and to be just the z coordinate of the ion relative to the Cartesian origin. For systems of this size, the transport coordinate is similar, if not identical, to a "relative position" transport coordinate, in which the distance between the ion and the channel or bilayer-channel center of mass is the restrained coordinate. Figure 1, taken from a study on the gramicidin channel in which the same restraint forces, equilibration parameters, window duration and spacing parameters, etc. were used, shows that usage of relative or absolute transport coordinates yields almost identical free-energy profiles. More detail on this comparison is provided in the report on that study.⁸⁵

During umbrella sampling with the test ion, a cylindrical restraint ($k_f = 20 \text{ kcal}/(\text{mol} \cdot \text{\AA}^2)$) was applied on the test ion to keep it within $10 \text{ \AA}$ of the z axis. The purpose of the cylindrical restraint was to assure a one-dimensional free-energy profile.^{86–89}

We used 201 windows with a window width of $0.2 \text{ \AA}$ to cover the transport coordinate $-20 \leq z \leq 20 \text{ \AA}$. For each window, the z coordinate of the test ion was set to the window position and the x and y initial values of the test ion were set to zero before performing minimization for 3000 steps, heating from 0 to 303.15 K, equilibration in the NPAT ensemble for 1 ns, and production in the NPAT ensemble for 1 ns. Because each window was initiated independently of the others, they could be run in parallel and there was no hysteresis associated with the direction of ion transport.^{90–91} We used the weighted histogram analysis method (WHAM) to unbias the umbrella potential on the test ion and extract the free-energy profile.^{92–93} The mathematical details for calculating or extracting the free-energy profile were the same as described previously.^{88–92–94–95}

The potential of mean restraint (PMR) is used as a test for the WHAM results. The PMR can be extracted as follows:⁹⁶

$$F(z_{\text{ion}}) = -k_f(z_{\text{ion}} - z_w), \quad (3)$$

$$PMR(z_w) = PMR(z_o) + \int_{z_o}^{z_w} \langle F(z_{\text{ion}}) \rangle dz_w, \quad (4)$$

where z_{ion} is the test ion position in the z direction (sampled every 2 fs for the ensemble average), z_w is the window-reference position, and $F(z_{\text{ion}})$ is the instantaneous restraint force applied in the z direction to the test ion. The Composite Simpson's Rule was used as a numerical integration method.⁹⁷

Results and Discussion

Preparation and Simulation Methods Stability

The preparation process generally produces stable and randomized systems compared with the initial crystal-like structures. For example, the simulating annealing, based on the deuterium order parameters [Figs. 2(a) and (b)], is sufficient to produce disordered lipid tails in the presence of either NaCl or NH_4Cl saline solution. Our order parameter results agree

with both the experimental^{98–100} and the simulation⁵⁹ results, except in the region close to the head group, where the order remains high in experiments,^{98–100} but commonly declines in simulation.⁵⁹ The consistency in the order parameters results among differently titrated M₂-TMD structures as well as different saline composition is an indication of the robustness of the preparation method.

Our RMSD results show reasonable channel-backbone structural stability within the production run time scale for all M₂-TMD structures in NaCl saline solution [Fig. 3(a)], whereas slight excesses occurred in the presence of NH₄Cl saline solution [Fig. 3(b)] after 3 ns, especially for the M₂-TMD_{His⁺₄³} structure. This indicates some structural variations, but these may be random in origin and are not necessarily caused by the presence of the NH₄Cl saline solution or the charged histamines. The RMSD results appear to be incomplete in convergence after 10 ns, but stable enough for the 2 ns umbrella sampling runs started from the end of the last equilibration run (Equilibration 10). Our RMSD results are in good agreement with previous simulation observations.^{33·40·43–45}

M₂-TMD Channel Structural Analysis

Some of the M₂-TMD channel structure properties in the presence of NaCl or NH₄Cl saline solutions are listed (Tables III and IV) and compared with those from experimental models using our calculations. The tilt angle averages in the presence of two different saline solutions are in a good agreement with experimental observations,^{21·30·31·38·101} even though there are large fluctuations in the protonated structures. Slight variances of our results with some experimental^{102–104} and simulation^{33·39·41–43} results are expected because of different M₂-TMD length, lipid bilayer environment, water model, hydration level, experimental source, equilibration level, or simulation length. However, the basic size and shape of the molecule is now established, and our results further attest to the stability of the 1NYJ structure in the lipid membrane environment.

