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Mapping the gating and permeation pathways in the voltage-gated proton channel Hv1

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Abstract

Voltage-gated proton channels (Hv1) are ubiquitous throughout nature and are implicated in numerous physiological processes. The gene encoding for Hv1 however was only identified in 2006. The lack of sufficient structural information of this channel has hampered the understanding of the molecular mechanism of channel activation and proton permeation. This study uses both simulation and experimental approaches to further develop existing models of the Hv1 channel. Our study provides insights into features of channel gating and proton permeation pathway. We compare open- and closed-state structures developed previously with a recent crystal structure that traps the channel in a presumably closed state. Insights into gating pathways were provided using a combination of all-atom MD simulations with a swarm-of-trajectories with the string method for extensive transition path sampling and evolution. A detailed residue-residue interaction profile and a hydration profile were studied to map the gating pathway in this channel. In particular it allows us to identify potential intermediate states and compare them to the experimentally observed crystal structure of Takeshita *et al*[1]. The mechanisms governing ion transport in the WT and mutant Hv1 channels were studied by a combination of electrophysiological recordings and free energy simulations. With these results we were able to further refine ideas about the location and function of the selectivity filter. The refined structural models will be essential for future investigations of this channel and the development of new drugs targeting cellular proton transport.

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Introduction

Voltage-gated proton channels were first discovered in the 1980's in snail neurons, where they play a role in extruding protons and controlling intracellular pH[2]. The function of the voltage-gated proton channel in the immune system has since been well established, where it plays an important role in charge compensation during the respiratory burst to maintain the NDAPH-oxidase mediated reactive oxygen species (ROS) production [3–5]. The molecular identity of the voltage-gated proton channel (Hv1), however, was not discovered until 2006 [6, 7]. Hv1 knock-out mice display impaired bacterial clearance, confirming Hv1 function in immune response [8]. Hv1 is categorized into the super-family of voltage-gated ion channels due to sequence homology. However, compared to other voltage-gated ion channels, Hv1 displays significant differences in its quaternary structure, organization of permeation pathway, and mechanism responsible for selective ion transport. In a classic tetrameric voltage-gated potassium (Kv) channel, four subunits come together to form a centrally located permeation pore [9, 10]. Each subunit in a Kv channel has 6 transmembrane domains (S1–S6) and is composed of two parts: S1–S4 forms the voltage-sensing domain (VSD) and S5–S6 is the pore-forming domain. In contrast, Hv1 is composed of only two subunits, containing only the VSD (S1–S4). Unlike other traditional water-filled pore containing ion channels, Hv1 does not have an obvious pore structure and the protons have been suggested to permeate through each of the two VSDs [5, 11, 12].

There are three conserved positively charged arginines in the S4 segment of Hv1. Membrane depolarization changes the positioning of S4 as though it were the voltage sensor[13]. Several groups have reported different models of the open-state structure of Hv1. The main difference of these models is the positioning of the positively charged S4 segment relative to other transmembrane domains [14–18]. Unlike the open state, there have been few models of the structure of the closed state. In the course of our earlier investigation, we proposed a closed-state model [14]. Our closed- and open-state models are consistent with our experimental data and clarify several aspects of the molecular mechanism underlying voltage-dependent gating of Hv1 channels. Recently, Takeshita *et al*[1] reported a putative closed-state crystal structure of the mouse voltage-gated proton channel, mHv1, which is in general agreement with our model (Fig. 1). Furthermore, in our prior work we modeled the gating behavior of the proton channel using Targeted Molecular Dynamics (TMD) [19]. While this method was sufficient to identify the large-scale movements of the channel, the individual salt-bridge formations were probably insufficiently represented due to the short time-span of the gating simulation, 100 ns. We here further improve this crude model and gain insight into the progression of salt bridge formation and breaking that occurs during gating by using a more rigorous, longer-time-scale sampling technique to propose a more definitive progression for the transitions from the closed state to the open state.

The protons have been suggested to be conducted in a hydrogen-bond chain mechanism through each VSD in Hv1 channels [5, 11, 12]. Hv1 is very selective for protons and the selectivity was proposed to be determined by a single negative charge D112 in the S1 transmembrane domain by the DeCoursey group [16]. The Isacoff group showed shortly thereafter that this residue is only part of the selectivity filter and a positive charge on the S4 transmembrane domain, R211, is also involved [20]. Both studies indicate the important role

of D112 in the proton permeation, but the molecular mechanism of proton permeation in Hv1 is still unclear. Due to the high proton selectivity of Hv1, the proton permeation pathway is believed to be very constricted [14]. Morgan *et al* [21] recently observed that single or double mutations of select residues believed to be in the pore of the channel appear to change the proton channel to be selective for Cl^- ions. They proposed that these residues (D112 and V116 in hHv1, and D160 and V164 in *Ci*-Hv1; the latter notation will be used throughout this paper) must be part of the ion selectivity filter since mutation of the D112 site destroys perfect proton selectivity and in some cases the channel becomes selective for Cl^- . In this paper, we further study the permeation mechanisms using both experimental and simulation approaches.

