Dynamical model of the CLC-2 ion channel exhibits a two-step gating mechanism

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ABSTRACT

This work reports a Markov state model of the conformational dynamics involved in regulating the inner and outer gates of the CLC-2 ion channel. This dynamical model, based on 600 microseconds of molecular dynamics simulation, suggests a new mechanism for channel activation. Transition path analysis highlights a dominant pathway between a highly stable closed state (similar to known structures) and several new protein conformations not observed in any previously elucidated CLC structure. Movement from the closed state involves the intracellular rotation of the inner-gate backbone along residues S168-G169-I170. This rotated state is similar to that observed in the recent cryo-EM structure of the bovine CLC-K channel, but the volume of the intracellular (inner) region of the ion conduction pathway is expanded to a greater extent. From this state, which remains closed at the extracellular side, two additional states may be assumed, each involving a unique rotameric flip of the GLU₁₈₁ (E211) side chain. Both additional states involve conformational changes that orient GLU₁₈₁ away from the extracellular (outer) region of the ion conduction pathway. In the first additional state, the rotameric flip of GLU₁₈₁ results in an open, or near-open, channel pore. The equilibrium population of this state is low (≈1%), consistent with the low open probability of CLC-2 in the absence of a membrane potential stimulus (0 mV). In the second additional state, GLU₁₈₁ rotates to occlude the channel pore. This state, which has a low equilibrium population (≈1%), is only accessible when GLU₁₈₁ is protonated. Together, these pathways model the opening of both an inner and outer gate within the CLC-2 selectivity filter, as a function of GLU₁₈₁ protonation. Collectively, our findings build on and are consistent with known properties of CLC-2 and will guide future investigations into CLC-2 channel biophysics.

Introduction

The chloride channel (CLC) protein family plays a wide variety of physiological functions in a diverse array of organisms, ranging from bacteria to humans¹–⁷. This family of membrane proteins is composed of both channels and H⁺/Cl⁻ exchange transporters that share a structurally unique homodimeric architecture⁸,⁹. Each subunit within the homodimer is an independent functional unit¹⁰–¹² composed of 17 membrane-embedded α-helices¹³. These helices coalesce to form a narrow, electropositive ion-conducting pore that is highly selective for Cl⁻¹³. Such an architecture is unusual for ion channels and raises many questions about the mechanisms of ion channel opening and closing (gating).

High-resolution CLC structures provide invaluable starting points for understanding the structure-function relationships of CLC protein activation. The first CLC structure solved, a prokaryotic H⁺/Cl⁻ exchange transporter¹⁵, proved relevant for guiding structure-function studies on a wide range of CLCs, including eukaryotic CLC channels¹⁴–¹⁷. This structure represents a closed conformational state in which bound chloride (Cl⁻) is occluded by proteinaceous “gates” that block exit to either the outer (extracellular) or inner (intracellular) side of the protein. The outer gate is formed in part by a conserved glutamate residue, GLU₁₈₁¹⁵–²⁵. When mutated to GLN (to resemble the protonated GLU), this side chain rotates outwards and partially unblocks the Cl⁻ permeation pathway¹⁸. The inner gate is formed by conserved SER and TYR residues¹³,²⁶–²⁸ that, according to solved CLC structures, physically obstruct the Cl⁻ translocation pathway from the intracellular side. In the recently published structure of the bovine CLC-K channel (the first structure of a CLC channel homolog), the loop containing the SER gate residue is rotated to unblock the intracellular gate²⁹. Despite the advances in structural understanding afforded by these discoveries, additional analysis is required to turn these static conformational snapshots into a complete dynamic picture of CLC gating. Experimental biophysicists have shown that these mechanisms are complex, involving interdependent influences of transmembrane voltage, [Cl⁻], and [H⁺]³⁰–³⁴. To begin to resolve these complexities and reveal the dynamic, atomistic details of the CLC gating machine, it will be critical to pair structural studies with high-resolution conformational modeling³⁵–⁴⁴.
In this study, we investigate the molecular dynamics of CLC-2, a chloride ion channel of particular interest due to its high levels of expression in the brain, in both neurons and glia. In glia, the importance of CLC-2 was illuminated in studies of knockout mice, which develop white matter vacuolization resembling human leukoencephalopathy, and complementary studies. Indeed, mutations in human CLC-2 are known to underlie a specific form of the disease, although the mechanistic details of causation are still uncertain. In neurons, CLC-2 has been suggested to play an important role in regulation of excitability and/or chloride homeostasis. However, the details of these roles are controversial. A precise understanding of the atomic interactions and rearrangements involved in channel activation will be valuable to facilitate understanding of CLC-2 physiological behavior and its role in human disease.