The interhelical distances at the C- and N-termini have rather modest fluctuations around their averages and are higher than those from the experimental models. We speculate that these values might be moderated and stabilized for the whole polypeptide in simulations as they are in experiments.¹⁰⁴ On the other hand, the average distances between the COM of two neighboring helices are consistent with results from experimental studies.^{21·101·102} Previous studies suggested that an interhelical distance within the range of 9.5–10.2 Å is an indication of a channel in the closed form, where water molecules penetration is not possible.^{38·39·43·103} The slight increase in this distance, as we change the M₂-TMD_{His⁺₄⁰} to the M₂-TMD_{His⁺₄³} structure, may be caused by the electrostatic repulsion effect in the selectivity filter, which was also observed in other MD simulations.^{39·41·43·45·46}

A REDOR solid-state NMR spectroscopy study suggested that the distance between the C_γ in Trp41 from helix_{*i*} and N_{δ₁} in His37 from helix_{*i*+1} is < 3.9 Å and represents the channel in the closed form.³⁸ Our results for this distance are higher than 3.9 Å (Tables III and IV) and less than some other values measured experimentally,^{101–103} but consistent with a previous simulation observation.⁴⁶ Because the thermally relaxed version of the solid state NMR M₂-TMD structure is somewhat more open in the region of His37 cluster, there may be other components, such as the Trp41 residues, shuttering the channel (see below).

The Rotameric Basis of Trp41 Gating

Various rotameric states for His37 and Trp41 side chains are observed in our simulations with NaCl or NH₄Cl saline solution (Tables III and IV). The most commonly observed rotameric states are the *t*–105° [Fig. 4(a)], which blocks the channel from passing water

molecules or ions [Fig. 4(b)], and the $t90^\circ$ [Fig. 4(c)], which allows the channel to pass water molecules and ions with the size of water molecule or smaller [Fig. 4(d)]. This apparent gating function of Trp41 side chains in our simulations is in a good agreement with the mutagenesis study.³⁶ To our knowledge, the Trp41 side chain rotamer, $t-105^\circ$, has never previously been proposed to be the rotamer that blocks the M_2 -TMD channel.

The illustrations of side chains configurations [Figs. 4(a) and (c)] reveal that there is a possible favorable interaction between $N_{\epsilon 1}$ in Trp41 residue from helix_i and $H_{\epsilon 2}$ in His37 residue from helix_{i+1} as a hydrogen bonding (H-bonding) interaction. Although solid-state NMR results suggested that there is a possibility of H-bonding interactions as imidazole–imidazolium interactions,¹⁰⁵ an alternative interpretation could be H-bonding interactions between His37 and Trp41 residues.

The $t-160^\circ$ His37 rotameric state and the $t-105^\circ$ and $t90^\circ$ rotameric states for Trp41 side chains were also observed in restrained MD simulations.⁴⁶ The next discussion explores the importance of this rotameric state for spontaneous water molecule and ion distributions in the channel.

Distribution of Water Molecules and Ions

The zoomed-in water molecule densities inside the M_2 -TMD channel in the z direction for two different saline solutions are shown [Figs. 5(a) and (b)]. The water molecule density was calculated by averaging the water molecules located within 1.0 Å thick slabs parallel to the bilayer over all trajectories, then divided by the slab volume. In the channel, the density value is low because the slab is primarily occupied by lipid and channel atoms, but still demonstrates the presence of water molecules, on average, in each slab. In particular, there is an occasional presence of a few water molecules in the selectivity filter at $z = 5$ Å, even for the M_2 -TMD_{His⁴+0} structure.

The occasional presence of water molecules in the filter raises the possibility that ions can enter the channel as well. Therefore, we also examined the Cl^- molality profiles for 10 ns production runs [Figs. 6(a) and (b)] in NaCl or NH_4Cl saline solution, respectively. On the production run time scale, no Cl^- were able to enter the selectivity region (around $z = 5$ Å) for M_2 -TMD_{His⁴+0} and M_2 -TMD_{His⁴+2} structures, whereas some Cl^- were able to enter the selectivity-filter region for M_2 -TMD_{His⁴+3} structure. Neither Na^+ [Fig. 6(c)] nor NH_4^+ [Fig. 6(d)] ions have any occupancy in the selectivity-filter region. In all M_2 -TMD structures, the high cation occupancy in the region ($10 < z < 20$ Å) is due to the negatively charged C-terminus side. The high cation occupancy in the M_2 -TMD_{His⁴+0} structure in ($-4 < z < 4$ Å) is reduced by the repulsion effects from protonated imidazole rings for the +2 and +3 states. All anion and cations molality profiles show good homogeneity in the bulk region ($|z| > 30$ Å).

