An open state of a voltage-gated sodium channel involving a $\pi$-helix and conserved pore-facing asparagine

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ABSTRACT Voltage-gated sodium (Nav) channels play critical roles in propagating action potentials and otherwise manipulating ionic gradients in excitable cells. These channels open in response to membrane depolarization, selectively permeating sodium ions until rapidly inactivating. Structural characterization of the gating cycle in this channel family has proved challenging, particularly due to the transient nature of the open state. A structure from the bacterium Magnetococcus marinus Nav (NavMs) was initially proposed to be open, based on its pore diameter and voltage-sensor conformation. However, the functional annotation of this model, and the structural details of the open state, remain disputed. In this work, we used molecular modeling and simulations to test possible open-state models of NavMs. The full-length experimental structure, termed here the $\alpha$-model, was consistently dehydrated at the activation gate, indicating an inability to conduct ions. Based on a spontaneous transition observed in extended simulations, and sequence/structure comparison to other Nav channels, we built an alternative $\pi$-model featuring a helix transition and the rotation of a conserved asparagine residue into the activation gate. Pore hydration, ion permeation, and state-dependent drug binding in this model were consistent with an open functional state. This work thus offers both a functional annotation of the full-length NavMs structure and a detailed model for a stable Nav open state, with potential conservation in diverse ion-channel families.

INTRODUCTION

Voltage-gated sodium (Nav) channels are membrane proteins that play an important role in the propagation of action potentials in excitable cells during nerve impulse conduction, among other physiological processes. These channels are involved in cardiac, muscular, and neurological disorders, making it important to understand the mechanisms that underlie their function (1). When the membrane reaches a threshold potential in a prototypical nerve cell, Nav channels open to allow Na$^+$ ions to flow inward, down their electrochemical gradient. Key to their function is a subsequent rapid inactivation that stops the ion flow and leaves time for the slow voltage-gated potassium channels to open, letting K$^+$ ions out and ultimately returning the cell to its resting potential (2).

A eukaryotic Nav channel comprises a single polypeptide chain of about 2000 residues, with four homologous domains arranged in a pseudotetrameric architecture, as verified by a handful of recent cryoEM structures reported in recent years (3–8). In contrast, bacterial Nav channels are homotetramers with $\sim$270 residues per subunit that have a simpler architecture with smaller intracellular and extracellular domains (9–26). Despite their limited
Bacterial Nav channels consist of three main domains: a voltage-sensing domain (VSD), a pore domain, and a C-terminal domain (Fig. 1A) (28). Each subunit VSD consists of four helices labeled S1–S4, arranged in a bundle. S4 contains several basic residues, which are positively charged at neutral pH and are responsible for sensing the changes in potential difference across the membrane. The VSDs are assigned to an activated conformation when these basic residues are displaced toward the extracellular side, relative to the resting conformation (10). The pore domain consists of the tetrameric arrangement of the S5 and S6 helices from each of the four subunits. The primary sequences of the pore domain and VSD are connected by a ~20-residue-long S4–S5 linker, which connects the pore domain to the VSD. The C-terminal domain includes a partially disordered neck region and a coiled coil at the C-terminus of the protein. In the resting state of the channel, the VSDs are presumed to occupy a resting conformation, and the pore is closed. In the open state, the VSDs are activated, and the pore is open. In the inactivated state, the VSDs remain activated, but the pore no longer allows ions to pass. The role of the C-terminal domain in the functional cycle is not yet fully established.

Many efforts have been made to determine the structure of Nav channels in different functional states. Bacterial Nav channels have provided some insights, including structures of at least six subtypes (NavAb, NavMs, NavRh, NavCt, NaChBac, and NavAe) in apparently distinct states (9–26). Still, these structures were solved in a nonnative environment (at cryogenic temperatures, solubilized by detergents) and often in the presence of mutations, such that their functional assignments can be ambiguous.

The resolution of open-state structures has proved particularly challenging. Because this state undergoes a spontaneous transition to the inactivated state under physiological conditions, trapping it for timescales sufficient for structure determination has often made it necessary to resort to protein modifications. In the case of NavMs, a bacterial Nav from Magnetococcus marinus, a full-length channel structure thought to be trapped in a conductive state was determined, based on the radius of the pore (24). However, several other factors, besides the pore radius, dictate whether a pore is conductive, such as hydrophobicity, hydration, and interaction with ions (29–33). In addition, the open state should enable access of open-pore blocker drugs to their binding site via a hydrated pathway across the gate (34,35).