Results and Discussions

Swarm-of-trajectories with the string method suggests two transitions during Hv1 activation

To investigate the gating path between the open and closed states of the voltage-gated proton channel of *Ciona intestinalis*, *Ci*-Hv1, and the change in the hydration of the central pore, we used the swarm-of-trajectories with the string method. The same strategy was used recently with considerable success to study conformational transitions in a number of secondary amino-acid transporters and the Shaker K^+ channel [22–24]. In the swarm-of-trajectories with the string method [24], an initial transition path was identified using the 100ns long targeted molecular dynamics calculations that we reported in our prior paper. This path was then partitioned into 41 separate windows, such that the root mean squared difference between the Cartesian coordinates of the backbone atoms was separated by more than 0.2 Å from the neighboring window. These windows were then allowed to evolve under constraints, the final results of which were 41 separate trajectories, representing portions of the transition pathway from the closed to the open state. For the purpose of this paper, we will refer to each portion of the transition pathway as a “window,” which are numbered from 0 (closed state) to 40 (open state). The swarm-of-trajectories with the string method simulation suggests that the gating transition from the closed to the open state in our Hv1 model involves two distinct transitions. The first transition (TS1) appears around window 14 and the second transition appears around window 29 (TS2) (see Movie M1 in Supporting Information, and Fig. 2A).

During the first transition (TS1), S4 moves perpendicular to the plane of the membrane (Fig. 2A), largely independent of motions by the other transmembrane regions. In addition, S4 moves into place adjacent to S3 and forms new bonds. There is little change in the salt bridges between residues of the S1–S3 domains during this period. In the second transition TS2 (Fig. 2A), S1–S2 and S3–S4 pairs both tilt slightly relative to the axis of the membrane. Due to the simultaneous movements of all the domains in this second transition, the S4 domain, as well as the other domains, form new salt bridges (Figs 2B–2C). The gating behavior observed in the relaxed string path further supports the general scheme proposed earlier from TMD studies [14]. The primary motion of the proton channel upon gating appears to be the movement of the S4 domain. The interaction energies between positively charged residues involved in stabilization of the open and closed states and other charged

residues of the proton channel are shown in Fig. 3 (K205 – Fig. 3A, R211 – Fig. 3B, R255 – Fig. 3C, R258 – Fig. 3D). The role of R255 and R258 in S4 in this process of transitioning from the closed to the open state appears more active than the roles of K205 and R211, which serve to maintain connections between the S1, S2, and S3 domains. We did not get sufficient sampling of the interactions between third charge in S4, R261, and the other residues of the channel and, therefore, we cannot provide conclusive analyses for R261. In the closed state, R255 engages D222 and E201 in salt bridges (Fig. 3C), in direct competition with K205 (which is apparent from the significant repulsion between the two cationic residues). These interactions with R255 disappear near window 7 and are replaced by weak attractive interactions of R255 with D160 and D233, which grow stronger and stabilize around window 18. In contrast, R258 remains in a stable salt bridge with D222 and in competition with K205 for a considerably longer span (Fig. 5D). This mechanism might be likened to the crawling of a caterpillar, where the leading feet, R255, stabilize S4 while the trailing feet, R258, remain anchored and only move during the second transition. Beginning around window 28, R258 changes its bridging pairs and engages D233 and D160 in salt bridges. Meanwhile, as the interaction of R258 with D160 becomes stronger, around window 38, the interaction between R255 and D160 becomes weaker. Simultaneously, the interaction between R255 and E167 becomes stronger (not shown). The remaining two cationic residues, K205 and R211, form the primary salt bridges between the S1, S2, and S3 domains (Fig. 2B). While they do exhibit changes in the salt bridges between the closed and open states, these changes appear to be fewer than those involving the S4 domain. In the first transition near window 14, the stabilizing interactions between basic residue K173 in S2 and acidic residues E167 in S1 and E243 in S4 are weakened by competition with D160 (not shown). During the first transition, there is a rearrangement in the primary stabilizing salt bridges between the basic residues K205 and R211 and the acidic residues E201, E219, and D222 (Fig. 3A–3B, and Fig. 2B). These residues are involved with stabilizing the S2 and S3 helices, which move very little relative to one another between the closed and open states. During the second transition, these displaced bridges between the S2, and S3 domain return to their original state. During the time between the first and second transitions, the interaction between K205 and E219 becomes stronger and then, after the second transition, the interaction returns to the same energies as before the first transition. Concurrently, the R211-E219 bond is strong before the first transition and after the second, but is weak between the two transitions (Fig. 3B). This apparent change in the interactions of K205 with two residues that are merely separated by a single turn may in part be to allow the S2 and S3 helices to move slightly in the course of gating and to counterbalance the repulsion between K205 and R255 in the closed state.

Crystal structure of mHv1 is similar to intermediate state of Ci-Hv1 model

To directly compare the structure of our model system with that of Takeshita *et al* [1], we created a homologue of mHv1 using the sequence of residues for Ci-Hv1. The structure of the crystal homologue was built using the SCWRL4 program [25]. We used the alignment described in our prior paper, which is based on the one Musset *et al* [16] used. However, in our model, the first arginine in S4, R255, aligns with R312 rather than with R315 in the Kv1.2–2.1 channel. This is analogous to the sequence alignment reported by Gonzalez *et al* [13]. Having built this homologous model, we compared it to the structures of the open,

closed, and intermediate states derived from the transition path computed using the swarm-of-trajectories with the string method. The root-mean squared difference (RMSD) fit between the structures was used only for the positions of the C α -backbone atoms of the four transmembrane helices, to avoid ambiguities related to poorly resolved coordinates of flexible loops, linkers, and side chains. The overlap between the crystal structure homologue and the best fitting intermediate state is shown in Fig. 1.

