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RESEARCH ARTICLE

Proteins: Structure, Function and Bioinforma

DOI 10.1002/prot.25951

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Cumulative hydropathic topology of a voltage-gated sodium channel at atomic resolution

short running title: *NavAb's cumulative hydropathic topology*

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/prot.25951

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Received: Jun 25, 2019; Revised: Mar 11, 2020; Accepted: May 14, 2020

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May 18, 2020

Abstract

Voltage-gated sodium channels (NavChs) are biological pores that control the flow of sodium ions through the cell membrane. In humans, mutations in genes encoding NavChs can disrupt physiological cellular activity thus leading to a wide spectrum of diseases. Here, we present a topological connection between the functional architecture of a NavAb bacterial channel and accumulation of atomic hydropathicity around its pore. This connection is established via a scaling analysis methodology that elucidates how intra-channel hydropathic density variations translate into hydropathic dipole field configurations along the pore. Our findings suggest the existence of a non-random cumulative hydropathic topology that is organized parallel to the membrane surface so that pore's stability, as well as, gating behavior are guaranteed. Given the biophysical significance of the hydropathic effect, our

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study seeks to provide a computational framework for studying cumulative hydropathic topological properties of NavChs and pore-forming proteins in general.

keywords: voltage-gated sodium channels; scaling; cumulative hydropathic effects; topology; NavAb; hydrophobic gating

Introduction

Voltage-gated sodium channels (NavChs) are fundamental components of electrically excitable cells such as neurons and muscle cells [1]. They belong to the superfamily of ion channels and their primary function is to facilitate the transport of sodium ions across a cell's membrane in response to changes in the membrane potential. Genetic dysfunction of NavChs has profound implications for physiological neuronal or muscle activity, leading to inherited human disorders [2]. For example, genetic and functional studies have shown that missense mutations in the SCN9A gene encoding the Nav1.7 channel are causally related to neuropathic pain syndromes such as inherited erythromelalgia [3–12], paroxysmal extreme pain disorder [13–16] and small fibre neuropathy [17, 18]. Although molecular mechanisms underlying missense SCN9A mutations remain largely unexplored, structural modeling of the Nav1.7 revealed that disrupting hydropathic lining properties of the Nav1.7's pore can act as a disease-causing molecular trigger [19, 20].

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(PDB code: 3RVY) [21] provides a prototype for understanding the functional architecture of the NavCh family. The NavAb channel consists of four homologous subunits, each of which comprises a pore domain (PD) interlinked with a voltage- sensing domain (VSD). This conserved architectural motif results in an ion-conducting central pore lined by hydropathically-diverse and, hence, functionally-distinct structural channel components (see Fig. 2d in [21]). Hydropathic effects not only contribute to the stability of an ion channel's native structure [22, 23] but also regulate ion conduction through the pore via "hydropathic gates" [24–29]. However, the spatial complexity underlying hydropathic interactions [30–32] poses a significant obstacle in the development of computational tools for hydropathic analysis of protein structures.

Hydropathic moments (usually referred to as "hydrophobic moments" [33]) provide a simple, yet efficient tool for modeling of molecular mechanisms (e.g., see [34]). The method of cumulative hydropathic moments [35] has been used for analyzing the spatial profile of cumulative hydropathicity relative to a function-relevant geometry (e.g., the geometric center of the residues distribution) [36–39]. Interestingly, it was shown that data extracted from the vanishing, i.e., zero-crossing, behavior of the cumulative zero- and second-order residue hydropathic moments exhibited invariance over a large number of non-redundant globular proteins [37]. This finding highlighted the universality of the spatial transition from a protein's hydrophobic core to its hydrophilic exterior implying the existence of hydropathic scaling laws

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spanning all different molecular scales.

In principle, hydropathic characteristics along a NavCh's pore are studied at a microscopic scale, i.e., by focusing on the hydropathic profile of pore-lining atomic structures (e.g., see [21, 40]). In this work, we extend this methodology to incorporate hydropathic characteristics of the spatial transition from the pore's microenvironment toward a macroscopic molecular regime that includes the VSDs. This methodological extension allows for a continuous mapping of hydropathic, as well as, structural properties of a NavCh on two dimensions. Specifically, instead of performing a three-dimensional (3D) mapping of hydropathic characteristics on the NavCh structure at residue-level resolution (as already implemented by available tools, e.g., pymol [41] and chimera [42]), we present a two-dimensional hydropathic mapping procedure of atomic resolution by exploiting pore-forming geometrical principles. The main advantage of this approach is that it elucidates how hydropathic dipole accumulation occurs with respect to pore's geometry thus providing information about the pore's gating behavior [24]. In order to achieve that we utilized the tool of cumulative hydropathic moments and developed an atomic sampling algorithm that adapts to geometrical characteristics of the pore so that hydropathic density variations, as well as, their corresponding hydropathic dipole field configurations are visualized and, consequently, analyzed across different molecular scales. Accordingly, the relevant dimensions of the presented methodology are the pore axis coordinate and the molecular scale. We illustrate our methods for the pre-open

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I217C NavAb channel where we report implications of our observations on molecular stability, as well as, on the formation of hydropathic gates along the pore.

Methods

I. 3D structure preparation.

The I217C NavAb model (PDB code: 3RVY) was selected for analysis as it provides a crystallography of the NavAb with the highest resolution (2.7 Å resolution). The structure was protonated using the WHAT IF software [43, 44] and its principal axes were estimated using the VMD software [45]. A coordinate system $(\hat{\mathbf{x}}, \hat{\mathbf{y}}, \hat{\mathbf{z}})$ with origin O was introduced and the protonated structure was placed within it, so that the principal pore axis, i.e., the axis approximating the direction of the channel's pore, is aligned with the z-axis. Orientation of the structure was set from the extracellular side (ES) to the intracellular side (IS) with respect to $\hat{\mathbf{z}}$ and the channel's molecular mass center $\mathbf{e} = \frac{1}{M}\sum_{i=1}^{N_c} m_i \cdot \mathbf{c}_i$ was set to coincide with O where $\mathbf{c}_i = (\mathrm{c}_{x,i}, \mathrm{c}_{y,i}, \mathrm{c}_{z,i})$ is the atomic center of the i-th atom, m_i is the mass of the i-th atom, $N_c = 14776$ is the total number of atoms and $M = \sum_{i=1}^{N_c} m_i$ is the total molecular mass.