Molecular dynamics (MD) simulations enable the direct visualization of atomistic interactions in and around a channel pore, including water, ions, or drugs, and can thus contribute to the functional assignment of experimental structures (30). MD simulations of the full-length NavMs structure have indeed questioned its functional assignment as an open state: its pore gate was shown to lose hydration over time, even in the presence of stabilizing interactions with the other channel domains (36). Thus the functional annotation of this structure, and its relationship, if any, to a stable open state remain unclear.

In this work, we used molecular modeling and MD simulations to test the open-state properties of NavMs models. Consistent with previous indications, the full-length x-ray structure was dehydrated; furthermore, even supraphysiological electric fields did not trigger wetting of the activation gate. Based on spontaneous transitions observed in these simulations, and sequence and structural features of related channels, we then constructed an alternative open-state model by introducing a π-helix in S6, N-terminal to the activation gate. Hydration, ion permeation, and drug binding in this model supported its annotation as a putative open state, providing a newly detailed testable mechanism for gating in the Nav family.

**MATERIALS AND METHODS**

**Model and simulation systems building**

Simulations in this work were based on the full-length x-ray structure of NavMs (PDB: 5HVX, UNIPORT: A0L5S6), with incomplete loops built using MODELLER 9.22 (37). The π-model was also built using MODELLER by shifting the sequence alignment in S6 of this template structure. Specifically, one gap was introduced in the template sequence immediately before Thr-207, and a second in the target sequence following Thr-234, thus shifting the target one position upstream from Thr-207 to the inner end of S6 (Fig. S1). This shift disrupted canonical i+4–i backbone hydrogen bonds of the first few residues in the shifted region, repositioning residues Thr-207 to Leu-210 to donate i+5–i backbone hydrogen bonds to the preceding unshifted residues. These interactions, characteristic of π-helices, were positioned to mimic a similar π-helix transition observed in TRP channels (38). Mutations were inserted using Visual Molecular Dynamics.
(VMD) (39). Each protein model was embedded in a homogenous lipid bilayer consisting of 362–400 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) molecules using the CHARMM-GUI Membrane builder (40). Each system was hydrated by adding a ~45 Å layer of water to each side of the membrane. Lastly, systems were ionized to reach a 150 mM NaCl concentration. Complexes of NavMs bound to lidocaine or flecainide were prepared by randomly placing the drug molecule in the central cavity and building the remainder of the system as described earlier. The Charmm36 force field was used to describe interactions between protein (41), lipids (42), and ions. Nonbonded fixes (NBFixes) were considered in the description of interactions between Na⁺ and carboxyl and carbonyl groups. The TIP3P model was used to describe the water particles (43).

**Drug molecules parametrization**

The parameters for lidocaine and flecainide (Fig. S2) were generated using CGENFF (44). Lidocaine has a pKa of 7.56, so it is 70% charged and 30% neutral at physiological pH. Flecainide has a pKa of 9.3, so it is 99% charged and 1% neutral at physiological pH (45). The charged forms of these drugs block the pore by entering it from the intracellular side via the so-called hydrophilic pathway (46–48). To evaluate the propensity of open-pore models to allow the binding of open-pore binders, we thus chose to model the charged form of lidocaine and flecainide. The lidocaine parameters were checked by calculating the free energy change between aqueous and membrane environments (Figs. S2 and S3). The system used consisted of one lidocaine molecule randomly placed in a homogenous lipid bilayer consisting of 200 POPC molecules, built using the CHARMM-GUI Membrane builder. These free energies were estimated using the accelerated weight histogram (AWH) method in GROMACS, simulating six walkers for 200 ns each. The collective variable was defined as the z component of the distance between the center of mass of lidocaine and a sodium ion localized in the lipid headgroup region restrained to its initial position. The average potential-of-mean-force (PMF) profile was calculated by averaging profiles estimated every 10 ns over the last 40 ns of the simulation, and the error bars were estimated by calculating the standard deviation over this data set. The free-energy profile and the preferential drug localization below the lipid-solution interface agreed with previous studies (49). In addition, the drug-membrane partition coefficient for charged lidocaine (log(P)) is 1.49 (50), yielding a free energy of lipid-water partitioning ΔG ~ −8.5 kJ/mol, approaching the free-energy difference determined in our simulations (ΔG ~ −11 kJ/mol). Although experimental partition coefficients were not readily available for charged flecainide, based on this validation with lidocaine, we proceeded with CGENFF parameters for both drugs.