When the structures are aligned using the minimum RMSD across all 4 domains, the average error across all the windows was ~ 5.5 Å (Fig. 4A). However, the error in the alignment drops dramatically when just the S1 and S3 pair is considered (Fig. 4B). The S2 and S3 pair, however, maintained a RMSD comparable to that of the RMSD of aligning all 4 domains (Fig. 4B). Further testing revealed that removal of S2 alone and alignment of the two structures using the S1, S3, and S4 domains was sufficient to eliminate the majority of the difference between our model and the crystal structure. Fig. 4A shows that the RMSD between the crystal structure and homology model using only these three domains is ~ 2.3 Å at the minimum. More interestingly, the minimum in the overall fit (best match between modelled and X-ray structure) appears to be an intermediate state between the open and closed states. Figure 4 shows the overlap between the crystal homologue and a structure with the minimum RMSD over all four helices taken from window 29, corresponding to an intermediate state in the gating cycle. From Fig. 1, it is apparent that the overlap between the S2 domains, however, is poor. This is very likely due to the chimeric protein used in the crystallization. In the crystal structure, the S2–S3 loop and a significant part of the S2 and S3 transmembrane helices of Hv1 are replaced by the corresponding segment from VSP (voltage-sensing phosphatase). The offset appears to be ~ 4 residues or 1 turn of the helix. The primary effects of an offset in the S2 domain would be in the packing and interactions of F198 and E201 with residues in the other domains.

We observe some weak interactions between D160 and D233 and R255(R205 in hHv1) in the closed state that become significant around window 29, the window we identify as most resembling the crystal structure of Takashita *et al*[1]. Interestingly, it should be noted that our earlier double-mutant cycle analysis shows that the interactions between D233 and R255 tend to stabilize the open state rather than the closed state[14], suggesting that the crystal structure may be an intermediate state more closely resembling the open state.

Intermediate states are more hydrated than closed and open states

One of the key concerns regarding proton-conducting channels is the existence of the proposed hydrated pockets involved in the proton hopping. To investigate hydration properties along the transition path, we analyzed several metrics of the degree and locations of these hydration pockets. The time-averaged number of waters present in the channel within 5 Å of F198 is shown in Fig. 5A. It is apparent from the figure that the open state contains more water molecules than the closed state. Surprisingly, the degree of hydration is found to be highest in the intermediate states. Most notably, there appears to be an increase in the number of waters present in the channel between window 7 and window 14. This may in part be due to the fact that this period also correlates with discontinuities in the interaction energies of salt bridges of several key residues responsible for stabilizing the structure of the

channel. This is particularly evident with the positive charges in the S4 domain (Fig. 2, Fig. 3). Similarly, we see a reduction in the average hydration number in the channel from 11 waters to 8 waters following the second transition (Fig. 5A), corresponding to reformation of salt bridges in the channel.

In the course of investigating the role that hydration might play in the process of gating, we tabulated the water occupancy in the channel for each window. It should be noted that in Figs. 5B and 5C the 0 of the z-axis corresponds to the average position of the side chain of F198, which we have shown corresponds to the center of a consistent constriction in the center of the pore domain [14]. However the position of F198 relative to the center of the membrane does vary by ~ 5 Å between the closed and open states. The hydration profiles, calculated as the number of water molecules present in 1 Å bins along the z-axis, of the Hv1 channel indicate that the central pore region does exhibit a constriction (Fig. 5B). However, for intermediate windows 14 and 29 there are a significantly larger number of waters present in and around the central constriction.

In the closed state, there are two plugs in the center of the subunit that exclude water molecules. The first dry region is formed by a cluster of hydrophobic groups in S1 and S4 and the aromatic side chain of F198, as proposed previously [14]. The second dry area is formed by a ring of charged residues located at the mouth of the pore on the cytosolic side. These residues are the same that form the strong salt bridges that stabilize the closed state. In the open state, however, most of the hydrophobic residues have moved out of the center of the subunit leaving only F198 as the constriction. Analysis of the probability of being dry is shown in Fig. 5C, which measures the fraction of the total number of time steps in a given trajectory for which a water molecule is present in a given 1 Å bin along the z-axis. Window 0, closed state (CS), and window 40, open state (OS), both have a constriction with a low probability of hydration. However, TS1 and TS2 states of the system have a significantly higher probability of hydration, indicating that there are waters present even in the central constriction for substantial periods of time. This indicates a much higher likelihood of a continuous water wire forming in the channel. Further analysis of the breaks present in the central region of the pore (Fig. 5D) show that both the open and closed states possess wide dry regions of ~ 5 and ~ 7 Å, respectively, whereas the other windows exhibit smaller breaks and in the case of window 20 (not shown) continuous water wires. However, since these are intermediate states, it is difficult to infer whether continuous water wires contribute in any way to the overall ion conductivity of proton channels. However, the presence of these waters in the channel may indicate a secondary function of water in pore. Namely, water may serve to stabilize the unpaired charged side chains present in the pore domain during the conformational rearrangements and so to speak “lubricate” the transition.