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II. Geometrical characteristics of the pore.

We considered P to represent the principal pore axis and $\mathbf{p}=(\mathrm{p}_x, \mathrm{p}_y, \mathrm{p}_z) \in P$ to be a pore point. The radius of the smallest sphere that can be squeezed through the pore at $\mathbf{p}$ i.e., the pore radius at $\mathbf{p}$, is given by [46]

$$R(\mathbf{p}) = \underset{i=1,2,..,N_c}{min}\{||\mathbf{c}_i - \mathbf{p}|| - vdW_i\} \tag{m1}$$

where $||\cdot||$ is the euclidean norm and vdW_i is the Van der Waals radius of the i-th atom (see Suppl. Table S1). Consequently, the distance between $\mathbf{p}$ and its nearest neighbor atom is given by

$$\bar{R}(\mathbf{p}) = \underset{i=1,2,..,N_c}{min}\{||\mathbf{c}_i - \mathbf{p}||\} \tag{m2}$$

In analogy to equation m1, we introduced the outer surface radius at $\mathbf{p}$ with

$$L(\mathbf{p}) = \underset{i=1,2,..,N_c}{max}\{||\mathbf{c}_i - \mathbf{p}|| + vdW_i\} \tag{m3}$$

The unit of measurement for $R(\mathbf{p})$, $\bar{R}(\mathbf{p})$ and $L(\mathbf{p})$ is expressed in [Å].

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III. Atomic sampling around the pore.

In order to investigate cumulative atomic properties with respect to P, we introduced the **p**-dependent atomic sampling radius

$$l_\alpha(\mathbf{p}) = \bar{R}(\mathbf{p}) + \alpha \cdot \frac{L(\mathbf{p}) - \bar{R}(\mathbf{p})}{K_\alpha} \text{ for } \begin{cases} K_\alpha \in \mathbb{Z}^+ \\ 0 < \alpha \leq K_\alpha \end{cases} \quad \text{(m4)}$$

where K_α is the total number of sampling spheres centered at $\mathbf{p}$, α denotes the index of the sampling sphere. Note that $l_\alpha(\mathbf{p})$ plays the role of the molecular scale, i.e., it provides a **p**-dependent measurement of the size of the channel in [Å]. The number of sampled atoms within a sphere of radius $l_\alpha(\mathbf{p})$ centered at $\mathbf{p}$ is given by

$$N(\mathbf{p}, l_\alpha(\mathbf{p})) = \sum_{i=1}^{N_c} \theta(l_\alpha(\mathbf{p}) - ||\mathbf{c}_i - \mathbf{p}||) \quad \text{(m5)}$$

where $\theta(\cdot)$ is the heaviside function.

IV. Cumulative hydropathic pore moments.

We employed the zero-order hydropathic pore moment [33]

$$h^{(0)}(\mathbf{p}, l_\alpha(\mathbf{p})) = \sum_{i=1}^{N_c} \theta(l_\alpha(\mathbf{p}) - ||\mathbf{c}_i - \mathbf{p}||) \cdot HI_i^\chi \quad \text{(m6)}$$

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and the first-order (or dipole) hydropathic pore moment [33]

$$\vec{\boldsymbol{h}}^{(1)}(\mathbf{p}, l_\alpha(\mathbf{p})) = \sum_{i=1}^{N_c} \theta(l_\alpha(\mathbf{p}) - ||\mathbf{c}_i - \mathbf{p}||) \cdot HI_i^\chi \cdot \vec{\boldsymbol{r}}_{\mathrm{p},i} = \underbrace{h_x(\mathbf{p}, l_\alpha(\mathbf{p})) \cdot \hat{\mathbf{x}} + h_y(\mathbf{p}, l_\alpha(\mathbf{p})) \cdot \hat{\mathbf{y}}}_{\vec{\boldsymbol{h}}_{xy}(\mathbf{p}, l_\alpha(\mathbf{p}))} + h_z(\mathbf{p}, l_\alpha(\mathbf{p})) \cdot \hat{\mathbf{z}} \tag{m7}$$

where $HI_i^\chi = HI_i + \chi_i$ is the hydropathic value of the i-th atom set according to the Kapcha-Rossky atomic hydropathic indices [47] (see Suppl. Table S2) with additive gaussian noise $\chi_i \in \mathcal{N}(\mu = 0, \sigma = 0.001)$, $\vec{\boldsymbol{r}}_{\mathrm{p},i}$ is the vector from $\mathbf{p}$ to $\mathbf{c}_i$ and the measurement units of $h^{(0)}(\mathbf{p}, l_\alpha(\mathbf{p}))$ and of $||\vec{\boldsymbol{h}}^{(1)}(\mathbf{p}, l_\alpha(\mathbf{p}))||$ are roughly given by $[kcal/mol]$ and $[kcal \cdot \text{Å}/mol]$, respectively. Division of equations m6 and m7 with m5 provided with an estimation of the hydropathic density [37]

$$m^{(0)}(\mathbf{p}, l_\alpha(\mathbf{p})) = \frac{h^{(0)}(\mathbf{p}, l_\alpha(\mathbf{p}))}{N(\mathbf{p}, l_\alpha(\mathbf{p}))} \tag{m8}$$

and of the hydropathic imbalance [37]

$$\vec{\boldsymbol{m}}^{(1)}(\mathbf{p}, l_\alpha(\mathbf{p})) = \frac{\vec{\boldsymbol{h}}^{(1)}(\mathbf{p}, l_\alpha(\mathbf{p}))}{N(\mathbf{p}, l_\alpha(\mathbf{p}))} \tag{m9}$$

pore functions for increasing molecular scale $l_\alpha(\mathbf{p})$, respectively. Due to the spherical sampling procedure all scalar and vector pore functions remain invariant to rotations of the channel around P.

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V. Discretization of the principal pore axis.