The spatial and temporal resolution afforded by computer simulations allows for the atom-scale examination of protein structure and function. Here we apply molecular dynamics (MD) simulations and Markov State Modeling (MSM) to study the conformational dynamics of CLC-2 gating. MSMs allow for the rigorous statistical characterization of biophysical simulations. Prior to constructing the MSM, the simulation data is first transformed into its kinetically slowest representation via the method of time-structure based independent component analysis (tICA). Such a transformation allows us to identify the slowest conformational changes in our dataset, and to build the MSM over these processes. We queried this MSM in order to predict the thermodynamics and kinetics of CLC-2 channel activation. Our model suggests that the top activation pathway begins in the closed state, near the template structure on which we based our model. The slowest timescale conformational change involves the rotation of the backbone along the S168-G169-I170 residues on the intracellular side of the entrance to the ion conduction pathway. The second major motion describes the flip of the GLU\textsubscript{ex} side chain on the extracellular vestibule. This second motion displays a sensitivity to residue protonation that correlates well with experimental evidence that the conserved glutamate residue, GLU\textsubscript{ex}, regulates the CLC “fast” gate in CLC-2. Furthermore, the model suggests that these “inner” and “outer” gates are nontrivially coupled, providing a novel depiction of CLC conformational change and a new mechanistic hypothesis that describes the stepwise process of channel activation. Together, these data provide a high-resolution mechanistic model of CLC-2 gating.

Results and discussion

Ion channel “gating” involves the conformational rearrangement of the protein, such that transmembrane ion flow through the channel pore is either increased or decreased. These gates are highly regulated so as to avoid dissipation of cellular ion gradients. High-resolution structures of CLCs have revealed two “gates” within each subunit. These gates physically occlude the permeation pathway, one towards the extracellular (outer) side and one towards the intracellular (inner) side. Outer-gate opening is thought to describe the circumstance where the GLU\textsubscript{ex} residue (E211 for rCLC-2, see Fig. 1 for reference) swings upward, such that it ceases to block the ion conduction pathway. Several lines of experiments support this model. This conformational change may be influenced by a variety of factors, including but not limited to, external membrane potential, thermal fluctuation, local ion concentration, or residue protonation. The conformational changes involved with inner gate opening are less precisely understood, though experiments are consistent with the narrowest constriction being localized near the SER/TYR site and not further towards the intracellular side.

We have applied dynamical modeling in order to identify and piece together the major conformational changes characterizing inner- and outer-gate opening. First, a homology model was built for the rCLC-2 sequence, using the 3ORG structure of the cmCLC transporter as a template structure. This template is 75% identical to CLC-2 within the pore-forming (selectivity filter) regions of the protein. The sequence conservation between the target and template is depicted in Fig. 1, bottom right. From this model, two MD datasets were generated, identical with the exception of GLU\textsubscript{ex} protonation. To improve computational sampling, and because each subunit contains an independent and identical pore, only one subunit of the homodimer was simulated, as depicted in Fig. 1. These datasets were analyzed with respect to the selectivity filter conformation (Fig. 1 inset).
Figure 1. Illustration of the simulated rCLC-2 monomer. The protein is shown as a transparent surface. The planar surfaces represent the system membrane bilayer (planes intersect the average z-coordinate of the lipid phosphorus atoms). The selectivity filter (SF) is highlighted in a ribbon representation. A larger view of the SF is given by the inset with a dashed border. Mechanistically important Cl- binding sites, $S_{cen}$ and $S_{ext}$, are labeled, as are nearby residues GLU$_{ex}$, SER$_{cen}$, and TYR$_{cen}$. Helices F and N help form the $S_{ext}$ binding site. GLU$_{ex}$, on Helix F, is positioned at the $S_{cen}$ ion binding site in this conformation. Chloride ions are shown in green. Sequence conservation between the CLC-2 and 3ORG selectivity filter residues is displayed on the bottom right. Hydrophilic residues are highlighted in blue, neutral residues in green, and hydrophobic residues in black. Residue height denotes the relative frequency of the amino acid at a given position. When there is a residue discrepancy, the CLC-2 residue is given on top. Each region of the SF has been segmented by the red lines.
Selectivity filter conformational dynamics