Interaction between D160 and R261 is important for ion permeation

To provide further insights into the organization of the open state in the Hv1 channel, we investigated several mutant channels that are capable of other (than proton) ion transport. As has been shown by previous studies [14, 20], the selectivity filter/bottleneck of the pore domain of the voltage-gated proton channel is most likely composed of the interaction between D160 (D112 in hHv1) in S1 and R261 (R211 in hHv1) in S4 in the open state. Our

molecular dynamics modeling also showed strong electrostatic interactions between these two residues [14]. Here we further characterize how these two residues play roles in ion permeation using electrophysiological recordings. First, we did cysteine mutations on these two residues individually in *Ci*-Hv1. Single mutations of D160 (D160C or D160A) did not yield any functional current (e.g. D160C in Fig. 6A top). We have previously shown that fluorophores attached to S242C in S4 allow us to measure S4 movement in *Ci*-Hv1 channels [13, 26]. Combining D160C with S242C in S4 allowed fluorescent labeling of the channels (Fig. 6A bottom). In response to voltage steps, the fluorescence changed in the D160C/S242C channels (Fig. 6A), as if the voltage sensor S4 still moves in response to changes in voltage. This indicates that the D160C mutation prevents the ion conduction, but not membrane expression or S4 movement of this mutant channel. However, the S4 movement is slowed down. In addition, the second channel transition, which is represented by the hook in the fluorescence signal upon membrane repolarization in *Ci*-Hv1 channels[26], is not seen in this mutant. This suggests that besides a slowing of the outward movement of S4, the channel opening transition is also impaired in the D160C mutant.

The addition of mutation R261C to D160C rescued the channel activity from D160C alone (Fig. 6C–D), suggesting that the interaction between D160 and R261 is important for proton permeation or the opening transition. Mutation R261C shifted the voltage dependence of channel opening to a more depolarized voltage range, while the voltage dependence of voltage sensor movement was not affected (Fig. 6B). The double-cysteine mutant preserved part of the pH sensitivity of Hv1: 0.7 unit of pH change shifts the voltage dependence of channel opening about 16 mV (Fig. 7A). However, the reversal potential of this mutant did not change significantly (Fig. 7B and insert), which indicates that the proton is not the predominant permeant ion in this mutant channel. Therefore, we further investigated the selectivity of this mutant using the classic sucrose dilution approach [16], where 90 percent of the extracellular solution is replaced by isotonic sucrose. In sucrose-diluted solution, the reversal potential of D160C/261C is shifted negatively compared to in regular ND96 solution (Fig. 7C–D and insert), showing that this mutant channel is more cation selective. This mutant is Na⁺ permeable: when 90 percent of the Na⁺ ions in ND96 is replaced with Choline, the reversal potential is shifted negatively to the similar extent as in the sucrose-diluted solution (Fig. 7E–F and insert). To further test whether this mutant channel still conducts protons, we performed BCECF experiment. BCECF is a pH sensitive dye and therefore can detect the pH changes caused by proton currents. We loaded the Hv1 expressing cells with 50uM BCECF and measured the fluorescence signal upon voltage clamping. Depolarization-induced fluorescence changes were seen in the WT channel, but not in the non-conducting D160C mutant (Supplementary Fig. S1A–B). The fluorescence signal from the D160C/261C double mutant shown in Supplementary Figure S1C indicates that this channel still conducts proton current. Therefore, the double mutant is a cation channel that allows both sodium and proton permeation.

D160C/R261C double mutation creates a Na⁺ binding site in Hv1

To characterize the energetics of ion permeation in some of the mutants described above, we calculated the free energy changes for ion transport across the open state of wild-type (WT) and mutant channels (D160A, D160C/R261C and D160V/V164E in *Ci*-Hv1). Unlike the

WT hHv1 channel, which is purely proton selective, the D112A (corresponding to D160 in *Ci-Hv1*) single mutant pore has been reported to result in an anion-selective channel [16, 21]. Therefore, we calculated the free energy changes for chloride transport in WT and mutant *Ci-Hv1* channels. The resulting PMFs for Cl^- are shown in Fig. 8. The highest barriers for the WT and the mutant channels are near the position of F198. In the WT, there is an energy barrier with a height of ~ 30 kcal/mol. The D160A mutation increases the height of this barrier, whereas the D160V/V164E mutation increases the width of this barrier (Fig. 8). The double-cysteine mutant (D160C/R261C) decreases the energy barrier, relative to the WT, to ~ 10 – 15 kcal/mol (Fig. 8).

We also studied the PMFs for Na^+ transport (as shown in Fig. 9). For the WT, the energy barrier is in accord with results reported by the Pomes and DeCoursey laboratories [15, 21]. However, the D160C/R261C double mutant changes the PMF for single cation transport dramatically. In a sharp contrast with Cl^- PMFs, D160C/R261C displays a very pronounced binding site (~ 14 kcal/mol) for a Na^+ , suggesting Na^+ permeation in agreement with the electrophysiological recordings reported above. We performed contact analysis to identify residues responsible for stabilization of the permeant cation. At $z = -19$ Å, the electrostatic interactions from K205 and H145 create a barrier for sodium entrance (Supplementary Fig. S3). Crossing over the barrier, sodium ion binds to a very favorable site at $z = -8$ Å. In contrast to the WT channel which forms an extended, essentially “dry” region from $z = -8$ Å to $z = 1$ Å, the double mutant (D160C/R261C) allows for a large water-filled cavity and the formation of a binding site capable of ion accommodation (Supplementary Fig. S2). The bound cation coordinates E201, N264, D222 and 3 water molecules (Fig. S3). As it was pointed out above, the D160C/R261C double mutation allows for a more hydrated pore and effective screening of electrostatic repulsion due to H145 and K205. In the double mutant, permeant cations remain semi-hydrated (~ 3) even near the constriction zone centered at F198. We observe that the central peak, corresponding to the position of F198, is shifted towards the extracellular surface in the D160C-R261C mutant relative to the WT and the side chain of residue 160 lies on the extracellular side of the filter (Fig. 9). Partially, this shift may be due to reduced steric pressure from the side chain of residue 160. Moving deeper, the ion loses most of the water molecules but remains well coordinated by residues in the channel near $z = -1$ Å, offsetting the dehydration penalty. Subsequently, the ion crosses another barrier, caused by the low coordination at $z = 7$ Å. Given the multi-ion cooperative mechanisms observed in voltage-gated sodium or potassium selective channels, multi-ionic effects may also be important in permeation mechanisms enabled in the proton channel mutants [27–29], thus limiting accuracy in resolution of barrier heights. Nevertheless, the drastically different PMF profiles for Na^+ and Cl^- in WT and D160C/R261C provide an excellent correlation with experimental observation of preferential Na^+ transport in the double mutant and provides additional evidence for the usefulness of a Hv1 model developed using a combination of computer modeling and double-mutant cycle analysis[14].