We discretized P by introducing the equidistant grid $Q=\{\mathbf{p}_1, \mathbf{p}_1+\Delta\mathbf{p}, .., \mathbf{p}_{N_\mathrm{p}}-\Delta\mathbf{p}, \mathbf{p}_{N_\mathrm{p}}\} \subset P$ where N_p is the total number of grid pore points, $||\Delta\mathbf{p}||$ is the sampling distance between two consecutive pore points and $\mathbf{p}_1 = (0, 0, \mathrm{p}_{z,1})$, $\mathbf{p}_{N_\mathrm{p}} = (0, 0, \mathrm{p}_{z,N_\mathrm{p}})$ are boundary pore points. Consequently, Q was constructed by setting $\mathrm{p}_{z,1} = round(\min\limits_{i=1,2,..,N_c}(c_{z,i}), 1) = -27.1$ and $\mathrm{p}_{z,N_\mathrm{p}} = round(\max\limits_{i=1,2,..,N_c}(c_{z,i}), 1) = 26.8$ with $round(x \in \mathbb{R}, n \in \mathbb{Z}^+)$ returning the value of x rounded up to the n-th decimal digit and by setting $N_\mathrm{p} = 540$ so that $||\Delta\mathbf{p}|| = 0.1$ Å.

VI. Detection of zero-crossing points of a scalar pore function along the principal pore axis.

We consider $f(\mathbf{p}, l_\alpha(\mathbf{p}))$ to represent a scalar pore function. If for a given scaling index α there is a pair $\{\mathbf{p}' = \mathbf{p} - \Delta\mathbf{p}, \mathbf{p}\} \in Q$ for which the sign-change condition $f(\mathbf{p}', l_\alpha(\mathbf{p}')) \cdot f(\mathbf{p}, l_\alpha(\mathbf{p})) < 0$ is satisfied, then the four-dimensional point

$$(\mathbf{s} = \mathbf{p}' + \frac{|f(\mathbf{p}', l_\alpha(\mathbf{p}'))|}{|f(\mathbf{p}', l_\alpha(\mathbf{p}'))| + |f(\mathbf{p}, l_\alpha(\mathbf{p}))|} \cdot \Delta\mathbf{p}, l_\alpha(\mathbf{s})) \qquad \text{(m10)}$$

represents a zero-crossing point of $f(\mathbf{p}, l_\alpha(\mathbf{p}))$ along $\mathbf{p}$-direction where $|\cdot|$ returns the absolute value of f and $\mathbf{s}$ is obtained by linear interpolation. The set of all detected zero-crossing points of $f(\mathbf{p}, l_\alpha(\mathbf{p}))$ along $\mathbf{p}$-direction for a given scaling index α is represented as $\Gamma(\alpha)$. Note that due to the addition of noise to the hydropathic indices, the probability of $f(\mathbf{p}, l_\alpha(\mathbf{p}))$ being zero was

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practically eliminated so that $f(\mathbf{p}', l_\alpha(\mathbf{p}')) \cdot f(\mathbf{p}, l_\alpha(\mathbf{p})) \neq 0$ is always satisfied.

Results

I. Hydropathic density variations around pre-open I217C NavAb's pore

The first step in this study was to investigate how atomic hydropathicity of the pore's microenvironment changes for increasing molecular scale, $l_\alpha(\mathbf{p})$ (see eq. m4). To do so, we employed the hydropathic density pore function, $m^{(0)}(\mathbf{p}, l_\alpha(\mathbf{p}))$ (see eq. m8), and illustrated it as a contour map (see Fig. 1(a)). "Reading" of the contour map was performed from left to right, namely, for a given pore point, $\mathbf{p}$, we investigated the zero-crossing behavior of the hydropathic density pore function for increasing $l_\alpha(\mathbf{p})$ based on a color gradient that illustrates negative and positive values of $m^{(0)}(\mathbf{p}, l_\alpha(\mathbf{p}))$ as blue as red, respectively. Accordingly, blue and red contour map domains represent hydrophobic and hydrophilic atomic structures, respectively. Observations were interpreted with respect to the conserved architectural motif dictating the structural separation of the PDs from the VSDs [48]. For that, a geometrical representation of the structural position of the PDs relatively to the VSDs (and vice-versa) was introduced by approximating the scales $\nu(\mathbf{p})$ for which an equilibrium of the corresponding radial distribution functions is achieved (see Suppl. S2). In that way, the line $\nu(\mathbf{p})$ appearing in

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Fig. 1(a) (and in Fig. 2(a)) indicates that the number of PD atoms within a sampling radius $l_\alpha(\mathbf{p}) \leq \nu(\mathbf{p})$ is larger than that of VSD atoms thus roughly approximating the structural boundary between PDs and the VSDs. On the other hand, for $l_\alpha(\mathbf{p}) > \nu(\mathbf{p})$ the number of VSD atoms gradually increases so that the spatial transition from the PDs to the VSDs is realized.

At first glimpse, the contour map of Fig. 1(a) reveals that the spatial organization of PD atoms around P results in the formation of a hydrophobic centrally-located cavity (CC). This becomes evident if we focus on the contour area enclosed within $-4.4 \leq \mathrm{p}_z \leq 7.3$ and $l_\alpha(\mathbf{p}) < 10.0$ Å (Fig. 1(a)). Deviating from the center of the pore toward the IS, we observe the narrowing and, eventually, occlusion of the pore by a hydrophilic microstructure as illustrated in Fig. 1(a) for $\mathrm{p}_z > 19.0$ and $l_\alpha(\mathbf{p}) < 10.0$ Å. Macroscopically, occlusion of the pore translates into a qualitative change in the profile of the outer pore radius, $L(\mathbf{p})$ (see eq. m3), as $L(\mathbf{p})$ monotonically decreases and increases for $\mathrm{p}_z \leq 19.0$ and for $\mathrm{p}_z > 19.0$, respectively. In that way, a funnel-like outer pore surface with an opening at the IS is formed by 2π-rotation of $L(\mathbf{p})$ around P (Fig. 1(a)). Deviating from the center of the pore toward the ES, we observe a widening of the pore so that an extracellular opening (or "mouth") is formed (see Fig. 1(a) for $\mathrm{p}_z < -12.0$). For $l_\alpha(\mathbf{p}) > 30$ Å hydropathic density variations cannot anymore be deduced via visual investigation of Fig. 1(a), indicating that the structural combination of the PDs with the VSDs tends to increase hydropathic uniformity of the atomic environment around the pore. This tendency results macroscopically in a down-regulation

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of hydrophobicity as the channel appears to be weakly-hydrophilic with

$$\begin{aligned} m^{(0)}_{total} =& m^{(0)}(\mathbf{p}, L(\mathbf{p})) \\ =& N_c^{-1} \sum_{i}^{N_c} HI_i^{\chi} = 0.00487 \sim \text{kcal/mol} \end{aligned} \tag{r1}$$

that accounts for the total, i.e., whole-channel, hydropathic density.