Because chloride ions have a better chance of entering the selectivity filter than their counterions due to electrostatic attraction to the titrated histidine side chains, we next examine the height of the free-energy barrier in the selectivity-filter region.

Ion Potentials of Mean Force

Generally, the free-energy barrier in the selectivity-filter region (Fig. 7) does not exceed 8 kcal/mol for any of the three ions or any of the three-charged states, which would probably be low enough to allow significant permeation.¹⁰⁶ Therefore, the partially open state used in these umbrella sampling simulations does not fully exclude water molecules and ions, as we would expect the fully closed state would do.

Free-energy profile convergence depends on many factors, such as adequacy of sampling of test ion positions in the umbrella windows, variations in surrounding structures on the umbrella window time scale (which, in turn, is very different for usage of serial processions along the transport coordinate rather than uniform starting structures for each umbrella window), and statistical fluctuations affecting relative positioning of PMF components from adjacent windows. We examined free-energy profile convergence by comparing free-energy profiles obtained with increasingly longer portions of the umbrella window trajectories. Convergence, determined from increasingly complete data sets, accuracy, judged from the proximity of the free-energy integral endpoint to the reference potential on the opposite side of the bilayer, and methodological independence, judged from comparison of the WHAM method to the PMR method [Eq. (4)], were all excellent. In one demonstration of convergence [Fig. 7(a)], the PMF converged to within 0.5 kcal/mol over the entire extent of the transport coordinate, whether based on 0.5, 0.75, or 1.0 ns, and to within 0.5 kcal/mol of the reference energy (0 kcal/mol at $z = -20$ Å) at the opposite z position ($z = 20$ Å). Other sources of uncertainty,⁹⁰⁻⁹¹ such as polarizability effects, structural fluctuations during ion transport, and real-time titration of polypeptide or test ion (i.e., ammonium), have not been examined and might be interesting topics for future research.

Changing from the $M_2\text{-TMD}_{\text{His}_4^{+0}}$ to the $M_2\text{-TMD}_{\text{His}_4^{+3}}$ structure, the selectivity-filter free-energy barrier in the Cl^- PMF profiles [Fig. 7(b)] gets lower. As expected, this behavior is in the opposite sense for the Na^+ PMF profiles [Fig. 7(c)] and for the NH_4^+ PMF profiles [Fig. 7(d)], except for the $M_2\text{-TMD}_{\text{His}_4^{+3}}$ structure, where the more open form of the channel allows a lower barrier in spite of the electrostatic repulsion of the tetraimidazole selectivity filter.

The electrostatic attraction between the Cl^- and the protonated imidazole rings is the apparent cause for the decrease in the Cl^- translocation free-energy barrier. The cation results imply that the channel has a higher permeability for NH_4^+ than for Na^+ , which is in a good agreement with some of the electrophysiological results.⁸ This prediction, based on the free-energy profile for a nondissociable model for ammonium, should be conservative, considering that proton dissociation in the channel may enhance effective ammonium conductance. However, the simulations make the prediction that the channel would have a higher permeability for Cl^- than for either Na^+ or (nondissociable) NH_4^+ , which, although might be expected on electrostatics grounds, was not seen in whole-cell clamping of mouse erythroleukemia (MEL) cells.⁷⁻¹⁵ Of course, in contrast to the simulations with covalently titrated imidazole groups, experimentally, the protons titrating the His37 residues could flee in advance of incoming cations and might not substantially inhibit cation flow, and likewise a fourth titration might stabilize and be stabilized by Cl^- in the filter. So, the remarkable finding here is that non-protonic current would appear to be moderately high for this structure in any of the selectivity-filter states, provided that all of the Trp41 side chains are in the open configuration.

