On the role of water density fluctuations in the inhibition of a proton channel

Eleonora Giani*, Lucie Delemotteb1, Michael L. Kleina,2, and Vincenzo Carnevalea,2

*Institute for Computational Molecular Science, Department of Chemistry, Temple University, Philadelphia, PA 19122; and Laboratory of Computational Chemistry and Biochemistry, Institute of Chemical Sciences and Engineering, École Polytechnique Fédérale de Lausanne, CH-1015 Lausanne, Switzerland

Contributed by Michael L. Klein, November 16, 2016 (sent for review June 21, 2016; reviewed by Thomas E. DeCoursey and Sergei Y. Noskov)

Hv1 is a transmembrane four-helix bundle that transports protons in a voltage-controlled manner. Its crucial role in many pathological conditions, including cancer and ischemic brain damage, makes Hv1 a promising drug target. Starting from the recently solved crystal structure of Hv1, we used structural modeling and molecular dynamics simulations to characterize the channel’s most relevant conformations along the activation cycle. We then performed computational docking of known Hv1 inhibitors, 2-guanidinobenzimidazole (2GBI) and analogs. Although salt-bridge patterns and electrostatic potential profiles are well-defined and distinctive features of activated versus nonactivated states, the water distribution along the channel lumen is dynamic and reflects a conformational heterogeneity inherent to each state. In fact, pore waters assemble into intermittent hydrogen-bonded clusters that are replaced by the inhibitor moieties upon ligand binding. The entropic gain resulting from releasing these conformationally restrained waters to the bulk solvent is likely a major contributor to the binding free energy. Accordingly, we mapped the water density fluctuations inside the pore of the channel and identified the regions of maximum fluctuation within putative binding sites. Two sites appear as outstanding: One is the already known binding pocket of 2GBI, which is accessible to ligands from the intracellular side; the other is a site located at the exit of the proton permeation pathway. Our analysis of the waters confined in the hydrophobic cavities of Hv1 suggests a general strategy for drug discovery that can be applied to any ion channel.

Hv1 | drug design | pore waters | binding site discovery | confined waters

The voltage-gated proton channel 1 (Hv1) is a dimeric voltage-sensing protein essential for many cellular functions, including proton extrusion, pH homeostasis, and production of reactive oxygen species (ROS) (1–3). Hv1 plays major roles in controlling B-cell proliferation, human sperm motility, and acid extrusion in lung epithelial cells (4, 5), and it is involved in immunological processes, such as phagocytosis (1, 6) and histamine secretion (7). Unlike other voltage-gated ion channels (VGICs), Hv1 is composed of two identical transmembrane (TM) subunits. Each monomer is formed mainly by a voltage-sensing domain (VSD) and is fully functional (8–10). Therefore, compared with other VGICs, Hv1 lacks the conventional pore domain (Fig. 1 A and B) and proton permeation occurs through each VSD (9, 11, 12). In the context of the dimer, the transition to the conductive state occurs through a cooperative conformational change of the two protomers (13, 14).

The TM region of Hv1 is constituted of four helices, segments S1 through S4 (15) (Fig. 1C). Three arginine residues, called gating charges or gating arginines (R1–R3), are located on S4 and confer voltage sensitivity to Hv1. The other segments (S1–S3) carry acidic residues that serve as countercharges. In other VSDs, these countercharges interact with the gating charges so that the latter can translate and occupy several distinct positions (16–19). Similar to what is observed in other VGICs (20, 21), S4 of Hv1 is believed to respond to a depolarizing pulse by moving along the normal axis to the membrane from the innermost (resting state) to the outermost (activated state) configuration (22, 23). In Hv1, the activated state corresponds to the conductive (open) conformation. Other states along the activation pathway (resting and intermediate-resting) are not conductive (14).

Hv1 activity has been shown to exacerbate severe pathological conditions, such as ischemic brain damage (24), by facilitating the production of ROS. Furthermore, Hv1 is implicated in cancer cell survival (25–27) and is dysregulated in chronic lymphocytic leukemia (28). These discoveries, and the ensuing potential therapeutic strategies, ignited extensive searches for effective Hv1 inhibitors; these efforts led to the identification of positively charged binders of the activated state (8, 29, 30). The lack of available experimental structures, only recently overcome by the discovery of the X-ray structure (31) of mouse Hv1, has motivated the generation of several comparative homology models using various VGICs as templates (32–38). Nonetheless, none of these studies focused on a systematic characterization of Hv1 as a pharmacological target using a structure-based approach for drug design.