A detailed examination of hydropathic density variations around the pore was performed by analyzing the zero-crossing behavior of $m^{(0)}(\mathbf{p}, l_\alpha(\mathbf{p}))$ for increasing molecular scale $l_\alpha(\mathbf{p})$. To do so, we detected zero-crossing points of $m^{(0)}(\mathbf{p}, l_\alpha(\mathbf{p}))$ for increasing scaling index α and constructed the sets $\Gamma^{(0)}(\alpha)$ (see Methods). A zero-crossing point of $m^{(0)}(\mathbf{p}, l_\alpha(\mathbf{p}))$ along $\mathbf{p}$-direction identifies a pore point around which the hydropathicity of the atomic environment becomes vanishingly small (see Suppl. S3) and changes polarity, and is illustrated as a blue-to-red contour map transition (see Fig. 1(a)). The arrangement of zero-crossing points on the contour map of Fig. 1(a) revealed the boundaries among visually distinguishable, but also among visually indistinguishable contour domains as indicated by black arrows for $l_\alpha(\mathbf{p}) \leq \nu(\mathbf{p})$ and $l_\alpha(\mathbf{p}) > \nu(\mathbf{p})$, respectively. Accordingly, the contour map was partitioned into four distinct domains, namely, the hydrophobic contour domain $T_3^{(0)}$, the hydrophilic contour domains $T_1^{(0)}$ and $T_2^{(0)}$, and the weakly hydrophilic contour domain $T_4^{(0)}$. This partitioning indicates that the CC is a smaller part of a larger domain that covers the largest area of the contour map, namely of $T_3^{(0)}$ (Fig. 1(a)). Around $T_3^{(0)}$ the hydrophilic $T_1^{(0)}$, $T_2^{(0)}$ and $T_4^{(0)}$ are formed

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in accordance with the overall structural organization of the PDs relatively to the VSDs as minimization of $\nu(\mathbf{p})$ roughly coincides with the formation of the CC (Fig. 1(a)). On the other hand, deviating toward the ES or toward the IS we observe an expansion of the PDs, as indicated by the monotonic increase of $\nu(\mathbf{p})$ for deviating from the CC, in accordance with an increase in atomic hydrophilicity that is expressed by the formation of $T_1^{(0)}$ with $T_2^{(0)}$ (Fig. 1(a)). Taken together, these observations reflect the channel's strategy to maintain a hydrophobic core, i.e., a hydrophobic CC, by increasing the density of hydrophobic atoms around its mass center, which in turn places hydrophilic atoms elsewhere. The advantage behind this configuration might be an increase in NavAb's stability due to reduction of water-accessible hydrophobic surface achieved by "burying" hydrophobic atoms around the mass center.

In order to obtain a statistical summary of our observations, we introduced the distributions $\psi^{(0)}$ and $\phi^{(0)}$, respectively (see Suppl. S4 for construction). This allowed for identifying molecular scales and locations along P where hydropathic density variations tend to increase or decrease as implied by extremization of $\psi^{(0)}$ and $\phi^{(0)}$, respectively. As we can see in Fig. 1(b), $\psi^{(0)}$ can be decomposed into two sub-distributions, namely, $\psi_A^{(0)}$ and $\psi_B^{(0)}$ (Fig. 1(b)), revealing that hydropathic density variations within the PDs occur more frequently as the majority of zero-crossing points (approximately 65%) are found in the contour map for $l_\alpha(\mathbf{p}) \leq \nu(\mathbf{p})$. Thus, separation of $\psi_A^{(0)}$ from $\psi_B^{(0)}$ is another indication of the structural and functional separation of

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the PDs from the VSDs.

The minimum structural information required in order to navigate through the pore's microenvironment is to identify pore-lining residues. To do that, we partitioned P into distinct pore regions based on a minimal-distance geometrical criterion that takes into account only direct-neighboring residues (see Suppl. S5). This approach revealed that the global minimum of $sm(\phi^{(0)})$ at $p_z = -12.0$ identifies a hydrophilic and narrow pore region that co-localizes with the hydrophilic-to-hydrophilic $S178$-$E177$ side chain transition that is part of the selectivity filter (SF) (Fig. 1(a),(c) and Suppl. Table S3). The strongly-hydrophilic and narrow environment that is locally formed creates favorable conditions for dehydration of incoming ions (Fig. 1(a)). On the other hand, the global maximum of $sm(\phi^{(0)})$ at $p_z = 19.0$ indicates that closing of the channel at the IS requires a hydropathically-diverse atomic environment as indicated by the hydrophilic-to-hydrophobic $C217$-$M221$ side chain transition (Fig. 1(a),(c) and Suppl. Table S3). The local minimum of $sm(\phi^{(0)})$ at $p_z = 7.3$ identifies the CC and co-localizes with the hydrophobic-to-hydrophobic $I210$-$V213$ side chain transition (Fig. 1(a),(c) and Suppl. Table S3). The local maximum of $sm(\phi^{(0)})$ at $p_z = -4.4$ co-localizes with the hydrophilic-to-hydrophobic $E177$-$L176$ side chain transition and identifies a transient pore region located between the narrow, hydrophilic pore region and the CC (Fig. 1(a),(c) and Suppl. Table S3). Finally, the local maximum of $sm(\phi^{(0)})$ at $p_z = -20.3$ found at the ES mouth of the pore co-localizes with the hydrophobic-to-hydrophilic $M181$-$S178$ side chain transition (Fig. 1(a),(c)

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and Suppl. Table S3). Notably, the hydrophobic-to-hydrophilic $M181$-$S178$ side chain transition becomes evident in Fig. 1(a) as a color change from blue-to-red occurring in the vicinity of the $\bar{R}(\mathbf{p})$ line with respect to $\hat{\mathbf{z}}$ (Fig. 1(a)). Accordingly, the location $\mathrm{p}_z = -20.3$ captures the transition from the wide ES mouth shaped by the hydrophobic $M181$ side chain toward a narrow and hydrophilic pore region surrounded by the hydrophilic $S178$ and $E177$ side chains.