Mutations alter hydration distribution and pore width

To further investigate the role of constrictions in the channel, we analyzed the hydration properties and pore width of the wild-type (WT) and the mutant channels (D160A, D160C/

R261C and D160V/V164E). To obtain proper sampling of the structures of these channels, we performed 100-ns equilibrium simulations on all 4 channels. These simulations were set up using CHARMM-GUI[30]. As with the channels modeled using the swarm-of-trajectories with the string method, we used 3 different metrics to investigate the hydration of the channel. The position of F198 however is sensitive to the identity of the residues in the selectivity filter, which mutations of D160 perturb. To provide a consistent axis for comparison of the pore properties, we set the value of $z = 0$ to be the average position of the backbone atoms of the four helical domains, S1–S4, of a representative structure taken from the equilibrium simulations of the WT channel. All frames and trajectories for the WT and mutants were aligned to the positions of the backbone atoms of the S1–S4 domains of the representative structure.

The hydration properties of the WT and mutants in the open state are shown in Fig. 10A–C. In all cases, the number of waters remains comparable to that of the open state of WT, as opposed to the intermediate states of the WT channel during gating. D160A, which is non-conducting, shows very little water between positions +5 and –5 Å (Fig. 10A). This barrier to water (Fig. 10B, D160A) is comparable to that of the closed-state structure both in “dryness”, ie absence of water in the channel, and the width of the dry region. Fig. 10B shows the fraction of sampled structures without a water present inside the channel at a given position along the z -axis, termed here as “dryness”. The samples are computed using 1 Å bins along the z -axis where any water O-atom present in the bin counts as 1 water. In the two conducting mutants, the dry regions are shifted towards the extracellular surface relative to the wild type. The “driest” point in the channel shifts from –17 (WT) to –15 (D160V-V164E) and –13 (D160C-R261C). Fig. 10C shows the “width” of the break in the water wire. The width is measured as the number of consecutive bins without a water molecule present. For the WT channel, the most frequent width for the dry region is around 4 Å. The distribution of widths has a long tail with a maximum width of 22 Å. This is comparable to the equilibrium simulations for the open state reported previously [14]. The two functional mutant channels, D160V-V164E and D160C-R261C, exhibit broader distributions of widths for the dry region averaging around 10 Å. It should be noted that these distributions have significant representation of frames with much narrower widths for the dry region, potentially allowing for the transport of protons by side chains that could not span the entire 10 Å. However the non-functional channel, D160A, exhibits a distribution with an average width of 14 Å and with very little representation below 6 Å, which is the same as that of the closed state. This suggests that the width of the dry region plays a role in the ion permeation across the channel. However, it should be noted that even the narrowest width, 4 Å, still represents a significant barrier for ion transport.

The single substitution of D160A likely increases the barrier to positive charges, given the loss of a negative charge to counterbalance the positive charge on R261 in the selectivity filter. This increased barrier, along with the significantly larger width of the dry region around the selectivity filter, likely accounts for the non-conducting behavior of D160A. To rescue function, it is necessary to either neutralize the charge on R261, as in the case of functional channel D160C-R261C, or to substitute a negative residue at another site present in the selectivity filter, for example V164E (V116E in hHv1[21]). However from the hydration analysis, it seems that position V164 is less ideal, since it increases the width of the dry

region. In conclusion, the presence of a negatively charged side chain is required in the selectively filter to maintain channel function. This side chain appears to reduce the width of the dry region allowing deeper penetration of water into the pore, as well as reducing the barrier to positively charged ions passing through the filter.

The pore radius calculations shown in Fig. 10D further support the idea of a constriction in the pore domain near the position of F198. For the mutants, the constriction, which tracks well with the position of F198, is shifted towards the extracellular surface. However, a second smaller constriction towards the intracellular surface, which roughly corresponds to the position of the second constriction found in the closed state in WT channels, appears to be reduced or disappears in the case of the mutants (Fig. 10D). The significant increase in the overall pore width in the channel likely accounts for the reduction in the barrier to competing cations (Na^+) and the change in the pore selectivity to become cation selective rather than solely proton selective.