**Simulation parameters**

Simulation systems without drugs were energy minimized for 15,000 steps using steepest descent and equilibrated with a constant number of particles, pressure, and temperature (NPT) for 36–45 ns in several steps, as also reported in Kasimova et al. (65). During the first 4 ns, the temperature was set at 340 K to allow for appropriate relaxation of lipid tails. The protein and lipid headgroup positions were restrained with a force constant of 1000 kJ/mol nm². In the next 4 ns, the temperature was set to 300 K and the position restraints on the lipid headgroups were released. In the remaining steps (40 ns), the position restraints on the protein were gradually released by varying the force constant from 1000 kJ/mol nm² to 0 kJ/mol nm². The protein-drug systems, equilibration was performed following the CHARMM-GUI default equilibration protocol, with the length of each step increased (to 2 ns for each of the first three steps and 10 ns each for the last three steps). In the first two steps, NVT equilibration was carried out, followed by NPT equilibration in subsequent steps. The restraints on the protein were gradually released by varying the force constant from 4000 kJ/mol nm² to 0 kJ/mol nm² for the protein backbone and varying the force constant from 2000 kJ/mol nm² to 0 kJ/mol nm² for side chains. During equilibration, pressure was maintained at 1 bar through Berendsen pressure coupling; temperature was maintained at 300 K/340 K through Berendsen temperature coupling (51) with the protein, membrane, and solvent coupled. Finally, unrestrained equilibrium simulations were run using a timestep of 2 fs, Parrinello-Rahman pressure coupling (52), and Nose-Hoover temperature coupling (53). The LINCS algorithm (54) was used to constrain bonds involving hydrogen atoms. For long-range interactions, periodic boundary conditions and particle mesh Ewald were used (55). For short-range interactions, a cutoff of 12 Å was used. Simulations were performed using GROMACS (versions 2016, 2019, and 2020) (56,57). In certain simulations (Table 1), a transmembrane voltage of +750 mV or −750 mV was applied in the form of an external electric field to the equilibrated NavMs z-model to study electrowetting. The transmembrane voltage was calculated as the product of the electric field by the simulation box length along the membrane normal. Note that in those simulations, volume was kept constant (sampling the NVT ensemble), thus avoiding fluctuations in the z dimension of the simulation box and hence in the transmembrane voltage applied. Equilibrium simulations described in this work are summarized in Table 1.

**Ion permeation free energy calculations**

The free energy of permeation of a Na⁺ ion along the channel pore axis was calculated using the AWH method (58). For each equilibrated structure, we applied an independent AWH bias and simulated six walkers for ~200 ns each (representing a total of ~1200 ns), sharing bias data and contributing to the same target distribution. The bias acts on the z axis defined using the center-of-mass distance between one central sodium ion and the L177 residues in NavMs. The target distribution was chosen to be flat. The sampling interval was chosen as the entire box length. To keep the sodium ion close to the pore, its distance from the pore central axis was restrained below 10 Å by adding a flat-bottom umbrella potential. The rate of change of each AWH bias was initialized by setting the average free-energy error to 20 kJ/mol and the diffusion constant to 0.00005 nm²/ps. The positions of all Cα atoms of the protein were restrained by imposing harmonic potentials with force constants of 1000 kJ mol⁻¹ nm⁻². Conversion was assessed by monitoring the evolution of the free energy profile and target distribution over time. The average PMF profile and associated uncertainties were

**TABLE 1 Summary table of equilibrium simulations performed**

| System                      | Equilibration | Production          |
|-----------------------------|---------------|---------------------|
| NavMs z-model               | 48 ns         | 400 ns              |
| NavMs z-model               | 2 ns          | 90 ns (with backbone restraints) |
| NavMs z-model +750mV        | start from end of NavMs z-model | 800 ns production |
| NavMs z-model −750mV        | start from end of NavMs z-model | 8000 ns production |
| NavMs π-model               | 48 ns         | 800 ns              |
| NavMs π-model lidocaine     | 36 ns         | 200 ns              |
| NavMs π-model flecainide    | 36 ns         | 200 ns              |
| NavMs π-model flecainide    | 36 ns         | 200 ns              |
| NavMs π-model N212A         | 48 ns         | 505 ns              |
| NavMs π-model N212L         | 48 ns         | 452 ns              |
| NavMs π-model N212C         | 48 ns         | 456 ns              |