II. Cumulative hydropathic topology of pre-open I217C NavAb's pore

In this section we investigated the dipole field topology underlying hydropathic density variations illustrated in the contour map of Fig. 1(a). In order to do so, we utilized the hydropathic imbalance pore function, $\vec{\boldsymbol{m}}^{(1)}(\mathbf{p}, l_\alpha(\mathbf{p}))$ (see eq. m7) that can be equivalently understood as the mean (or average) cumulative hydropathic dipole moment (see connection of eq. m7 with eq. m9) thus providing information about accumulation of hydropathic dipoles around the pore. Given that the gating behavior of a nanopore is largely determined by its geometry and dipole accumulation parallel to its lining [24], we adopted here a similar approach to the previous section and analyzed how microscopic, pore-lining dipoles scale up and form larger polarized structures that incorporate, both, the PDs and the VSDs. By exploiting radial structural symmetries underlying NavAb's tetrameric con-

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formation it was straightforward to reduce $\vec{\boldsymbol{m}}^{(1)}(\mathbf{p}, l_\alpha(\mathbf{p}))$ to its z-component, $\vec{\boldsymbol{m}}_z^{(1)}(\mathbf{p}, l_\alpha(\mathbf{p})) = m_z^{(1)}(\mathbf{p}, l_\alpha(\mathbf{p})) \cdot \hat{\mathbf{z}}$, as the amplitude of the radial component of dipole hydropathic moment, $||\vec{\boldsymbol{h}}_{xy}(\mathbf{p}, l_\alpha(\mathbf{p}))||$ (see eq. m7), is shown to be vanishingly small (see Suppl. S6). Accordingly, our analysis focused on the spatial profile of $m_z^{(1)}(\mathbf{p}, l_\alpha(\mathbf{p}))$ that is illustrated on the contour map of Fig. 2(a). A zero-crossing point of $m_z^{(1)}(\mathbf{p}, l_\alpha(\mathbf{p}))$ along $\mathbf{p}$-direction accounts for a change in dipole field's orientation, as well as, for a vanishingly small hydropathic imbalance effect (see Suppl. S7), i.e., for the formation of a hydropathic dipole center on P for a given scaling index α. From a topological viewpoint, this vanishing event corresponds to an equilibrium point of the hydropathic imbalance field acting along P for a given α. Depending on the orientation of the local field, an equilibrium point acts either as molecular attractor (i.e., "sink") or molecular repeller (i.e., "source") so that a hypothetical molecule (e.g., a partially-hydrated sodium ion) would be either attracted toward or repelled from it, respectively (see Suppl. S8). The importance of the topological interplay between hydropathic "sinks" and "sources" in ion permeation dynamics and, specifically, ion selectivity was recently highlighted in [49]. Similar to the previous section, we introduced the expression

$$\Omega^{(1)} = \bigcup_{\alpha} \{\Gamma^{(1)}(\alpha)\} \tag{r3}$$

that incorporates topological information extracted from every molecular scale $l_\alpha(\mathbf{p})$ where $\Gamma^{(1)}(\alpha)$ represents the set of all detected dipole centers

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for a given scaling index α (see Methods). Consequently, the contour map of Fig. 2(a) was partitioned into four domains; the pair $T_1^{(1)}$, $T_4^{(1)}$ and the pair $T_2^{(1)}$, $T_3^{(1)}$ corresponding to configurations of $\vec{\boldsymbol{m}}_z^{(1)}(\mathbf{p}, l_\alpha(\mathbf{p}))$ with orientation toward the ES, i.e., $m_z^{(1)}(\mathbf{p}, l_\alpha(\mathbf{p})) < 0$, and with orientation toward the IS, i.e., $m_z^{(1)}(\mathbf{p}, l_\alpha(\mathbf{p})) > 0$, respectively. Notably, $T_3^{(1)}$ and $T_4^{(1)}$ cover the largest contour area so that the map can be roughly split into two parts, namely, an ES and an IS part, revealing a topological dichotomy of the pore (Fig. 2(a)). The structural advantage behind this topological configuration is the stabilization of CC via an accumulation of dipoles around it in a direction roughly parallel to P (Fig. 2(a)). Following this line of thought, $T_1^{(1)}$ and $T_2^{(1)}$ might guarantee stabilization of hydrophilic atomic structures at the ES and IS contributing to the formation of $T_1^{(0)}$ and $T_2^{(0)}$, respectively (Fig. 1(a), 2(a)). For $l_\alpha(\mathbf{p}) > \nu(\mathbf{p})$ a qualitative change in the spatial profile of $m_z^{(1)}(\mathbf{p}, l_\alpha(\mathbf{p}))$ takes place where the number of dipoles decreases, as indicated by flattening of $\psi^{(1)}$ (Fig. 2(a),(b)), and the topological dichotomy of the pore is disrupted. Specifically, the structural combination of the PDs with the VSDs causes a displacement of dipole centers toward the IS, as indicated in Fig. 2(a) by the "$(*)$" arrow, so that the size of $T_3^{(1)}$ over $T_4^{(1)}$ increases. Consequently, a unidirectional dipole field configuration is macroscopically established with

$$m_z^{(1)}(\mathbf{p}, L(\mathbf{p})) = -m_{total}^{(0)} \cdot \mathrm{p}_z - 0.1368 \tag{r4}$$

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that is in accordance with the molecular field theory presented in [50] (see Suppl. S9). In summary, these topological characteristics result in a skewed distribution $\psi^{(1)}$ indicating that the vast majority of dipole centers (approximately 90%) are characterized by $l_\alpha(\mathbf{s}^{(1)}) \leq \nu(\mathbf{p})$. This finding highlights the prominent role that PDs play in shaping properties of the hydropathic imbalance field acting along P.