Despite being perceived as challenging targets, ion channels are amenable to rational drug design, as shown by recent success stories (39, 40). A prominent example is the M2 proton channel of the influenza A virus, a drug target showing striking similarities to Hv1. In both cases, a bundle of four helices lines a proton conduction pathway, both channels show exquisite proton selectivity (41, 42), and the regulation of both channels is sensitive to pH gradients (43). In addition, positively charged fragment-like molecules, such as amantadine (44) and 2-guanidinobenzimidazole (2GBI) (30), are known to act as pore blockers of M2 and Hv1, respectively. Many groups, including ours, have successfully pursued drug discovery projects with M2 as a target (45–53). In particular, we have recently shown that scaffold replacement of hydrogen-bonded

**Significance**

Hv1, a voltage-gated proton channel, is an emerging pharmacological target implicated in many pathological conditions, including cancer and ischemic brain damage. We used the recently published experimental structure of Hv1 to generate structural models of relevant conformational states. Thermodynamic analyses of pore waters shed light on the molecular underpinnings of Hv1 druggability. We exploit this information to suggest possible optimizations of known inhibitors and identify a potential binding site located at the exit of the proton path. The resulting molecular picture paves the way for the discovery of novel Hv1 inhibitors and outlines a general approach for identifying druggable binding sites in ion channels.

Author contributions: E.G., L.D., M.L.K., and V.C. designed research, performed research, contributed new reagents/analytic tools, analyzed data, and wrote the paper.

Reviewers: T.E.D., Rush University; and S.Y.N., University of Calgary.

The authors declare no conflict of interest.

*Present address: Science for Life Laboratory, Department of Theoretical Physics, Royal Institute of Technology, SE-171 21 Solna, Stockholm, Sweden.

†To whom correspondence may be addressed. Email: mlklein@temple.edu or vincenzo.carnevale@temple.edu.

This article contains supporting information online at www.pnas.org/cgi/doi/10.1073/pnas.1609964114/-/DCSupplemental.
water clusters in the M2 channel is key to optimize ligand binding and selectivity (S4).

Because ion channel modulators can operate at different stages of the activation cycle, we consider the characterization of the Hv1 structural ensemble a prerequisite for the design of pore blockers. Hence, starting from the recently published crystal structure of murine Hv1 solved in an intermediate-resting (closed) state (31), we have combined structural modeling and molecular dynamics (MD) simulations to model relevant conformational states of the human Hv1 monomer: resting, intermediate-resting, and activated states. To generate this structural ensemble, we used the same approach as Wood et al. (33) and Kulleperuma et al. (34): We started from the alignments shown in Fig. 1 and generated initial configurations for MD simulations (SI Appendix, Figs. S1A and Table S1; ∼2.5 μs of total simulation time).

**Salt-Bridge Patterns Define Activation States.** In the VSDs of VGICs, the outward movement of S4 proceeds through successive transitions across well-defined conformational states (19, 20, 56). In contrast to all other VSD examples, Hv1 populates only two major states in our simulations: activated and intermediate-resting states. Indeed, despite the fact that we started the simulations from three structural models showing distinct S4 configurations, the final equilibrated models reveal only two distinct salt-bridge patterns: The first one is common to the resting and intermediate-resting states, whereas the second one is distinctive of the activated state (Fig. 2A–C and SI Appendix, Figs. S1 and S2). The resting and intermediate-resting states show, however, a structural difference: a rearrangement of S3, which causes a change in the position of D112 on S1 (Fig. 2).

A network of interactions involving R2 and R3 characterizes the activated state: Both residues face the channel lumen and interact with D185 and/or D112, the channel’s selectivity filter right at the center of the membrane (57–59), with D112 and D185 being the closest countercharges to R3 and R2, respectively (Fig. 2). Consistent with this picture are the results by Berger and Isacoff (58), who used experimental mutagenesis to show that D112 interacts with R2 and that although both R1 and R2 are readily accessible to Zn²⁺ applied externally, R3 is not, suggesting that R3 faces the intracellular crevice in the activated state. In our simulations, R1 points away from the channel lumen and faces the lipids.