From our simulations of Cl^- and NH_4^+ transport, it appears that the 1NYJ-based structure would not fully exclude non-protonic ions like the whole polypeptide in MEL cells does. Of course, the whole polypeptide may have slightly different tilt angles and His37–Trp41 rotamer positions.¹⁰⁴

We were unable to confirm the high barrier to Na^+ obtained with this structure in the initial report of proton-selective PMF profile²¹. We would speculate that, given the similarities of the most recent structural assessments for peptides in crystal¹⁰¹ and micelle¹⁰³ to that

obtained previously in bilayer38·102, the proton-selective and closed state (if any) may be yet to be identified.

Conclusions

Three M₂-TMD structures with two different saline solutions were studied using MD and umbrella sampling simulations. The M₂-TMD_{His⁴+0} has been thought to represent a closed form, whereas the M₂-TMD_{His⁴+2} and M₂-TMD_{His⁴+3} represent intermediate forms.

Generally, our M₂-TMD structures, after 25 ns preparation time with the CHARMM force field, have several of the features observed in NMR and X-ray studies.

The t-105° rotamer for Trp41 blocks the channel from passing water molecules, whereas the t90° rotamer allows the channel to pass them.

Cl⁻ have a lower free-energy barrier in the selectivity-filter region of the M₂-TMD channel than either Na⁺ or NH₄⁺, and NH₄⁺ have a higher expected permeability than Na⁺.

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Figure 1. Symmetrized one-dimensional K^+ PMF profiles in the gA system as a function of the transport coordinate.⁸⁵ The PMF profiles shown are from independent transport coordinate (black line) and relative transport coordinate (grey line) implementations.

Figure 2.

Deuterium order parameters for the DMPC tails in $\text{M}_2\text{-TMD}_{\text{His}_4^{+0}}$ (black — line), $\text{M}_2\text{-TMD}_{\text{His}_4^{+2}}$ (black -- line), and $\text{M}_2\text{-TMD}_{\text{His}_4^{+3}}$ (grey — line) systems with (a) NaCl and (b) NH_4Cl saline solutions.

Figure 3.

RMSD versus time for the polypeptide backbone in $M_2\text{-TMD}_{\text{His}_4^{+0}}$ (black line), $M_2\text{-TMD}_{\text{His}_4^{+2}}$ (red line), and $M_2\text{-TMD}_{\text{His}_4^{+3}}$ (grey line) systems with (a) NaCl and (b) NH_4Cl saline solutions.

Figure 4.

Selected Rotameric states for the (His37, Trp41) conformations; ($t-80^\circ$, $t-105^\circ$) in (a) and (b), ($t-80^\circ$, $t90^\circ$) in (c) and (d), are demonstrated using the initial 1NYJ structure.

The His37 and Trp41 residues from two adjacent helices are shown by the Licorice drawing method, whereas the whole polypeptide is shown by the Maximal Speed Molecular Surface (MSMS) drawing method using a probe radius of 1.4 Å. The colored objects represent: C (cyan), H (white), N (blue), and O (red). In the Licorice images, the left-side of the image points toward the N-terminus side, whereas the right-side of it points toward the C-terminus side. In the molecular surface view, the perspective is looking from the C-terminus end.

Figure 5.

A zoomed in water molecules density profiles inside a channel in the z direction in M_2 -TMD_{His⁴+0} (black — line), M_2 -TMD_{His⁴+2} (black - - line), and M_2 -TMD_{His⁴+3} (grey — line) systems with (a) NaCl and (b) NH₄Cl saline solutions.

Figure 6.

Ions molality profiles in the z direction: Cl^- molality profiles in $\text{M}_2\text{-TMD}_{\text{His}_4^{+0}}$ (black — line), $\text{M}_2\text{-TMD}_{\text{His}_4^{+2}}$ (black — — line), and $\text{M}_2\text{-TMD}_{\text{His}_4^{+3}}$ (grey — line) systems with (a) NaCl and (b) NH_4Cl saline solutions. Cation molality profiles in $\text{M}_2\text{-TMD}_{\text{His}_4^{+0}}$ (black — line), $\text{M}_2\text{-TMD}_{\text{His}_4^{+2}}$ (black — — line), and $\text{M}_2\text{-TMD}_{\text{His}_4^{+3}}$ (grey — line) systems for (c) Na^+ and (d) NH_4^+ .

Figure 7.