Markov State Models (MSMs) have emerged as a powerful tool for robustly estimating the statistics of biophysical systems from MD datasets. This technique first partitions a dataset into a set of discrete conformational states, and then parameterizes a transition matrix describing the probability of transition for each state pair. Prior to constructing the MSM, it is advantageous to featurize the data according to a structural metric, and then transform the featurized dataset using time-structure independent component analysis (tICA). tICA determines the slowest-decorrelating dynamical degrees of freedom. This improves the MSM statistics, and additionally provides a set of unbiased reaction coordinates that are useful for separating and visualizing important conformational states. These reaction coordinates can be thought of as dynamical modes, and are referred to as “tICs”. A tIC is given by a linear combination of input features, and can be interpreted by identifying the features with the highest linear coefficients (referred to as loadings). After the MSM is constructed, the state representation can be simplified via macrostating. This simplification allows for the identification of the most thermodynamically stable conformational states.

Here, we employ these techniques to characterize the conformational dynamics sampled in our datasets.

Our analysis identified four major conformational states: $C_{oi}$, $C_o$, $O$, and $U$. These states were named to reflect whether the channel appears closed (C) or open (O) at the inner (i) or outer (o) gate, with the exception of $U$, a postulated non-conducting state. A representative conformation for each thermodynamic macrostate is illustrated in Fig. 2. For a detailed labeling of each selectivity filter component, see Fig. 1. Each of these states can be kinetically distinguished from the others by tIC 0, tIC 1, and tIC 3. Note that tIC 2 was found to be redundant with tIC 1 and hence will not be discussed. These tICs were interpreted by analyzing the input features with the highest loadings. In Table 1 we have detailed how each tIC (dynamical mode) separates a particular two-state kinetic process, and which features distinguish each tIC. Greater detail describing the tIC loadings is given in the supplementary information.

Along each major dynamical mode, we observe two-state behavior. tIC 0 involves the rotation of the backbone dihedrals of the S168, G169, and I170 residues. This implies that the bottom strand of the SF can exist in two unique conformations. tICs 1 and 3 both involve flipping the GLU intrusion $\chi_1$ side chain dihedral, a motion that is coupled to the residues involved in the tIC 0 conformational change. Although tICs 1 and 3 begin in the same initial state ($C_o$) and describe the rotameric flipping of the same residue, the end states ($O$ and $U$) differ in conformation. This suggests that the GLU$^{\alpha}$ rotamer can exist in three possible states.

![Figure 2](image-url)

**Figure 2.** Structural visualization of each CLC-2 selectivity filter macrostate conformation. The S168-G169-I170 residues, located at the inner region of the SF, exist in two orientations, either pointing toward the ion conduction pathway ($C_{oi}$), or away ($C_o$, $O$, $U$). The GLU$^{\alpha}$ residue side chain, located at the outer region of the SF, exists in three orientations, either occluding the $S^{\text{ext}}$ Cl$^-$ binding site, located in the gap between helices F and N ($C_{oi}$, $C_o$), or in one of the two distinct outward rotamers ($O$ and $U$). Note that in the $U$ state, the geometry of the $S^{\text{ext}}$ binding site is distorted by the conformation of helix N.

| dynamical mode | conformational change | top residue features | kinetic process |
|----------------|-----------------------|---------------------|----------------|
| tIC 0          | SER$_{cen}$ backbone rotation | $\psi$(I170,G169), $\psi$(G169,S168) | $C_{oi}$ $\leftrightarrow$ $C_o$ |
| tIC 1          | GLU$^{\alpha}$ side chain flip | $\chi_1$(E211), $\phi$(S168,G167) | $C_o$ $\leftrightarrow$ $U$ |
| tIC 3          | GLU$^{\alpha}$ side chain flip | $\chi_1$(E211), $\phi$(G169,S168) | $C_o$ $\leftrightarrow$ $O$ |