Similarly to the previous section, we employed the smoothed profile of the distribution $\phi^{(1)}$, $sm(\phi^{(1)})$, in order to identify how geometrical and hydropathic topological characteristics correlate with each other. We found that the global maximum of $sm(\phi^{(1)})$ at $\mathrm{p}_z = 2.8$ not only co-localizes with the $L176$-$I210$ side chain transition but also with the local maximization of $R(\mathbf{p})$, both identifying the CC (Fig. 2(a),(c) and Suppl. Table S3). This observation illustrates how microscopic, pore-lining dipoles around the NavAb's mass center co-aggregate into a larger structure that holds the CC open and is consistent with experimental findings presented in [24] showing that opening of a hydrophobic pore can be achieved by adding dipoles parallel to its lining. From the perspective of a hypothetical molecule, the global maximum of $sm(\phi^{(1)})$ indicates the formation of a molecular repeller approximately in the center of the pore as indicated by the relative orientation of $T_3^{(1)}$ with respect to $T_4^{(1)}$. This topology does not favor entrance of molecules to the CC from the SF or from the AG while, on the other hand, facilitates a pathway for waters or small, hydrophobic compounds penetrating the lipid membrane to enter the pore through the NavAb's side fenestrations (see

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Fig. 4 in [21]). Accordingly, the repeller dynamics is practically reversed for the case of lipid-membrane penetrating molecules; they can easily access the center of the pore through the fenestrations as they are attracted and, consequently, "trapped" into CC under the influence of $T_3^{(1)}$ or $T_4^{(1)}$, respectively (Fig. 2(a),(c)). This "trapping" mechanism would then allow to small, hydrophobic drug agents to reach and interact with the $T206$-$M209$-$V213$ drug-binding pore site [21, 51–53] (see Fig. 3(b) for the location of this pore site). On the other hand, the global minimum of $sm(\phi^{(1)})$ at $\mathrm{p}_z = -17.9$ accounts for the formation of $T_1^{(1)}$ and $T_3^{(1)}$ and co-localizes with the $M181$-$S178$ side chain transition indicating a decrease in dipole field topological variability at the ES mouth of the pore (Fig. 2(a),(c) and Suppl. Table S3). This topological configuration facilitates a passage for an incoming hydrated ion through the ES mouth toward the SF under the influence of a strong, bi-directional field effect originating from the combination of $T_1^{(1)}$ and $T_3^{(1)}$. The local maximum of $sm(\phi^{(1)})$ at $\mathrm{p}_z = -11.7$ co-localizes with the narrowing of the pore thus capturing the local minimization of $R(\mathbf{p})$, as well as, with the global minimum of $sm(\phi^{(0)})$ (Fig. 1(a), 2(a),(c)). Given that dipole accumulation around $\mathrm{p}_z = -11.7$ occurs roughly parallel to P, the relative orientation of $T_1^{(1)}$ with respect to $T_3^{(1)}$ generates a local attractor favoring binding and, consequently, dehydration of ions arriving at the extracellular end of the SF (Fig. 1(a), 2(a),(c)). Formation of local ion-binding sites (such as of the $\mathrm{site}_{\mathrm{HFS}}$ [21]) is attributed to the spatial organization of the SF $T175$-$L176$-$E177$-$S178$-$W179$ residue complex [21] and, especially, of $E177$

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side chains playing a key-role in ion selectivity in sodium and calcium channels [54]. The local maximum of $sm(\phi^{(1)})$ at $\mathrm{p}_z = 22.7$ identifies with high precision the pore point where pore occlusion takes place revealing a microscopic dipole accumulation mechanism occurring roughly parallel to P, as we can visually deduce from Fig. 2(a) for $\mathrm{p}_z = 22.7$ in the vicinity of $\bar{R}(\mathbf{p})$ line, which would favor molecular localization. Thus pore occlusion of the pre-open NavAb might not only be the outcome of a purely structural effect but also of a hydropathic gating process. The local minima at $\mathrm{p}_z = -7.1$ and $\mathrm{p}_z = 10.8$ reflect the formation of $T_3^{(1)}$ and $T_4^{(1)}$ thus identifying locations on P around which dipole field topological variability is minimized, respectively (Fig. 2(a),(c)). In that way, molecules resting or entering CC through its fenestrations (see Fig. 4 of [21]) would be transported toward the SF and the AG under the influence of the strong, unidirectional field effect originating from $T_3^{(1)}$ and $T_4^{(1)}$, respectively.

What are the lessons to be learned from cumulative hydropathic topological analysis? A summarizing read-out of the cumulative hydropathic topological analysis is presented in Fig. 3 alongside with targeted conservation analysis [55, 56], as well as, 3D hydropathicity mapping of the pore walls. Specifically, a condensed output of our method was obtained in terms of a topological quasiprobability score (TQS) (see Suppl. S8, eq. [S12]) that quantifies the probability along the pore for a molecular attractor (or repeller) to occur (Fig. 3(c)). TQS essentially provides with a qualitative description of the relative size and location of energetic barriers to molecular permeation,