In this work, extended individual simulations were prioritized over shorter replicate conditions, to capture longer continuous time series and monitor the developing response of the system to perturbations. Where needed, we carried out enhanced sampling simulations to recover equilibrium probability distributions (Table 2).
calculated from a single AWH walker, since all walkers communicate with one another. The average PMF profile was calculated by taking the data from the last 100 ns in intervals of 10 ns, and the error bars were estimated by calculating the standard deviation over this data set. The free-energy profiles were then shifted to set the reference to the center of the bilayer and allow a direct comparison with the water density profiles estimated using the channel annotation package (CHAP).

Drug binding free energy calculation
The free energy of binding of pore blockers lidocaine and flecainide was calculated as described earlier, except for the following parameters. For drugs, the bias acted on the $z$ axis defined by the center-of-mass $z$ distance between all atoms of the drug and the E178 residues. The sampling interval was restricted to the interval 0.8–3.0 Å by imposing harmonic restraints. Other simulation details are the same as for the ion permeation free energy calculation described earlier. Convergence, average profiles, and error estimates were assessed as described earlier for the ion permeation free energy profiles.

Analysis
The water number density and hydration free energy were calculated using CHAP (30,59). The C-terminal domain was removed from the system for this analysis. The plots produced show the time-averaged water number densities and hydration free energies. Error bars represent the standard deviation over this data set. The free-energy profiles were computed for each system and are shown to alter the surface tension of water at hydrophobic surfaces and may result in the hydration of transmembrane constriction in this region (Fig. 2 B) (36). Given the close packing of S6 against the full length of S5 and the S4–S5 linker in this structure (Fig. 2 C and D), a local expansion sufficient to hydrate the activation gate does not appear possible, barring substantial rearrangement of the already fully activated VSD as well as pore domain. Thus, our simulations confirmed that the full-length NavMs structure—termed the $\alpha$-model, as described in subsequent sections—is unlikely to represent an open state.

Transmembrane potential is insufficient to consistently hydrate the pore
Having observed the experimental structure to be nonconducting at 0 mV, we further asked whether the application of a transmembrane potential might stimulate conformational transition to a hydrated, more plausibly open state. Indeed, the introduction of an electrical potential has been known to alter the surface tension of water at hydrophobic surfaces and may result in the hydration of transmembrane pores via an electrowetting phenomenon (61,62). We therefore probed whether electrowetting might also enable hydration of NavMs. To do so, we applied external electric fields resulting in transmembrane potentials of around $+750$ mV and $-750$ mV to the so-called $\alpha$-model, and we computed the time-averaged water density during further unrestrained simulations, initially up to 800 ns. Applying external

| System                  | Production          |
|-------------------------|---------------------|
| NavMs $\alpha$ model $\text{Na}^+$ | 200 ns $\times$ 6 walkers |
| NavMs $\pi$ model $\text{Na}^+$   | 172 ns $\times$ 6 walkers |
| NavMs $\alpha$ model lidocaine | 200 ns $\times$ 6 walkers |
| NavMs $\pi$ model lidocaine  | 200 ns $\times$ 6 walkers |
| NavMs $\alpha$ model flecainide | 200 ns $\times$ 6 walkers |
| NavMs $\pi$ model flecainide  | 200 ns $\times$ 6 walkers |
| NavMs $\pi$ model N214L $\text{Na}^+$ | 200 ns $\times$ 6 walkers |
| Lidocaine-membrane       | 122 ns $\times$ 6 walkers |