**Table 1.** Dynamical modes of the MD data, as determined by tICA.
Figure 3. Free energy of major CLC-2 conformational states, projected onto all major dynamical modes (tICs), as a function of GLU_ex protonation. Note that tIC 2 is redundant with tIC 1 and is not shown. These landscapes are shown in both 1D and 2D, for both the deprotonated dataset (GLU_ex, black solid lines) and the protonated dataset (GLU_ex^p, gray dashed lines). tIC 0 denotes the conformation of the S168-G169-I170 backbone, while tIC 1 and tIC 3 denote the conformation of the GLU_ex side chain. In all cases, C_o was found to be the global free energy minimum, and to not vary greatly with respect to GLU_ex protonation. Also insensitive to protonation are the basin free energies of C_o and O. The largest difference caused by GLU_ex protonation is the existence of U, which occurs only for GLU_ex^p (as isolated by tIC 1), and the structure of the kinetic barriers.
The thermodynamics of each conformational state was further analyzed using the MSM-derived free energies. This analysis is illustrated by Fig. 3, where the data have been separated by the GLU$_{ex}$ protonation state, and projected onto each major dynamical mode. We observe that in all cases, C$_{oi}$ describes the conformational free energy minimum. This state, so named because the channel pore appears closed to both the outer (o) and inner (i) sides of the membrane, is similar to the template (3ORG) structure with the exception that GLU$_{ex}$ has moved upwards out of the S$_{cen}$ site, towards the S$_{ext}$ site. This positioning at S$_{ext}$ is similar to that observed in all other CLC structures found in the PDB. The C$_{o}$ state is the next most stable state, with a free energy less than 1 kCal/mol greater than the C$_{oi}$ state. This state was labeled C$_{o}$ because the channel pore is closed to the outer side but open towards the inner side. In this state, the S168, G169, and I170 backbone strand has changed conformation, but the GLU$_{ex}$ residue remains in the same geometry. As can be seen by the landscapes in Fig. 3, GLU$_{ex}$ protonation does not affect the free energies of the C$_{oi}$ and C$_{o}$ states. The transition barrier height for the C$_{oi}$ ↔ C$_{o}$ conformational change is approximately 2 kCal/mol.

tICs 1 and 3 identify two higher energy conformational states, O and U, each connected to state C$_{o}$. As discussed previously, these states each represent rotamers of the GLU$_{ex}$ residue. In the O (“open”) state, GLU$_{ex}$ appears to move out of the way of the Cl$^-$ permeation pathway, whereas in the U state, GLU$_{ex}$ collapses into the pore, which remains occluded (as discussed below). Both states O and U are between 2 and 3 kCal/mol greater in energy than states C$_{o}$ and C$_{oi}$. The O state shows a mild sensitivity to protonation, while the U state exists only when GLU$_{ex}$ is protonated. This result can be seen by the absence of a U basin in the GLU$_{ex}$ 2D free energy plots of Fig. 3. The transition barrier height of C$_{o}$ → O, along tIC 3, is approximately 4-5 kCal/mol for both protonation states, though there is considerable uncertainty for GLU$_{ex}$ (shown by the error bar along tIC 3). The transition barrier height of C$_{o}$ → U, along tIC 1, for GLU$_{ex}$ is approximately 5 kCal/mol.