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i.e., "gates" [29], along the pore (see Suppl. S8). According to this interpretation scheme, a central gating mechanism keeps the hydrophobic CC "closed" by imposing large energy barriers to sodium ions entering from the ES or the IS side while facilitating an "opening" to small hydrophobes and waters entering via the side fenestrations. Hence, hydrated sodium ions escaping from the SF's local binding sites have to overcome a large energy barrier in order to arrive at the center of the pore (Fig. 3(c)). This barrier stems from the molecular influx into CC through the fenestrations which is in opposite direction to the ion escape trajectory thus preventing (or, at least, not favoring) ion entrance into the CC. Escape trajectory is thus likely to involve multiple, partial re- and dehydration cycles, i.e., an interplay of attractors-vs-repellers sites, so that ions are gradually transported from the narrow, strongly-hydrophilic and highly-conserved SF environment toward the wide, hydrophobic center of the pore (see Fig. 3(a),(b), and (c) where TQS attains its global negative and positive, respectively, maximum and in-between fluctuations of the weakly-smoothed TQS trace indicating the attractors-vs-repellers interplay). The advantage behind a long escape trajectory is that NavAb's specificity is optimized as entrance into CC is permitted only to ions that can re- and de-hydrate according to the local gradients of the energy landscape. Ions arriving in the center of the pore are fully-hydrated thus they can rapidly diffuse toward the hydropathically-diverse AG where a local attractor site is formed at $p_z \approx 19.0$ (see Fig. 3(a), and (c) where TQS attains its local negative maximum, and previous Section for properties of

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the atomic environment around $p_z \approx 19.0$). Finally, a weak, repeller mechanism at $p_z \approx -20.3$ attributed to the hydrophobic-to-hydrophilic interplay among the $M121$ and $S178$ side chains is likely to regulate ion access to the SF from the ES (see Fig. 3(a), and (c) where TQS attains its local positive maximum, and previous Section for properties of the atomic environment around $p_z \approx -20.3$).

Discussion

In this study we followed the trace of earlier works on cumulative hydropathic analysis of protein systems (see [36–39, 50]) and presented a computational scheme that allows for visual detection and analysis of hydropathic pattern formation around a NavCh's pore. Specifically, considering that structural symmetries play a fundamental role in biological pore (for a review see [57]), we developed a scaling analysis methodology that utilizes the tools of hydropathic density and hydropathic imbalance pore functions (see eq. m8 and m9, respectively) and returns two-dimensional spatial profiles (i.e., contour maps) of these experimental quantities where the relevant dimensions are unfolding perpendicular and parallel to the membrane surface. In the absence of structural pore symmetries, e.g., in the case of heteromeric eukaryotic NavCh channel such as the Nav1.7, our algorithm is still applicable but radial contributions to the hydropathic dipole field need to be taken into account. This can be done by introducing an appropriate color scheme for

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illustrating changes in orientation and magnitude of the radial hydropathic dipole field component (see eq. m7) so that a third contour map is constructed. For the sake of simplicity and due to limitations in computing time, we treated the pre-open I217C NavAb as a rigid entity by neglecting thermal atomic fluctuations. However, introduction of fluctuations at room temperature is not expected to affect contour pattern formation due to the linear additive scheme used for estimating cumulative effects. The same argument applies also for the choice of an atomic hydropathic value, namely, choosing a set of different atomic hydropathic indices, as well as, increasing the noise amplitude χ_i up to a threshold value is not expected to affect our findings as long as grouping of atoms into hydrophobic and hydrophilic ones remains unchanged.

We demonstrated that the spatial profiles of hydropathic density and hydropathic imbalance pore functions exhibit pseudo-symmetrical characteristics with respect to an axis parallel to the membrane surface so that the pore is roughly dichotomized with respect to the CC. This becomes evident if we focus on the relative positioning of the pairs $\{T_1^{(0)}, T_2^{(0)}\}$, $\{T_1^{(1)}, T_2^{(1)}\}$ and $\{T_3^{(1)}, T_4^{(1)}\}$ with respect to a symmetry axis that is placed perpendicular to the principal pore axis at $p_z \approx 2.8$ (Fig. 1(a), 2(a)). Accordingly, two qualitatively-different stability mechanisms coexist and co-determine I217C NavAb's functional placement within the membrane; at the microscopic regime, hydropathically-diverse pore-lining components are stabilized via an increase in their topological symmetry (see Fig. 2(a) formation of

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$\{T_1^{(1)}, T_2^{(1)}\}$ and $\{T_3^{(1)}, T_4^{(1)}\}$), while, toward the macroscopic regime, stabilization of the weakly-hydrophilic ensemble of PD and VSD atoms requires a gradual decrease in their topological similarity (see Fig. 2(a), shrinkage of $T_4^{(1)}$). The biophysical principle underlying this mode of organization is the optimization strategy dictating that burying of hydrophobic atoms around a protein's core contributes to protein stability [58] (the pre-open I217C NavAb's hydrophobic core was identified as the CC (see Fig. 1(a))).

Persistent extrema of the distributions $\phi^{(0)}$ and $\phi^{(1)}$ were utilized in order to investigate hydropathic gating properties of the pore. In particular, we demonstrated that the maxima of $sm(\phi^{(1)})$ identify pore locations where hydropathic topological and geometrical characteristics of the atomic environment favor the formation of a central hydrophobic gate (at $\mathrm{p}_z \approx 2.8$) regulating ion transport between the hydrophilic SF attractor (at $\mathrm{p}_z \approx -11.7$) and the hydropathically-diverse activation gate (AG) [21] (at $\mathrm{p}_z \approx 22.7$). Conservation analysis of the PDs highlighted the highly-conserved nature of the SF as already pointed out in [21] (see Fig. 3). Accordingly, the SF attractor topological configuration reflects an evolutionary-driven functional optimization of the PDs that might be relevant for other NavCh species as well. Given that these locations account for a smoothed, multiscale hydropathic effect, thermal fluctuations are expected to induce only small dislocations.

In summary, the strength of the presented methodologies is that they allow not only for a detailed mapping of multiscale hydropathic characteristics lining a NavCh's pore but also for elucidating how their underlying

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topology correlates with the pore's geometry at atomic resolution. This is a crucial step toward understanding where along a NavCh's pore hydropathic gates are likely to form and how they contribute to its functioning. A weaknesses, however, is that multiscale hydropathic mapping at atomic resolution is computationally expensive in comparison to traditional structural biology methods that operate at residue-level and that interpretation of observables can be far from trivial. To circumvent these complexities statistical summary measures, such as the distributions ψ and ϕ, can be employed alongside with targeted conservation analysis.