Conclusions

In our simulations of the voltage-gated proton channel Hv1, we found that there are two major structural rearrangements, manifested by salt-bridge breaking and reformation, in the transition pathway from the closed state to the open state. These two conformational changes might be the two conformational changes detected previous in our VCF (voltage-clamp fluorometry) experiments on the Hv1 channel [26]. The recent crystal structure of Hv1 reported by Takeshita *et. al.*[1], which is in a presumably closed state, is very close to an intermediated state in our modeling. In addition, we found that both the closed and open states did not exhibit complete water wires. However, we did observe transient water wires in intermediate states when the salt bridges stabilizing the structure were disrupted. We propose that these transient water wires are important for catalyzing the conformational changes during the transitions from the closed state to the open state. In agreement with previous studies [16], we find that an aspartate in S1 (D112 in hHv1; D160 in *Ci*-Hv1) is important for proton selectivity. Cysteine mutation of this residue in *Ci*-Hv1 did not yield anion selective channels, in contrast to in hHv1, suggesting that there are some subtle species differences for the Hv1 channel. The D160C/R261C double mutant showed high selectivity for Na^+ and the PMFs calculation confirmed the Na^+ permeation in this double mutant. It is most likely that the mutations of these two residues create a large cavity that allows the accommodation of larger cations. This result is consistent with previous studies [20] that D160 and R261 are in the narrowest region of the Hv1 channel and might serve as the selectivity filter for Hv1.

Methods

Molecular biology

The *Ci*-HV1 in the pSD64TF vector was kindly provided by Dr. Y. Okamura (Osaka University, Japan) [6]. Mutations were introduced using Quikchange site-directed mutagenesis kit (Qiagen) and were fully sequenced to ensure incorporation of intended mutations and the absence of unwanted mutations (sequencing by Genewiz). In vitro transcription of cRNA was performed using mMessageMachineSP6 RNA Transcription

Kit (Ambion). 50nl of cRNA is injected into the *Xenopus* oocytes two days before measuring.

Electrophysiology

We performed two-electrode voltage clamp (TEVC) recordings as described earlier[13]. Solutions for TEVC contained 96mMNaCl, 2mMKCl, 1mM MgCl₂, 1.8mM CaCl₂, and 100mM HEPES (pH = 7.5). We injected oocytes with 50nl of 1M HEPES (pH = 7.0) to minimize pH changes due to the proton currents. This results in approximately 100mM HEPES in the cytosol. Data were analyzed using pClamp10.2 and Origin 8.6. The voltage dependence of channel activation $G(V)$ was obtained by using fits of $A_2 + (A_1 - A_2) / (1 + \exp((V - V_{1/2})/k))$ to the tail current–voltage relationship, where $V_{1/2}$ is the voltage at which there is half-maximum activation, and k is a slope factor equal to RT/zF ; z is the apparent gating charge and F is Faraday's number. Data were normalized between the A_1 and A_2 values of the fit. A ramp voltage was used to determine the reversal potential of the channels.

Voltage-clamp fluorometry

We performed VCF experiments as described previously [13]. Briefly, we labeled oocytes for 30 min with 100 μ M Alexa-488 maleimide (Molecular Probes) in ND96 solution (96 mM NaCl, 2 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, and 5 mM HEPES (pH = 7.4)). For BECF experiment, we incubate the oocytes with 50 μ M BCECF dye (Life Technologies) in ND96 solutions for 30 min and wash before recording. Fluorescence was monitored through a FITC filter cube: exciter, HQ480/40; dichroic, Q505LP; and emitter, HQ535/50. Fluorescence intensities were low-pass filtered at 200–500 Hz and digitized at 1 kHz.

In silico mutations

A representative structure for the wild-type(WT) open state monomer of *Ci*-Hv1 was taken from results published by Chamberlin *et al*[14]. Mutations in the sequence were introduced using the mutation module of the Maestro Software Suite[31], and the structure was minimized using a built in minimizer. Three mutant forms of the WT were made, D160A, D160C/R261C and D160V/V164E. Subsequently the refined structures were equilibrated in a DMPC lipid bilayer solvated by 150 mM of KCl. TIP3P water model and CHARMM–27 ion parameters were used for the initial equilibration simulations. The solvent–protein–membrane systems were built using the CHARMM–GUI interface of Im *et al* [30]. Each system was equilibrated at 303.15 K and a pressure of 1 A using the Hoover thermostat with the NPaT ensemble. The system was modeled using periodic boundary conditions in a tetragonal box, 60×60×103 Å. An initial staged equilibrium was carried out with gradually decreasing harmonic constraints on heavy atoms. The unconstrained system was then equilibrated for 20 ns. The equilibrated structures were subsequently run for an additional 100ns. All MD simulations were performed with a program suite NAMD ver2.9 [32]. Subsequent analysis of the system was performed using the CHARMM program suite (35b1r1 and 38b1) [33].

Potentials of Mean Force

Free energy profiles for chloride ion permeation were calculated for WT, D160A single mutant, D160V/V164E and D160C/R261C double mutants by one-dimensional umbrella sampling methods, a powerful computational technique that was used with a considerable success in studies of K-channels[34, 35]. Free energy profiles for sodium ion permeation were also calculated for WT and D160C/R261C double mutants. Umbrella sampling simulations in these four systems were performed with harmonic biasing potentials with a force constant of 2.5 kcal/(mol·Å²) or 5.0 kcal/(mol·Å²) along the z-axis. The zero position along the z-axis is the center of mass (COM) of the backbone atoms of residues 157, 198, 225 and 261. The reaction coordinate for each ion was the distance along the z-axis between the ion and the COM. The final snapshots of the conventional MD were employed as the starting conformations for the umbrella sampling. The sampling windows were spaced every 1.0 Å from -47.0 Å to 41.0 Å resulting in 89 windows and the simulation time per window was set to 2ns for 1D PMF computations. Only the last 1.8ns were used to calculate the PMF. The energy profiles were rebuilt with the weighted histogram analysis method (WHAM)[36]. The tolerance for WHAM was set to 0.0001. The statistical uncertainties were estimated by separating the data into five blocks.