**Figure 4.** Kinetic rates describing the transition between major CLC-2 conformations, as a function of GLU$_{ex}$ protonation. Note that the size of each node is proportional to its equilibrium population (larger denotes a lower free energy), and the weight of each arrow is proportional to the speed of the kinetic transition (heavier weight denotes a faster rate). Additionally, it is possible that there exist transitions occurring over timescales longer than what was sampled in our datasets (which likely underlies the lack of an observed transition from C$_{oi}$ to U). As identified by the thermodynamic analysis, the U conformational state exists only for the GLU$_{ex}$ model. Both the GLU$_{ex}$ and GLU$_{ex}$ models show similar rates describing C$_{oi}$ ↔ C$_{o}$ and C$_{o}$ ↔ O. However, in the case of GLU$_{ex}$, state U may transition forward to any other state.
To examine kinetics in greater detail, continuous-time rate-matrix MSMs were computed for the macrostate model in order to estimate the relative rate of transition between each major conformational state (as the traditional MSM gives transition probabilities, rather than transition rates). The resultant kinetic network graphs are given in Fig. 4. We observe that the \( C_{oi} \) state transitions only to the \( C_o \) state, while the \( C_o \) state may transition to any of the other states. The forward and backward rate of \( C_{oi} \leftrightarrow C_o \) are comparable. For both the \( O \) and \( U \) states, the reverse rates from these states to the \( C_o \) state are faster than the forward rates. While the primary effect of GLU\( ex \) protonation is the addition of the \( U \) state, kinetic analysis highlights two additional, mechanistically relevant, features of protonation. First, the connectivity of the modeled closed state, \( C_{oi} \), to the modeled open state, \( O \), suggests that protonation of GLU\( ex \) is not essential for channel conduction. While this result is in contrast to a computational study of the 3ORG structure, which found that protonation of the GLU\( ex \) side chain is required for ion transport to occur\(^{38} \), it is in harmony with electrophysiological studies of CLC-2, which have demonstrated that protonation of GLU\( ex \) does not affect channel opening\(^{69} \). The second additional highlight of our kinetic analysis is that the transition rate from \( O \) to \( C_{oi} \) is slowed when GLU\( ex \) is protonated. While this result is uncertain due to the magnitude of the error bars of our calculations, it is worth highlighting given its consistency with experimental results, which demonstrate that the CLC-2 closing rate decreases with increasing extracellular \([H^+]\)\(^{70} \).

**Structural analysis**

Above, we computed estimates for the thermodynamic and kinetic behavior of four major CLC-2 conformational states. Now, we delve deeper into the structural analysis of these states. To further investigate the geometric structure of each macrostate, the volume of the ion conduction pathway was analyzed. For each macrostate, several conformations were sampled, and the radius of the ion conduction pathway computed. These results are given with respect to distance from the GLU\( ex \) residue (one path to the extracellular region, one path to the intracellular region), and are illustrated in Fig. 5. It was found that GLU\( ex \) is the bottleneck of the conduction pathway for all macrostates. States \( C_{oi} \), \( C_o \), and \( O \) display a comparable bottleneck of approximately 1.5 Å. In contrast, state \( U \) is more congested in this region, reducing to a radius of only 1 Å. It is unlikely that this especially constricted bottleneck would allow for chloride ion permeation.

While states \( C_{oi} \), \( C_o \), and \( O \) display bottlenecks of approximately 1.5 Å in radius (narrower than the \( \sim 1.8 \) Å radius of a Cl\(^-\) ion), the extent of these bottlenecks along the length of the channel pore varies significantly between the conformations. For \( C_{oi} \) and \( C_o \), the pore is constricted to less than 1.8 Å for a \( \sim 19 \) Å stretch, whereas for state \( O \), the pore is constricted for less than half that distance (Fig. 5). Comparing \( C_{oi} \) and \( C_o \), the most striking difference is along the intracellular pathway, which widens sufficiently to accommodate chloride in \( C_o \). This finding aligns with our initial observation that these states are distinguished by the conformational state of the inner gate, while the outer gate remains closed. Across both the extracellular and intracellular region, the \( O \) state is the most voluminous. This finding aligns with our observation that both inner- and outer-gate residues (GLU\( ex \) and SER\( cen \)) have rotated away from S\( cen \). The fact that a narrow constriction persists within the putative “open” state (the most open of the set of macrostates) suggests that further conformational change may be involved in reaching a fully open state. Such an additional conformational change (to widen the pore following the rotation of GLU\( ex \)) has been proposed based on experimental studies\(^{37,83,84} \).