As the S4 segment moves inward toward the intermediate-resting state, all of the gating arginines become involved in salt bridges with acidic amino acids of the protein. In the intermediate-resting state, R3 and R2 are primarily engaged with D174 and E153 and, alternatively, R1 is in interaction with D112 and D185. These observations are in qualitative agreement with the modeling and double-mutant cycle analysis of Chamberlin et al. (35), conducted in Ciona intestinalis (C) Hv1. Indeed, the pairs E119-R1, E119-R2, and D185-R2 (Hv1 numbering) were shown to stabilize the open state weakly, whereas E153-R1, E153-R2, D174-R1, and D174-R2 were shown to stabilize the closed-state conformations (consistent with what is shown in Fig. 2A–C). Furthermore, in general agreement with our model, it was previously suggested that D112 (D160 in C-Hv1 numbering) interacts with both R258 (R2) and R261 (R3), but not with R255 (R1) (35). Also, Berger and Isacoff (58) used experimental mutagenesis studies to show that D112 interacts with R3 in the open state, and that this interaction is crucial to confer stability to the channel (consistent with what is shown in Fig. 2C).
The similarities in the salt-bridge network between previously published models and our model, one of the crucial differences of putative functional relevance is the TM position of the negative countercharges. Specifically, here, D112 and D185, instead of being located in the intracellular and extracellular crevices in previously reported models, are located right at the center of the membrane region, which seems to be compatible with D112’s role as a selectivity filter.

**Conformational Substates Show Distinct Hydration Profiles.** Progressing from the intermediate-resting to activated state, no major wet/dry transitions are apparent (Figs. 2 D and E and 3 and SI Appendix, Fig. S1). In both cases, water penetrates throughout the channel lumen. In particular, two major hydration crevices are located at the innermost and outermost ends of the channel (34, 35, 37), whereas a restricted area (from ~6 to 6 Å along the z axis) is significantly less populated (Fig. 3). Both the size and structure of these compartments are specific to each activation state. In contrast to other known VSDs, each Hv1 transition shows a significant degree of conformational heterogeneity; discrete substates characterized by different structural features, as well as different hydration and electrostatic potential profiles are observed (Fig. 4 and SI Appendix, Figs. S2 and S3 and Table S2). Along the proton permeation pathway of the activated state of Hv1, two main constriction areas of varying width are defined in the proximity of residues S143 (extracellular crevice) and F150 (intracellular crevice). Salt bridges contributed by D112, R2, and D185 are responsible for tuning the former constriction; the latter is modulated by rearrangements of residues F150 and R3 (34). Our simulations suggest that three substates (A1–A3; Fig. 4 and SI Appendix, Results) can be defined on the basis of distances involving the signature residues R3 and F150 at the two constriction sites (34). Interestingly, such conformational heterogeneity is consistent with the electron paramagnetic resonance (EPR) experiments by Li et al. (37), showing that Hv1 is significantly more flexible than other VSDs.

We now describe in detail the conformational ensemble visited in the three A1 to A3 substates. In A1, the polar/hydrophobic ring made of residues V112, S143, L189, and L204 restricts water accessibility at the extracellular crevice. Note that two of these residues, V112 and L185, were previously shown to participate in the formation, in the closed-state mHv1 (60), of a site for Zn$^{2+}$ binding. The polar/hydrophobic ring is crucially located right at the top (extracellular side) of the bundle, toward the exit of the proton conduction path. We observe that the size of the upper ring controls water density, which, in turn, can affect proton conduction. In particular, we notice that water density is affected by the conformation of L189: when its side chain moves away from the lumen, the latter is occupied by water (Fig. 4). In A2, the distance between R3 and F150 is larger than in A3 and the polar/hydrophobic ring is open. This configuration allows hydration of both crevices, intracellular and extracellular, with water molecules populating the entire pore. In A3, movements of residue F150 toward the channel lumen reduce water accessibility at the intracellular crevice. In previous studies (35, 61), F150, a highly conserved phenylalanine among VSDs, was shown to act as the gating charge transfer center and to function as a hydrophobic plug, occluding the pore in the resting state. Our simulations show that F150 can also influence water accessibility in the activated state. Surrounding hydrophobic residues, including F149(S2), M151(S2), F182(S3), V109(S1), and W207(S4), affect the orientation of the F150 side chain. In particular, F182 and M151 establish a sulfur/aromatic interaction at the S2–S3 interface. Switching of the relative orientation of F182 and M151 (F182 moves upward to overhang M151) induces a movement of the S2 helix toward S3 and results in dragging along the bulky side chain of F150, the channel lid. Details of all substates are reported in SI Appendix.