Swarm-of-trajectories with the string method

In this work, we applied the recently developed and emerging string method combined with the swarm-of-trajectories to obtain optimal paths connecting the structurally available conformational states of the Hv1 channel developed in our previous work [37, 38]. The string method aims to find the minimum free energy path (MFEP) in the subspace of a large but finite set of coordinates, **z**, referred to as “collective variables”[39]. A path is ordered as a chain of *M* states or “windows”, connecting two stable conformations. In this work, we explore the transition paths between the open and closed states of the channel were considered. To study the conformational transitions of the channel, the collective variables include the Cartesian coordinates of the protein backbone atoms. The initial paths were obtained from 100ns long targeted molecular dynamics as described previously[14]. There were 41 intermediate states, or windows, for the path. The numbers of intermediate states were chosen so that the average RMSD of the collective variables between adjacent windows is less than 0.2 Å.

The iteration of the string method generally followed the procedures by Gan *et al*[37] and refined by Zhao *et al*[24]. Each iteration consists of 4 steps: generation of the swarm-of-trajectories, evolution of the window, run of constraint MD, and re-parameterization. First, for each window, 100 10ps-long MD simulations were carried out. Second, the coordinates of the collective variables for the 100 trajectories were averaged. Third, 750ps of MD simulations were then carried out with a strong harmonic constraint (20 kcal/mol Å) on the collective variables to evolve the collective variables to the average drift and relax the rest of the system other than the collective variables. Finally, the windows are re-parameterized to ensure that they are evenly distributed in terms of collective variables along the new path, which, in our case, means the RMSD difference of the atoms in the set of collective variables between adjacent windows are roughly equal. This final step ensures that the windows are not trapped in local minima.

The Following Ovchinnikov *et al* [40, 41], convergence of the string to the optimal path is evaluated each iteration by monitoring the average RMSD each window has moved from its initial conformation. In addition, we also monitored the average RMSD each window has moved from the same window 4 iterations before. Convergence is assumed when both lines reach a plateau. The transition paths are converged in 20–22 iterations. The path provides 41 relaxed windows for the entire gating cycle. Structures from the path were chosen as appropriate structures for analysis of salt-bridging reorganization, hydration profiles and comparisons to published crystal structure. The fully relaxed path was subjected to production simulation with 10ns per string window resulting in 410ns sampling along transition path.

Pore hydration

The hydration properties of the pore domains were all computed using the 10ns equilibrium runs from each of the windows in the gating simulation. The average number of waters per window, shown in Fig. 5A, was computed as the time-averaged number of water present within ± 5 Å of residue F198. The average number of water as a function of z-position, shown in Figs. 5B, was computed using 1 Å slabs lying in the plane of the membrane. The water molecules were counted based upon the position of the oxygen atom. The slabs ran from -30 to 30 Å centered on the position of F198. The probability being dry for each window, shown in Fig. 5C, is the fraction of frames out of the total analyzed in which no waters were present in a given slab. The histogram analysis of width of the break for each window, shown in Fig. 5D, is the number of consecutive slabs with no water present in them and is plotted against the number of frames in which a given width of the break occurs for a given window.

The hydration properties of the wild-type (WT) and mutant (D160A, D160C-R261C, D160V-V160E) channels were computed using the same approaches as described above with the distinction is that value of $z=0$ was set to be the average position along the z-axis of a representative structure taken from the WT equilibrium simulation. All WT and mutant structures and trajectories were aligned without rotation to the backbone atoms of the S1–S4 domains of a representative structure taken from the open state WT equilibrium calculations. Due to this, the z-axes of the WT and mutants are shifted relative to the axes of the structures from the gating calculations by ~ 10 Å.

Pore radius

The pore radius was computed using 100 structures sampled evenly over 100ns equilibration runs. The radius of the pore was sampled using a dummy atom with variable radius that was placed in the center of the pore in 0.25 Å incremental steps from the 10 to -50 Å along the z-axis. At each step the probe is placed in the 0 of the X and Y axes and the protein was moved such that the probe was in the center of mass of the backbone atoms of the residues within ± 5 Å of the probes position along the z-axis, and the radius of the probe was slowly increased while the positions of the residue side chains are minimized. This process continued until the repulsion between the probe and the surrounding residues either exceeded the 100 kcal/mol cutoff or the radius of the probe exceeded 30 Å. Then the position of the probe was reset in the center of the channel 0.5 Å further along the z-axis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance Statement

Our study reports on basic biophysical principles governing selective ion permeation in voltage-gated proton channels, which are membrane proteins with important roles in immune response and fertility. To further confirm and develop our model we compared it to recently reported crystal structures. To gain further insight into the mechanisms of ion selectivity in these channels were performed in-vivo and in-silico mutations on the channels and investigated their functioning. We found that targeted modifications around the constriction zone formed in an open state of the channel dramatically affects ion selectivity of the channel enabling transport of Na^+ . We also further investigate the gating behavior of the wild-type structures. Our in-silico predictions were confirmed experimentally both with regard to the mutant and the wild-type structures, further establishing the validity of the channel model for future applications in drug development targeting proton channels.