For both extracellular and intracellular pathways, the \( U \) state is the most constricted, despite the fact that the inner-gate SER residue is rotated to the “open” position. The GLU\( ex \) residue is in a unique rotameric state, rotated away from SER\( cen \), but this rotation does not increase the volume of the extracellular region of the channel, in part due to the distortion of helix N in this state (see Fig. 2). The constricted nature of the \( U \) state indicates that it is a non-conductive (closed) channel. That the \( U \) state is only accessible when GLU\( ex \) is protonated (neutral) is in agreement with experimental observation that low extracellular pH induces channel closure\(^{19,85,86} \) (though it is known that an extracellular histidine is also involved\(^{87} \)).
The radius of the ion conduction pathway demonstrates a strong dependence on macrostate. Distance along the pathway (x axis) is given with respect to the GLU\textsubscript{ex} residue. The plot on the left illustrates the pore radius from the extracellular side of the protein, descending into the pore to GLU\textsubscript{ex}, while the plot on the right illustrates the pore radius from GLU\textsubscript{ex} to the intracellular side of the protein. As an example, the top structures illustrate the volume of the C\textsubscript{oi} ion conduction pathway from multiple perspectives. All macrostates are relatively open near the extracellular mouth of the protein, but narrow in distinct ways to the bottleneck located at GLU\textsubscript{ex}. The O state is the most open in the region just above GLU\textsubscript{ex}, and U is the most narrow. Additionally, the minimum radius of the bottleneck is approximately the same for all states except U, which is highly constricted. Moving toward the intracellular opening from GLU\textsubscript{ex}, O is once again the most voluminous, while U is the most constricted.

Figure 5.
Now, we further interpret our model (Figs. 3, 4) structurally by visualizing representative conformations from each macrostate. Superpositions of these macrostate conformations are depicted in Fig. 6A. Observe that the inner residues, S168-G169-I170, assume two major conformational states, while the outer residues, GLU$_{ex}$ and those surrounding the $S_{ext}$ binding site, assume three major conformational states.

In Fig. 6B, we compare these structures to a set of relevant structures from the PDB. In the known structures, the GLU$_{ex}$ side chain has been observed to adopt three conformations, referred to as “down”, “middle”, and “up”\(^{26}\). The “down” conformation, with GLU$_{ex}$ pointing into $S_{cen}$, has been seen only in 3ORG, our template structure. In the CLC-2 model based on this structure, we found that GLU$_{ex}$ quickly equilibrated away from this conformation to a rotamer where it occupied the $S_{ext}$ binding site (the “middle” position). This positioning is similar to that observed in most CLCs in the PDB (overlay shown for representative structure 1OTS). The GLU$_{ex}$ “up” position, which has been observed when GLU$_{ex}$ is mutated to GLN (PDB 1OTU\(^{18}\)) has been considered to represent a more open pore conformation; however this conformation is more occluded than the $O$ macrostate\(^{37}\). The $O$ state demonstrates a SF-conformation similar to that of the 5TQQ structure, especially with respect to the intracellular region.
Figure 6. Structural overlays of selectivity filter (SF) conformations. **A.** Overlay of the four macrostates observed in this study. Left: The S168-G169-I170 backbone conformation distinguishes the $C_{oi}$ and $C_o$ states. Right: The GLU$_{ex}$ side chain conformation distinguishes the $C_o$, $O$, and $U$ states. These rotamers are depicted from the plane of the membrane (upper), and also from the extracellular plane (lower). The rotation of SF orientation is signified by the black arrow. **B.** Comparison to relevant structures in the PDB. As in A (right panel), these comparisons are shown from the plane of the membrane (upper), and also from the extracellular plane (lower). The stable $C_{oi}$ state adopts a GLU$_{ex}$ conformation different from that in the template structure 3ORG and more similar to that observed in most CLC structures in the PDB, as represented by 1OTS. The $O$ state adopts a GLU$_{ex}$ conformation not seen in any known structure, including 1OTU, where GLU$_{ex}$ is mutated to GLN. At the intracellular side, the conformation of the S168-G169-I170 backbone of the $O$ state resembles that of the 5TQQ structure.
Next, we analyze these conformations along each major dynamical mode (tIC). This is done by examining the structures along each tIC projection, as shown in Fig. 7. Here, the free energy surfaces for each two-state process are projected onto the major reaction coordinates (tICs, or dynamical modes) described earlier, and separated by GLU$_{ex}$ protonation state. The shading surrounding the free energy curves represents the error (1 standard deviation) calculated from statistical bootstrapping of the MSM. The gray structure along each tIC represents the process transition state.