**Electrostatic Potential Favors Conduction in Activated Hv1.** In VDSs, potential sites for cation binding have been extensively described (62). These potential sites coincide with the position of acidic residues on segments S1 to S3, and serve as countercharges for the gating arginines during outward movement of S4 (20). From the intracellular side of Hv1, the first site available to proton binding corresponds to D174. In the intermediate-resting state, D174 is stably engaged in interaction with R3 at the bottom of the intracellular crevice (Fig. 2B and SI Appendix, Fig. S2). During activation, the electrostatic field is reshaped in such a way as to...
favor proton permeation in both the A1 and A2 substates (Fig. 2G and SI Appendix, Fig. S2). Indeed, salt-bridge rearrangements result in freeing D174 and E153 from a positive countercharge, hence generating a configuration of the electrostatic potential that facilitates cation translocation. Conversely, in the A3 substate, the approach of R3 toward F150 induces a sudden increase of the electrostatic potential in the region flanked by R3 and D174, a feature unlikely supportive of proton conduction.

In contrast to hydration profiles, which have been shown to be poor predictors of proton conductance (34), the electrostatic potential identifies the activated state as potentially conductive.

**Pore Waters Bind at Discrete Locations.** In proton channels, water represents the primary vehicle for conduction (63). Hence, the distribution of pore waters is regarded as an important structural feature.

Analysis of hydration profiles of activated Hv1 reveals that the region flanked by F150 in the activated state (between −5 Å and 0 Å) experiences large-amplitude water density fluctuations, a behavior reminiscent of wet/dry transitions observed in solvent confined into hydrophobic environments (64–66). Interestingly, F150 is entirely surrounded by hydrophobic residues (SI Appendix, Figs. S4A and B).

Taken together, these data suggest the presence of a potentially druggable hydrophobic cavity lined by the benzyl moiety of F150 (65, 67, 68). Notably, F150 was shown to constitute a gating charge transfer center in Shaker-like VSDs (61) and a hydrophobic plug in Hv1 (35). As such, the conformation of the F150 bulky side chain depends on the activation state (35).

Because proton hopping occurs along hydrogen-bonded water chains (or water wires), it is crucial to characterize the distribution and persistence of water–water hydrogen bonds. Given the diffusive nature of pore waters, these hydrogen bonds are not necessarily localized: Distinct configurations of the protein can show water wires characterized by different positions of donor and acceptor waters. Thus, an interesting question concerns the possible presence of “hot spots” where hydrogen bonds preferentially occur. These hot spots can be informative of Grothuss-hopping conduction pathways and, possibly, of the mechanism of inhibition by pore blockers.

To identify the preferred location of water–water hydrogen bonds, we used cluster analysis (Fig. 4 and SI Appendix, Figs. S3 and S5). In brief, we treated each hydrogen bond as a diatomic molecule (with the two atoms representing the donor and acceptor atoms); thus, the distance between any two hydrogen bonds is the root mean square deviation (rmsd) between two configurations of a diatomic molecule. The goal of cluster analysis is to partition the set of hydrogen bonds (across all of the configurations of the protein and for all of the water molecules) in groups showing a large degree of internal similarity (using the rmsd as an objective measure). We term such groups “hydrogen-bonded water clusters.” The results of this analysis (shown in Fig. 4B and SI Appendix, Fig. S3) reveals a discrete nature of solvent distribution along the Hv1 channel lumen: Hydrogen bonds tend to be localized in the regions where the most populated clusters are found.

In both intermediate-resting and activated states, cluster distributions show hydration patterns that unambiguously correlate with structurally defined substates (Figs. 4 and 5 and SI Appendix, Fig. S3). In the activated state, major hydration sites span the entire

---

**Fig. 3.** (Left) Density profiles of water (solid lines) and F150 (dotted lines) along the channel pore (z axis). (Top) Intermediate-resting and activated states. (Bottom) Activated substates (A1–A3). (Right) Three-dimensional structure of the Hv1 activated state is shown as a reference. Gating charges (R1–R3), countercharges, and F150 are shown as sticks in blue, red, and white, respectively. As a reference, pink spheres highlight the position of selected alpha-carbon atoms. The center of the axis in the density profiles is the average position of membrane lipids.