Figure 1. Similarity of *Ci*-Hv1 model and mHv1 crystal structure

The putative closed-state crystal structure of the mHv1 proton channel (19) (**green**) is compared to the closest *Ci*-Hv1 structure taken from our swarm-of-trajectories with the string method (**teal**). The two structures were aligned using backbone carbons in the S1, S3, and S4 helical domains. For the purposes of this comparison, each domain was defined as: S1 = 150 – 164, S2 = 187 – 205, S3 = 220 – 237, S4 = 250 – 262.

Figure 2. Two transitions during Hv1 channel activation

(A) The large scale motions of the different domains in each part of the transition pathway are shown. (B–C) Salt bridges in closed, intermediate, and open states. (B) K205 in S2 and R211 in S3 are shown in blue, whereas E201 in S2 and E219 and D222 in S3 are shown in red. Weak (green) and strong (purple) attractive interactions are shown. (C) R255 and R258 in S4 are shown in blue, whereas D160 in S1, E201 in S2, and E219, D222, and D233 in S3 domain are shown in red. Weak (green) and strong (purple) attractive interactions are shown.

Figure 3. Salt bridges break and (re-)form during Hv1 channel activation

Color map of interaction energies (color coded in kcal/mol) for selected residues pairs graphed against the window numbers starting from the closed state (CS) at window 0, transition 1 (TS1) at window 14, transition 2 (TS2) at window 29, and open state (OS) at window 40 are shown. The charts are ordered by cationic residues. (A – K205, B – R211, C – R255, D – R258)

Figure 4. mHv1 crystal structure is most similar to an intermediate state of the Ci-Hv1 model
 The crystal structure of the mHv1 proton channel from Takeshita *et al*[1] is compared using the root mean squared difference from the closest Ci-Hv1 model structure taken from each window. The closed state (CS) at window 0, transition 1 (TS1) at window 14, transition 2 (TS2) at window 29, and open state (OS) at window 40 are shown on the z-axis. The RMSD was computed based upon alignment of the two structures and tabulation of the RMSDs using solely the specified domains. For example, the S1, S3, S4 fit shown is for the case where the structures are aligned using the three domains and RMSDs are tabulated only for those three domains. In the graphs, the residues included in each domain are as follows: S1 = 150 – 164, S2 = 187 – 205, S3 = 220 – 237, S4 = 250 – 262.

Figure 5. Intermediate states are more hydrated than the closed and open states

(A) Time averaged number of water molecules within 5 Å of F198. (B) Time averaged number of water molecules present in 1-Å bins along the z-axis, where the 0 point is set to be the position of F198 for selected windows. (C) Fraction of the frames out of 5000 with at least 1 water present in 1-Å bins along the z-axis, where the 0 point is set to be the position of F198 for selected windows. (D) Histogram of the number of frames out of 5000 with a given number of consecutive 1-Å bins without any waters present.

Figure 6. R261C rescues currents in D160C mutation

(A) Currents (top) and Fluorescence (bottom) in response to voltage steps between -40 mV and $+120$ mV from a holding potential of -60 mV for D160C channels. (B) Normalized steady-state fluorescence (F) and conductance (G) from wild-type (WT) and R261C/S242C Hv1 channels from experiments as in A. (C) Currents from D160C/R261C channels in response to voltage protocol shown above. (D) Normalized conductance (G) versus voltage for D160C/R261C measured as in C.

Figure 7. D160C/R261C retains pH-dependent gating, but not proton selectivity

(A) Normalized conductance (G) versus voltage measured as in Fig 6C in external pH= 7.5 and 8.2. (B) Currents in response to voltage steps between -40 mV and $+120$ mV from a holding potential of -60 mV in external pH= 7.5 and 8.2. (C–F) Currents in response to voltage steps between -40 mV and $+60$ mV from a holding potential of -60 mV in external (C) 100 mM NaCl (D) 10 mM NaCl + Sucrose, (E) 100 mM NaCl, (F) 10 mM NaCl + 90 mM CholineCl.

Figure 8. Mutations in Hv1 selectivity filter retain barrier to chloride

PMFs for chloride ion crossing wild-type, D160A, D160C/R261C, and D160V/V164E *Ci*-Hv1 channels. The starting and ending positions of the chloride ion relative to the channels are shown as blue spheres on the background. The error bars were estimated from block averaging with five blocks.

Figure 9. D160C/R261C mutations create Na⁺ binding site in Hv1 channel

PMFs for sodium ion crossing wild-type and D160C/R261C *Ci*-Hv1 channels. The starting and ending positions of the sodium ion relative to the channel are shown as blue spheres. The error bars were estimated from block averaging with five blocks.

Figure 10. Mutations of *Ci*-Hv1 channels shift position of selectivity filter

(A) Time averaged number of water molecules present in 1-Å bins along the z-axis, where $z = 0$ is set to be the average position of the backbone atoms of the S1–S4 domains. (B) Fraction of the frames out of 1000 with at least 1 water present in 1-Å bins along the z-axis, where $z=0$ is set to be the average position of the backbone atoms of the S1–S4 domains. (C) Histogram of the number of frames out of 1000 with a given number of consecutive 1-Å bins without any waters present. (D) The computed radius of the pore along the z-axis.