By examining the most significant tIC 0 feature loadings, we determine that the backbone of the S168, G169, and I170 residues determine the conformational states of the $C_o \leftrightarrow C_{oi}$ process. This is illustrated in Fig. 7, top. The $C_{oi}$ state (yellow) positions the S168 side chain toward the ion conduction pathway. The $C_o$ state positions the S168 side chain to the cytosol. In the tIC 0 transition state, the S168 side chain is oriented halfway between the $C_{oi}$ and $C_o$ macrostate structures. Hence, we observe a full 180 degree intracellular rotation of the S168-G169-I170 backbone. Recently, a cryo-EM structure of the bovine CLC-K channel (5TQQ) was found to uniquely (compared to previous CLC structures) display a cytosolic facing SER$_{cen}$ residue$^{29}$, similar to the conformation found here. The GLU$_{ex}$ residue adopts the same rotameric state in $C_{oi}$ and $C_o$, positioned directly in the way of the ion conduction pathway, as is found commonly in known CLC structures. This observation suggests that the outer gate is “closed” for the $C_{oi}$ and $C_o$ states.

Together, tIC 1 and tIC 3 separate two unique rotameric states of the GLU$_{ex}$ residue. Note that along these tICs, the S167-G169-I170 backbone is “out”, as can be seen by the outward orientation of the S168 side chain. As revealed by our model, accessibility to the $U$ rotameric state is possible only when the GLU$_{ex}$ residue is protonated. As visualized in Fig. 7 middle and bottom, states $O$ and $U$ describe rotamers of GLU$_{ex}$ where the side chain is no longer resting in the exterior ion binding site (this ion binding site is occupied by an ion for the $O$ state). The structures of the $O$ and $U$ states therefore represent two unique outer gate conformations.
Figure 7. Structural analysis of the major free energy basins along the top reaction coordinates illustrates the conformational changes involved in CLC-2 activation. For each coordinate, the transition state conformation is shown in gray. The conformational changes involve rotation of the S168, G169, and I170 residue backbone (tIC 0), as well as the rotameric flip of the GLU$_{ex}$ residue (tIC 1, tIC 3).
The structures identified for the major conformational states were arranged into a schematic network mechanism, given in Fig. 8. This network diagram summarizes the results presented in Fig. 7. We observe that there is a dominant motion from the $C_{oi}$ to $C_o$ state, which involves the rotation of the S168-G169-I170 backbone. Following this motion, the GLU$_{ex}$ side chain may rotate away from the ion conduction pathway. This GLU$_{ex}$ flip has a low equilibrium population under these conditions (zero millivolts), such that return to the resting GLU$_{ex}$ conformation is favored. The $O$ rotamer possibly activates the channel to the full conducting state, while the protonation-dependent $U$ rotamer constricts the conduction pathway to a non-conducting state. This hypothesized non-conducting state is consistent with experiments demonstrating the state-dependent effect of pH on the CLC-2 ion channel. Under a constant hyperpolarizing stimulus of -100 mV (to open the channel), application of pH 5.5 external buffer (to favor GLU$_{ex}$ protonation) followed by a depolarizing pulse to close the channel (while GLU$_{ex}$ remains protonated) renders the channel unable to re-open upon application of a second -100 mV stimulus. In contrast, the same experiment performed under basic conditions (pH 8.0, favoring GLU$_{ex}$ deprotonation) reduces current overall but does not prevent the channel from re-opening, suggesting that GLU$_{ex}$ protonation locks the channel in a unique non-conducting conformation that prevents immediate reactivation. This observation is consistent with our model in which the non-conducting $U$ conformational state of CLC-2 is only accessible when GLU$_{ex}$ is protonated.

Figure 8. Model of CLC-2 activation. Dashed rates signify transitions only accessible when GLU$_{ex}$ is protonated. The S168-G169-I170 backbone may exist in two possible conformational states. One points toward the ion conduction pathway, the other has the S168 side chain intracellularly solvent-exposed. The GLU$_{ex}$ residue side chain may exist in three possible conformational states. In the closed state, GLU$_{ex}$ rests directly in the S$_{ext}$ ion binding site, obstructing the ion conduction pathway. When GLU$_{ex}$ is protonated, or an ion permeates close to the residue, it flips outward. In the $O$ (putative open) state, GLU$_{ex}$ moves to the side, opening up space for a chloride ion. When GLU$_{ex}$ is protonated, the selectivity filter can adopt a third conformation (the $U$ state), which constricts the permeation pathway, to a non-conducting state.
Summary and conclusions