**Fig. 4.** Activated Hv1 state. (A) Structural determinants of the substates (1–3) are shown as distance pairs between signature residues. (B) Time series of cluster indicators: For each instantaneous configuration of pore waters, each hydrogen bond is classified and labeled according to the cluster to which it belongs (details about the clustering technique are provided in Methods). Only the 50 most populated clusters are shown.
proton permeation pathway (Fig. 5) and are populated by highly ordered solvent molecules (i.e., hydrogen-bonded water clusters) of varying occupancy. In A1 (Fig. 5 B and F), hydrogen-bonded water clusters are present in the intracellular vestibule between E153 (S2) and D112 (S1). Here, the solvent is surrounded by residues R2 and R3 (S4), F182 and D185 (S3), and F150 (S2). Given its location (i.e., the innermost crevice), we named it the internal site (I-site). In contrast, the extracellular crevice is less hydrated and the small area enclosed by the polar/hydrophobic ring is almost entirely dry (SI Appendix, Fig. S4C). In A2 (Fig. 5 C and G), highly structured solvent molecules are mainly confined toward the exit of the proton permeation pathway, within a compartment between residues D112 and E119 (S1), which we call the external site (E-site). In this substate, a continuous solvent structure penetrates the entire TM segment. In both A1 and A2, disordered waters (i.e., with large conformational entropy) are found in the regions of the pore not already occupied by hydrogen-bonded water clusters (Fig. 5). In A3, hydrogen-bonded water clusters are present in both compartments; however, a dry region is present between R3 and F150 (Fig. 5 D and H).

**Water Wires Are Intermittent.** The mechanism of proton conduction in the Hv1 channel is still a matter of debate (32, 33, 35, 69). Many authors have questioned whether proton translocation is achieved through a Grotthuss-like mechanism supported by continuous water wires spanning the entire TM segment; definitive evidence for this mechanism is still lacking. Our results suggest that water chains connecting the entire TM length of the Hv1 channel are improbable. However, continuous water wires of significant length (up to six consecutive hydrogen-bonded water molecules) are observed that bridge amino acids facing the Hv1 lumen (Fig. 6 and SI Appendix, Fig. S7). Remarkably, the longest upper wire forms in 1% of the frames, and our configurations show this long water chain can be separated by as long as 60 ns (Fig. 6). Continuous chains (22%) also populate the outermost compartment (E-site) and bypass the constriction defined by the polar/hydrophobic ring in the compartment connecting D112–D185 to E119 (SI Appendix, Fig. S7 and Table S3). Interestingly, the locations of the majority of water wires coincide with the sites of larger solvent fluctuations, namely, the hydrophobic pocket and the polar/hydrophobic ring (Fig. 6 and SI Appendix, Figs. S6 and S7); thus, water wires disappear concertedy, a collective behavior resembling diffusion of water in narrow hydrophobic channels (70).

Introducing a charge in the permeation pathway produces only minor perturbations in the solvent structure (SI Appendix, Table S1, simulations 9–11). Here, similar to what was observed in the absence of a charge in the channel lumen, abridged water wires span piecewise across main structural compartments along the proton permeation path and are located primarily where dry transitions occur. However, solvent becomes more structured in the presence of isotropic charge densities (SI Appendix, Table S3). Indeed, when a charge is introduced at the two innermost locations (I-site: E153, C-site: halfway between D112 and D185), water wires are long and frequently occurring (SI Appendix, Fig. S7 and Table S3). Most of the chains form within the I-site, in the proximity of charge density locations (75% and 80%). When a charge is introduced at the I-site, the site connecting E153 to F150 is almost entirely populated by lower water wires (71%). Instead, both lower and upper wires occupy the I-site when the charge density is applied at the C-location (53% and 27%, respectively). Interestingly the former wires, located closer to charge density, are longer and highly recurrent. Conversely, when a charge is introduced at the exit of the gating path (E-location: halfway between E119 and D123), the majority of wires form at the E-site (42%). The remaining waters are distributed between the upper and lower wires (33% and 25%, respectively). The number of water wires is in the lower range and, on average, the water wires connect four water pairs.

**Druggable Pockets and 2GBI Binding Mode.** In activated Hv1, 2GBI and analogs act as pore blockers and inhibit proton conduction
(30). Although double-mutant cycle analysis identified the residues interacting with these blockers (29, 30), experimental structures of 2GBI and/or analogs in complex with Hv1 have not been determined yet. We propose binding hypotheses of the activated state in complex with 2GBI and analogs via molecular docking. We used SiteMap (71, 72) to locate putative binding sites along the channel pore in activated substrates. Interestingly, the two most favorable binding spots were identified close to structural features likely crucial for proton permeation. The first binding pocket opens toward the intracellular crevice (I-site), in the area surrounded by residues E153 to D112, at the interface with F150 and F182 (SI Appendix, Fig. S4B). A second druggable pocket extends toward the extracellular crevice (E-site), and is a smaller hydrophobic area right below E119, the outermost charged residue in the channel lumen. Interestingly, both druggable pockets are located where major rearrangements of the structure of water across distinct substrates (i.e., A1–A3) are observed (Fig. 5 and SI Appendix, Fig. S4C).