Author contributions

M.N.X. conceived the idea, designed the study and performed the analysis; R.W. and P.L. helped with the refinement of algorithmic steps; R.W., P.L., D.K., Y.Y., J.H., B.H.S. and S.G.W provided with critical feedback and helped with the interpretation of the results; Y.Y. and S.G.W encouraged M.N.X. to focus on specific aspects of the findings; B.H.S. helped with the supervision of the project; G.L. and C.G.F. were in charge of overall direction; M.N.X. wrote the manuscript in consultation with all the co-authors; PROPANE study group provided with technical and scientific support.

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Acknowledgments

The study was partly funded by the European Union 7th Framework Programme (grant n602273).

Competing Interests

The authors declare that there is no conflict of interest.

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Figure 1: **Spatial profile of the hydropathic density pore function.** (a), Contour map of $m^{(0)}(\mathbf{p}, l_\alpha(\mathbf{p}))$ for $\mathbf{p} \in Q$ and $\alpha = 1, 2, .., K_\alpha = 800$. Blue and red color contour domains represent hydrophobic and hydrophilic atomic structures around P, respectively. Black lines $R(\mathbf{p})$, $\bar{R}(\mathbf{p})$ and $L(\mathbf{p})$ depict structural pore characteristics. Magenta dashed line $\nu(\mathbf{p})$ indicates the structural boundary between the PDs and the VSDs (see Suppl. S2). Black arrows indicate the clustering behavior of zero-crossing points of $m^{(0)}(\mathbf{p}, l_\alpha(\mathbf{p}))$ found in $\Omega^{(0)}$ resulting in the formation of the contour domains $T_1^{(0)}$, $T_2^{(0)}$, $T_3^{(0)}$ and $T_4^{(0)}$. (b), Histogram of $\Omega^{(0)}$ along $l_\alpha(\mathbf{p})$-direction, $\psi^{(0)}$ (see Suppl. S4 for construction). (c), Histogram of $\Omega^{(0)}$ along $\mathbf{p}$-direction, $\phi^{(0)}$ where $sm(\phi^{(0)})$ represents a smoothed version of $\phi^{(0)}$ (see Suppl. S4 for construction). Grey shaded areas in (a),(c) mark pore regions where a transition from one pore-lining residue side chain to the next one takes place along P (see Suppl. S5). Dashed black horizontal lines in (a),(c) correspond to extrema of $sm(\phi^{(0)})$. IS stands for intracellular side and ES stands for extracellular side.

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Figure 2: **Spatial profile of the hydropathic imbalance pore function.** (a), Contour map of $m_z^{(1)}(\mathbf{p}, l_\alpha(\mathbf{p}))$ for $\mathbf{p} \in Q$ and $\alpha = 1, 2, .., K_\alpha = 800$. Blue and red contour domains represent configurations of $\vec{\boldsymbol{m}}_z^{(1)}(\mathbf{p}, l_\alpha(\mathbf{p}))$ with orientation toward the ES and toward the IS, respectively. Black lines $R(\mathbf{p})$, $\bar{R}(\mathbf{p})$ and $L(\mathbf{p})$ depict structural pore characteristics. Magenta dashed line $\nu(\mathbf{p})$ indicates the structural boundary between the PDs and the VSDs (see Suppl. S2). Black arrows indicate the clustering behavior of dipole centers found in $\Omega^{(1)}$ resulting in the formation of contour domains $T_1^{(1)}$, $T_2^{(1)}$, $T_3^{(1)}$ and $T_4^{(1)}$. Black arrow labeled as "$(*)$" indicates the clustering behavior of dipole centers for $l_\alpha(\mathbf{p}) > \nu(\mathbf{p})$. (b), Histogram of $\Omega^{(1)}$ along $l_\alpha(\mathbf{p})$-direction, $\psi^{(1)}$ (see Suppl. S4 for construction). (c), Histogram of $\Omega^{(1)}$ along $\mathbf{p}$-direction, $\phi^{(1)}$ where $sm(\phi^{(1)})$ represents a smoothed version of $\phi^{(1)}$ (see Suppl. S4 for construction). Grey shaded areas in (a),(c) mark pore regions where a transition from one pore-lining residue side chain to the next one takes place along P (see Suppl. S5). Dashed black horizontal lines in (a),(c) correspond to extrema of $sm(\phi^{(1)})$. IS stands for intracellular side and ES stands for extracellular side.

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Figure 3: **Summary of cumulative hydropathic topological analysis; topological description of single-molecule permeation energy landscape.** (a), Residue-level 3D hydropathicity mapping of a single PD structural unit (residue sequence: $M130$:$M221$) based on the Kapcha-Rossky hydropathic scale (see Suppl., Table S2) performed in pymol [41] computational environment. (b), Conservation analysis of a PD structural unit. Analysis was performed online on the ConSurf Server (http://consurf.tau.ac.il/) according to the algorithmic implementations described in [55, 56]. (c), A summary read-out of cumulative hydropathic topological analysis is provided in terms of the strongly- and weakly-smoothed topological quasiprobability score (TQS) (see Suppl. S8, eq. [S12]) plotted for $\mathbf{p} \in Q$. Geometrical pore characteristics are represented in terms of the pore radius trace, $R(\mathbf{p})$, plotted for $\mathbf{p} \in Q$. Pore points belonging to the subsets $Q_{2\cdot i+1, i=0,1,..,7}$ minimize their distance from the pore-lining residue side chains $M181$, $S178$, $E177$, $L176$, $I210$, $V213$, $C217$ and $M221$, respectively. Vertical green zones in (c) highlight the locations of persistent attractors and repellers along P corresponding to negative-value and positive-value maxima of TQS, respectively (see also Suppl. S8), and indicating the location and relative size of energy barriers imposed to permeating molecules (see Suppl. S8). ES stands for extracellular side, EF for extracellular funnel, SF for selectivity filter, CC for central cavity and AG for activation gate. Note that the SF residue side chains complex $T175$-$L176$-$E177$-$S178$-$W179$ averages the highest conservation score with 2.4 (out of 4.0) with pore-lining residue conservation scores confined within $[0, 2.0]$.

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