We have applied computational modeling methodologies to predict the conformational dynamics of CLC-2 activation. Four major conformational states were identified and characterized with respect to thermodynamics, kinetics, and structure. Of the major states, two were found to be highly stable, and two to be less stable but still detectably populated the simulation conditions (zero millivolts). Progression through these states was found to follow a particular sequence. First, from the $C_{ex}$, or closed state, there is flip of the S168-G169-I170 backbone. This flip occurs via an intracellular rotation and results in a volume increase in the inner regions of the ion conduction pathway. Once this occurs, the GLU$_{ex}$ side chain is able to flip. Our model suggests that intracellular gate opening (mediated by the intracellular rotation of SER$_{cen}$) must occur prior to opening of the extracellular gate (rotation of GLU$_{ex}$). Such a mechanism is consistent with the picture emerging from mechanistic electrophysiological studies, which indicate that intracellular Cl$^-$ is the main factor driving CLC-2 channel opening$^{69}$. The intracellular rotation of the S168-G169-I170 backbone that we observe would allow intracellular chloride to access the inner channel pore and facilitate opening of the outer glutamate gate by forcing GLU$_{ex}$ into its open conformation as chloride traverses the membrane.

Our studies of GLU$_{ex}$ protonation reveal consistencies with the existing literature, and suggest potential areas of new investigation. Our observation that protonation of GLU$_{ex}$ is not required for opening is consistent with functional studies of CLC-2$^{69}$, as is our observation that protonation of GLU$_{ex}$ decreases the rate of transition from open to closed conformations$^{70}$. Our observation that protonation of GLU$_{ex}$ introduces the ability to adopt an alternative rotamer orientation suggests a potential link between CLC-2 inhibition by extracellular protons (which also involves an extracellular histidine residue$^{87}$). Given the complex relationship between H$^+$, Cl$^-$, and voltage in regulating CLC-2 gating, many questions remain. This CLC-2 activation model will complement current and future efforts toward furthering the biophysical understanding of CLC proteins.

Methods

Molecular dynamics

The initial structures consisted of a CLC-2 homology model built using the software Modeller$^{88}$. The rat CLC-2 monomer sequence was mapped onto a monomer of the 3ORG structure. The membrane system was assembled via CHARMMGUI$^{89}$. The TIP3P$^{90}$, lipid14$^{91}$ and AMBER14SB$^{92}$ forcefields were used. Equilibration was done using AMBER 14*, with restraints on the protein and lipids, which were slowly released as the system was heated gradually from 0 to 300 K. An additional 100 ns simulation was run, from which initial system configurations were sampled. The AMBER prmtop and inpcrd files of these structures were used to set up OpenMM$^{93}$ simulations in the NPT ensemble. From these initial structures, 100 unique velocity distributions were assigned. The distributed computing platform folding@home$^{94}$ was then used to perform 600 $\mu$s of aggregate MD simulation. The UCSF Chimera program$^{95}$ was used to visualize the MD trajectories and generate figures.

Markov state modeling

MSM analysis was accomplished using MSMBuilder 3.7$^{96}$. The MD datasets were featurized using the signed $\phi$, $\psi$, $\chi_1$, and $\chi_2$ dihedral angles for all residues in the CLC-2 selectivity filter. The SF residue numbers are as follows (using the rCLC-2 primary sequence numbering): 167, 168, 169, 170, 171, 209, 210, 211, 212, 213, 463, 464, 465, 466, 467, 468, 559. The featurized datasets were transformed using kinetic-mapping of all tICA tICs. The transformed data was clustered into 324 microstates via the mini-batch kmeans method. The same tICA model and state decomposition was used for both datasets. 100 bootstrap MSMs were constructed (with a lag-time of 28.8 ns), from which the MLE model has been highlighted. Error bars for the free energy estimates were calculated using all MSM models. Macrostates were determined using the PCCA+ algorithm. A continuous time-rate-matrix MSM was used to estimate the kinetic rates of macrostate conversion. The Caver 3.0 software package$^{97}$ was used to calculate the pore radii for each macrostate.

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Author contributions statement

All authors designed the experiments. K.A.M. performed the research. M.M. and V.S.P. supervised the research. All authors edited and reviewed the manuscript.

Additional information

VSP is a consultant and SAB member of Schrodinger, LLC and Globavir, sits on the Board of Directors of Apeel Inc, Freenome Inc, Omada Health, Patient Ping, Rigetti Computing, and is a General Partner at Andreessen Horowitz. Other authors declare no competing financial interest.