We performed induced fit docking of 2GBI against the activated Hv1 state, scanning the entire intracellular crevice for favorable ligand orientations (Methods). The binding mode of 2GBI in activated Hv1 is shown in Fig. 7, and is consistent with double-mutant cycle analyses (30). In the favored orientation, 2GBI binds Hv1 at the I-site in a configuration that occludes the pore (Fig. 7 and SI Appendix, Figs. S8 and S9 and Table S4). A number of hydrophobic residues (V109, L108, I146, I105, and V178) contribute to stabilize the benzimidazole ring that engages in π–π interactions with F150 and R3 (R211). Whereas N1 on the benzimidazole ring (International Union of Pure and Applied Chemistry notation) serves as hydrogen bond donor to D112, N3 accepts a hydrogen bond donated by R3 (R211). Additional interactions involve F182 and the guanidinium moiety. Both D112 and D185 stabilize the charged group via hydrogen bonding, with D185 also engaging in a salt bridge. Alternative binding modes of 2GBI fall into two major clusters, both potentially in agreement with experimental data (30) (SI Appendix, Results and Figs. S8 and S9).

**Pore Blockers Displace Waters and Interrupt Water Wires.** Displacement of water molecules from druggable pockets is one of the crucial determinants of ligand binding affinity. During activated Hv1 transitions (A1–A3), water density fluctuations result in intermittent occupancy of discrete solvation sites (Fig. 5), namely, the I-site and E-site. In its most favorable bound conformation (Fig. 7), 2GBI overlaps with ordered water molecules found in the I-site, the compartment connecting E153 to D112–D185 at the intracellular crevice. The clusters’ population in the I-site shows extreme variability: Hydrogen-bonded pairs that are stable and localized in the A1 substrate are completely absent in A3. Arginine R3 and the hydrophobic plug (F150) line the resulting dry volume. Interestingly, the ligand scaffold fits perfectly in the region of space vacated by these water clusters (Fig. 7D). Binding of 2GBI is likely favored by the displacement of these confined waters: A stabilizing contribution to Gibbs free energy likely results from the gain in entropy that these localized and orientationally restrained waters experience upon 2GBI binding.

Binding of active analogs (compounds 4 and 6; SI Appendix, Results and Fig. S10) confirms this picture: The bulky substituents on the benzimidazole ring improve ligand target interactions and increase hydrophobicity by filling the region devoid of waters in A3.

In the activated state, short-lived water wires can form that presumably connect protonatable residues located along the proton permeation pathway (Fig. 6 and SI Appendix, Figs. S6 and S7). Even if short and transient, these wires indicate that proton transfer events during conduction are easy and fast. Interestingly, 2GBI and active analogs hinder formation of the water wires connecting E153 to D112 (SI Appendix, Fig. S11). These amino acids can exchange protons only if a structural fluctuation brings them in close proximity or if one or more intervening hydrogen-bonded waters act as a shuttle. Thus, we conclude that 2GBI acts as a plug to prevent proton diffusion from the intracellular to extracellular milieu (29, 30, 73).

**Pore Blockers Displace Highly Fluctuating Waters.** It is well known that hydration fluctuations play a crucial role in hydrophobic cavity/ligand binding (74, 75). Large density fluctuations of solvent atoms are indicative of potential bistable, wet/dry solvation free energy, and, as such, can be taken as a measure of hydrophobicity. Therefore, detecting the regions of maximal water density fluctuations amounts to identifying the region of the pore where displacement of the waters by a hydrophobic scaffold is most favorable. The density fluctuations of proteins’ atoms carry similar information in that they highlight regions putatively “soft,” which are amenable to rearrangement upon ligand binding. We analyzed protein and solvent fluctuations as well as orientational preferences of water clusters along the channel lumen of activated Hv1, and we compared the relative density maps against the binding modes of 2GBI along with analogs with comparable/superior (compounds 6 and 4) or significantly reduced (compound 11) potency (Figs. 8 and 9). By orientational preference of hydrogen-bonded clusters, we mean their average directionality (i.e., how the donor and acceptor are arranged in the majority of the cases).

Thanks to this analysis, we can show that two driving forces guide ligand binding. First, the ligand hydrophobic moiety is entirely surrounded by fluctuating solvent, prone to hydrophobic scaffold replacement, and by fluctuating protein side chains, easily rearrangeable upon binding. Indeed, the bulky benzimidazole ring lies in a hydrophobic cavity in the channel lumen where the main dry transition occurs (Fig. 7D), and the phenyl ring engages in π–π interactions with the side chain of the hydrophobic plug F150, the one showing the highest fluctuations. Second, the 2GBI scaffold is oriented so to maximize the ligand complementarity with the hydrogen-bonded donor and acceptor locations in the regions where water density fluctuations are minimal (and thus waters are hardest to displace; Fig. 9).