One-dimensional ions PMF profiles as a function of the transport coordinate. (a) Na^+ PMF profiles in $\text{M}_2\text{-TMD}_{\text{His}_4^{+0}}$ including different simulation times; 0.5 ns (grey — line), 0.75 ns (black — — line), and 1.0 ns (black — line). In the other panels, the PMF profiles shown are from the $\text{M}_2\text{-TMD}_{\text{His}_4^{+0}}$ (black — line), $\text{M}_2\text{-TMD}_{\text{His}_4^{+2}}$ (black — — line), and $\text{M}_2\text{-TMD}_{\text{His}_4^{+3}}$ (grey — line) systems for (b) Cl^- , (c) Na^+ , and (d) NH_4^+ .

Table I**Ammonium Ion Parameters**

Stretching	Elements	Energy constant (K_b) ^a (kcal/(mol·Å ²))	Bond length (Å)
	N-H	403.0	1.003
Bending	Elements	Energy constant (K_θ) ^a (kcal/(mol·rad ²))	Bond angle (°)
	H-N-H	44.0	109.471
Electrostatic	Elements	Partial charge	
	N	−0.625490	
	H	+0.406372	

^aExtracted from version 31 of the CHARMM force field

Summary of System Preparation Approach

Table II

Process	Ensemble ^a	Restraints ($k_f \bar{b}$, kcal/(mol·Å ²))		Steps			
		Lipids ^c	Channel ^d				
				P	bb	non bb	com
Minimization 1	NVT	200	F	F	F		10,000
Heating 1	NV	200	F	F	F		20,000
Equilibration 1	NVT	200	F	F	F		100,000
Annealing ^e	NV	200	F	F	F		200,000
Minimization 2	NVT	200	F	F	F		3000
Heating 2	NV	20	F	F	F		50,000
Equilibration 2	NPAT	20	F	F	F		100,000
Equilibration 3	NP- γ T	10	F	F	F		10,000,000
Equilibration 4	NPAT	2	100				100,000
Equilibration 5	NPAT	1	40				100,000
Equilibration 6	NPAT		10				100,000
Equilibration 7	NPAT		2				100,000
Equilibration 8	NPAT		0.4				100,000
Equilibration 9	NPAT		0.1				267,000
Equilibration 10	NPAT					1000	1,250,000

^aThe symbols in this column stand for; N: constant number of atoms, V: constant volume, T: constant temperature, P: constant pressure, A: constant area, γ : constant surface tension

^b k_f : force constant

^cThe symbol in this column stands for; P: phosphorus atoms

^dThe symbols in this column stand for; bb: backbone, com: center-of-mass, F: fixed atoms

^eIt consists of three stages with each stage: 40 ps of heating to 1000 K followed by 80 ps of cooling to 303.15 K

Some of M₂-TMD Channel Structure Properties in NaCl Saline Solution

PDB Structure		1NYJ		1NYJ	2H95	2RLF ^a	3C9J
	M ₂	M ₂	M ₂				
	TMD _{His⁺4}	TMD _{His⁺2}	TMD _{His⁺3}				
Source	MD Simulations			NMR	NMR	NMR	X-ray
Residues		22–46		22–46	26–43	18–60	22–46
Lipid Bilayer		DMPC		DMPC	DMPC	DHPC ^b	
Reference		This work		(38)	(102)	(103)	(101)
Tilt Angle (°)	36.9 ± 3.9	39.6 ± 5.6	38.0 ± 2.8	37	23	16	32
Interhelical	C ^c	17.6 ± 0.9	19.5 ± 2.2	17.8 ± 1.1	17	12	24
	M ^d	11.4 ± 0.8	11.7 ± 1.3	11.6 ± 0.8	10	13	12
Distance (Å)	N ^e	18.9 ± 2.1	18.9 ± 1.1	19.5 ± 1.3	17	16	11
Trp _{C_γ} ⁴¹ – His _{N₆₁} ³⁷	H _{A–B}	5.5 ± 0.4	8.1 ± 0.8	4.8 ± 0.5	3.8	10.9	9.3
	H _{B–C}	5.6 ± 0.7	5.4 ± 1.0	7.4 ± 0.8	3.7	10.9	9.0
Distance (Å)	H _{C–D}	6.8 ± 1.0	5.3 ± 0.4	5.6 ± 0.5	3.7	10.9	8.6
	H _{D–A}	5.8 ± 0.9	4.6 ± 0.4	6.1 ± 0.6	3.8	10.9	8.6
His37	H _A	t-80°	t-80° <i>f</i>	t-80° <i>f</i>	t-160°	t-160°	t-80°
	H _B	t60°	m-170° <i>g</i>	t-80° <i>f</i>	t-160°	t-160°	t-80°
Rotamer ^h	H _C	t60°	t60° <i>f</i>	t-80° <i>f</i>	t-160°	t-160°	t-160°
	H _D	t-80°	t-80°	t60°	t-160°	t-160°	t-80°
Trp41	H _A	t-105°	t-105°	t90°	t-105°	m95°	t-105°
	H _B	t90°	m0°	t-105°	t-105°	m95°	t-105°
Rotamer ^h	H _C	t90°	t90°	t-105°	t-105°	m95°	t-105°
	H _D	t-105°	t90°	t-105°	t-105°	m95°	t-105°