In the 2GBI active analogs 6 and 4, potency is maintained or increased (30) as the hydrophobic moieties grow and expand at the interface with major hydration and protein fluctuation sites (Fig. 8 B and C), a clear indication that highly fluctuating solvent...
regions coincide with thermodynamically favorable pockets for ligand binding. Conversely, this behavior is not observed in the binding mode of 2GBI analog 11, with disruptive effects on activity (29, 30). Furthermore, in all active 2GBI analogs, N1 is donating a hydrogen bond to a cluster of waters, with a clear preference to accept hydrogen bonds, located in the proximity of the selectivity filter D112. Interestingly, this complementarity is lost in the poor inhibitor 2GBI analog 11, suggesting a crucial role for this interaction in stabilizing the GBI moiety. Notice that our docking results cannot exclude alternative binding modes of compound 11, with flipped orientations of the oxazole ring where the oxygen points toward R211 and the nitrogen faces D112 (29, 30). Importantly, in all these orientations of compound 11, interactions with the hydrogen/bond acceptor waters are hindered by the absence of a strong donor feature on the oxazole moiety.

**Detection of an Externally Accessible Binding Site.** The identification of pockets suitable for ligand binding is a challenging task, particularly in the case of ion channels because they lack traditional binding cavities buried into hydrophobic enclosures, such as the ATP binding pocket in protein kinases. Here, we exploit the thermodynamic properties of pore waters, combined with molecular docking of fragment molecules, to detect the most favorable “druggable” pockets in activated Hv1 channels. We showed above that a wet/dry transition occurs at the E-site, in the space enclosed by the polar/hydrophobic ring (V116, S143, L189, and L204; Fig. 5), where water wires are also detected (SI Appendix, Figs. S4 and S6). Furthermore, analysis of pore waters identified highly fluctuating solvent at two spots in the proximity of the ring (SI Appendix, Fig. S12 A–C). The concomitant presence of a wet/dry transition of water wires and high hydration fluctuations was also observed at the I-site within the 2GBI binding pocket. Taken altogether, these data provide a strong indication that the E-site accommodates an additional druggable pocket, potentially suitable for ligand binding. To corroborate our hypothesis further, we collected a set of fragment molecules, or probes, to be used in binding pocket detection via molecular docking. The rationale is that probe ligands preferentially bind at druggable hot spots (76, 77). We generated binding modes of all these fragments within the Hv1 pore in the volume between the I-site and the E-site. Results show that probes bind preferentially at the E-site at two locations, namely, the polar/hydrophobic ring and a second spot immediately preceding the ring at the extracellular side (Fig. 10 and SI Appendix, Figs. S12 and S13). It is noteworthy that both spots coincide with the hydration fluctuation sites.

**Molecular Underpinnings of Hv1 Druggability.** Our work suggests novel insights into the molecular underpinnings of Hv1 druggability. First, our analysis of the hydration properties of activated Hv1 rationalized the binding mode of 2GBI as well as analogs and suggested strategies for scaffold optimization. Specifically, rational expansion of active analogs (or novel chemical classes by scaffold hopping) should be achieved along three different directions. One is the benzo-ring (or an equivalent position on a generalized scaffold), where substituents on position 5 are known to increase/maintain potency by stabilizing highly fluctuating solvent (compounds 4 and 6; Fig. 8). Introducing acceptor groups at position 3 and/or of the benzimidazole ring should offer stabilizing interactions to structured waters with strong preferences for donating hydrogen bonds (Fig. 9). Expansions of the guanidine moiety toward highly fluctuating regions are also promising (Fig. 8), as suggested by docking (Fig. 10). Second, extensive analyses performed on pore waters suggested the presence within the E-site of a second pocket suitable for ligand binding. Ideally, a chemical scaffold matching this pocket should exploit favorable interactions with residues in the polar/hydrophobic ring and should benefit by positioning a (possibly hydrophobic) moiety within the extracellular hydration-fluctuation site (Fig. 10 and SI Appendix, Fig. S12). Alternatively, modifications of the guanidine moiety are also promising, as suggested by binding modes of probes (Figs. 8–10 and SI Appendix, Fig. S13).

**Conclusions**

Ion channels are an important class of integral membrane proteins. Encoded by more than 400 genes, these channels are expressed in virtually all cell types, where they exert a number of complex physiological roles, from controlling the ion flux across the cellular membrane to establishing its resting potential. Ion channels are responsible for a number of channelopathies (i.e., pathological conditions of various origins), including genetic channelopathies caused by malfunctioning ion channels (78). Nevertheless, despite recent efforts resulting in the discovery of novel potential drugs targeting ion channels (39, 40), this class of proteins remains quite underexploited in drug discovery because it poses significant challenges (78–81). For many years,
networks and electrostatic potential maps are the signatures of activation and proton conduction in which voltage activation and proton conduction contribute to ligand binding (74, 75, 103), our docking results show that this site has a high propensity to be targeted by ligands that could be exploited to expand the current repertoire of Hv1 modulators.

Methods

Comparative Homology Modeling. We used the recently published crystal structure of Hv1 (31) as a template to generate models of the monomeric Hv1 in intermediate-resting, activated, and fully resting states (SI Appendix, Methods). Briefly, all models were generated using MODELLER (104) using a previously described procedure to model fully resting and activated states (33, 34).

Classical MD Simulations. We performed classical MD simulations of the resting, intermediate-resting, and activated states of Hv1. All systems were embedded in a 1-palmitoyl-2-oleoylphosphatidylcholine bilayer to generate a simulation box of \(100 \times 100 \times 100 \) Å\(^3\), and then hydrated in a 150 mM NaCl water solution. All simulations were conducted in the isothermal/isobaric (NPT) ensemble at 303.5 K and 1 atm. All systems were simulated using NAMD2 (105) implementing the particle mesh Ewald approach (106). The CHARMM36 (107, 108) and TIP3P (109) force fields were used for the treatment of protein and lipids and for the treatment of water, respectively. The simulation details are provided in SI Appendix, Methods and Table S1.
MD Simulations with External Potential. We performed MD simulations in presence of an electrostatic potential resulting from a Gaussian charge density located at a fixed position in space (SI Appendix, Methods). Forces, resulting from the electrostatic potential \(g(r)\), were added to equations of motions. Three simulations were performed in parallel (SI Appendix, Table S1).

Volumetric Maps of Densities and Density Fluctuations. We calculated density and density fluctuation maps for water, proteins, and the donor and acceptor oxygen of the hydrogen-bonded water clusters. We calculated linear density profiles of water, F150, and lipids (Fig. 3 and S1 Appendix, Fig. S1) upon setting the origin at the center of the lipid bilayer (details are provided in SI Appendix, Methods).

Electrostatic Potential Maps. We generated 3D maps of the electrostatic potential using the PMEPot module implemented in Visual Molecular Dynamics (VMD) (110) (SI Appendix, Methods).

Clusters of Hydrogen-Bonded Waters and Water Wires. We implemented a multistep procedure based on clustering and minimum distance path detection to identify hydrogen-bonded water clusters and detect persistent hydrogen-bonded water wires (SI Appendix, Methods). In summary, hydrogen-bonded water pairs were first represented as distance vectors between donor and acceptor atoms and then clustered according to the results of an agglomerative algorithm developed by Jarvis and Patrick (111). Clustering parameters were chosen using an information theoretical criterion to select the best partitioning of the dataset (112) (SI Appendix, Fig. S5). Water wires were detected by monitoring the occupancy of contiguous representative vectors from the intracellular side to the extracellular side of the channel and then by calculating the minimum distance path on the percolation pathway according to the Floyd–Warshall algorithm (sections 26.2 and 26.4 in ref. 113).

Binding Site Predictions, Induced Fit Docking, and Docking of Probes. Representative snapshots of activated Hv1 were used for protein preparation (114–116). Ligands 2GBI and analogs (compounds 4, 6, and 11), as well as molecular fragments (29, 76), were prepared for docking studies. Induced fitting (117) and Glide-XP (118) were used to dock 2GBI and analogs and to dock molecular fragments, respectively (details are provided in SI Appendix, Methods).

ACKNOWLEDGMENTS. We thank the Swiss National Supercomputing Centre and National Center for Supercomputing Applications for the computer time. We also thank Sari C. van Keulen (École Polytechnique Fédérale de Lausanne) and Christopher MacDermid (Temple University) for many stimulating scientific discussions. This work was supported by NIH National Institute for General Medical Sciences Grant P01 GM55876 (to M.L.K.) and by National Science Foundation Grant ACI-1614804 (to V.C. and M.L.K.) and major research instrumentation Grant CNS-09-58854. This work received funding from European Union Framework Program Grant PIOF-GA-2012-329534, “Voltsoins” (to L.D.).