- ^aThe analysis for this structure was performed on the residues 22–46
- ^bDHPC stands for 1,2-Dihexanoyl-sn-glycero-3-phosphocholine
- ^cC Stands for the distance between two neighboring helices using the C-terminal end of the principle axis
- ^dM Stands for the distance between two neighboring helices using the COM of each helix
- ^eN Stands for the distance between two neighboring helices using the N-terminal end of the principle axis
- ^fThis is for a protonated histidine residue
- ^gThis is not a common rotamer in the Lovell et al. library.⁸²
- ^hH_{*i*} stands for helix *i*

Table IV

Some of M₂-TMD Channel Structure Properties in NH₄Cl Saline Solution

PDB Structure	1NYJ				2H95	2RLFa	3C9J
	M ₂	M ₂	M ₂	M ₂			
	TMD _{His4+0}	TMD _{His4+2}	TMD _{His4+3}				
Source	MD Simulations				NMR	NMR	X-ray
Residues	22–46				22–46	18–60	22–46
Lipid Bilayer	DMPc				DMPc	DHPC ^b	
Reference	This work				(38)	(102)	(101)
Tilt Angle (°)	37.2 ± 3.8	36.1 ± 3.5	37.9 ± 6.4		37	23	32
Interhelical	C ^c	17.1 ± 1.3	18.0 ± 2.4	17.7 ± 1.2	17	12	24
	M ^d	11.2 ± 0.8	11.5 ± 1.8	11.8 ± 0.6	10	13	12
Distance (Å)	N ^e	18.9 ± 2.4	18.5 ± 3.1	20.0 ± 1.5	17	16	11
Trp ⁴¹ – His ³⁷ _{N₆₁}	H _{A-B}	4.5 ± 0.4	5.5 ± 0.9	5.0 ± 0.7	3.8	10.9	9.3
	H _{B-C}	5.4 ± 0.3	6.6 ± 1.3	4.9 ± 1.0	3.7	10.9	9.0
	H _{C-D}	5.7 ± 0.3	5.4 ± 0.9	5.8 ± 0.7	3.7	10.9	8.6
	H _{D-A}	5.6 ± 0.5	7.2 ± 1.2	7.3 ± 0.9	3.8	10.9	8.6
His37	H _A	t-80°	t-160° <i>f</i>	t-160° <i>f</i>	t-160°	t-160°	t-80°
	H _B	t60°	t-80°	t-160° <i>f</i>	t-160°	t-160°	t-80°
Rotamer ^g	H _C	t60°	t-160° <i>f</i>	t-160° <i>f</i>	t-160°	t-160°	t-160°
	H _D	t-80°	t-80°	t60°	t-160°	t-160°	t-80°
Trp41	H _A	t-105°	t-105°	t90°	t-105°	m95°	t-105°
	H _B	t90°	t-105°	t-105°	t-105°	m95°	t-105°
Rotamer ^g	H _C	t-105°	t-105°	t-105°	t-105°	m95°	t-105°
	H _D	t90°	t90°	t-105°	t-105°	m95°	t-105°

^aThe analysis for this structure was performed on the residues 22–46

^bDHPC stands for 1,2-Dihexanoyl-sn-glycero-3-phosphocholine

^cC Stands for the distance between two neighboring helices using the C-terminal end of the principle axis

^dM Stands for the distance between two neighboring helices using the COM of each helix

^eN Stands for the distance between two neighboring helices using the N-terminal end of the principle axis

^fThis is for a protonated histidine residue

^gH_i stands for helix *i*