### RESULTS

The full-length NavMs x-ray structure has a dehydrated pore
To test the functional annotation of NavMs as a model for gating, we first examined the hydration of the channel pore using MD simulations. The full-length x-ray structure of NavMs (PDB: 5HVX) was initially proposed to represent the open state, based on its radius at the activation gate (Fig. 2 E) (24). However, pore hydration has been considered a prerequisite for conduction in ion channels and may provide more informative metrics than geometric radius alone (30). Indeed, the capacity of this structure for conduction was recently challenged based on its propensity to become dehydrated in MD simulations (36). In our hands, the pore radius of full-length NavMs contracted slightly (~0.5 Å) during backbone-restrained equilibration in a lipid bilayer (Fig. S4 C). In addition, the pore remained contracted and dehydrated during an independent 90-ns equilibrium simulation conducted in the presence of backbone restraints (Fig. S5). During subsequent unrestrained simulations, the pore maintained a stable profile over 400 ns (Figs. 2 F, S4 A and B, S6 A). Throughout this run, water density was effectively zero near the activation gate (designated $-1$ nm along the pore axis; Fig. 2 A). The molecular determinants of this dehydration appeared to be the pore-facing Leu-211 and Ile-215 residues, which created a hydrophobic constriction in this region (Fig. 2 B) (36). Given the close packing of S6 against the full length of S5 and the S4–S5 linker in this structure (Fig. 2 C and D), a local expansion sufficient to hydrate the activation gate does not appear possible, barring substantial rearrangement of the already fully activated VSD as well as pore domain. Thus, our simulations confirmed that the full-length NavMs structure—termed the $\alpha$-model, as described in subsequent sections—is unlikely to represent an open state.
electric fields did indeed increase the pore hydration at the activation gate (Fig. 3), but water density remained substantially less than that of the bulk solvent. In fact, the pore repeatedly alternated between wetted and dewetted states at both +750 mV (depolarized, blue) or −750 mV (hyperpolarized, green), with standard deviations (gray) and bulk-water density (dashed blue). Inset on the right shows an aligned view of the pore-lining S5–S6 helices from two subunits. The −1 nm region (activation gate) is only partially hydrated by application of an electric field.

**Formation of a helical defect in S6 and rotation of a conserved asparagine at hyperpolarized potentials**

Interestingly, during extended simulations (>8 μs) of NavMs at −750 mV a kink formed spontaneously in the S6 helix of one subunit, with disruption of the backbone hydrogen bond between residues Val-210 and Phe-214 (Video S1). Rotation of S6 C-terminal to this kink led the hydrophobic side chains of residues Leu-211 and Ile-215 to reorient away from the activation gate. In their place, an asparagine residue (Asn-212) facing the S4/S5 linker rotated inward toward the pore (Fig. 4 A–C). This asparagine is the only hydrophilic residue in the lower S6 segment of NavMs and is conserved across several bacterial and eukaryotic sodium channels, raising the possibility of its importance for channel function (63) (Fig. 4 D). Indeed, the equivalent asparagine has also been shown to mediate coupling between the VSD and pore domain in the eukaryotic channels Nav1.4 and Nav1.5 (64) and to come in contact with the inactivation Ile-Phe-Met (IFM) particle of the channels solved in an inactivated state (3,4,7,8). Distortions in S6 have also been observed, where the classical α-helical backbone hydrogen bonding pattern is disrupted and a π-helix formed, along with inward orientation of the asparagine residue, in various subunits of structures of eukaryotic Nav channels (NavEe1, NavPaS, Nav1.4, Nav1.5, and Nav1.7; Fig. 4 E and G). Interestingly, a π-helix transition and asparagine reorientation were recently proposed to be involved in pore opening in the related TRP channel family (65–67). Based on the spontaneous behavior observed in our simulations, and collective evidence for a conserved gating mechanism, we hypothesized that an S6 distortion and coordinated rotation placing Asn-212 in a pore-facing orientation (Fig. 4 F and G) might contribute to stabilizing a conductive pore in NavMs (Fig. S8, Video S1).

**π-Helix formation in S6 enables pore hydration**

To test the plausibility of an open NavMs state containing a π-helix and a pore-facing Asn, we prepared a symmetrized...
system—termed hereafter the \( \pi \)-model—containing a \( \pi \)-helix and inward-facing asparagine in all four subunits. This model was prepared by introducing a \( \pi \)-helix six positions before the conserved asparagine, using homology modeling to the original structure with gaps at strategic positions in the alignment to shift the sequence by one residue (Fig. S1). This realignment resulted in the disruption of backbone hydrogen bonding from the –NH group of residue Thr-207, and rotation of the remainder of S6, leading Asn-212 to reorient toward the pore (Fig. S4 E). During equilibration and production MD simulations, the pore profile was largely stable (pore radius standard deviation within 1 Å; Figs. S4 D, S6 B, and D); notably, Asn-212 remained in a pore-facing position (Fig. S4 F), with the unpaired hydrogen bond characteristic of the \( \pi \)-helix motif varying between Thr-207 and Phe-210. Moreover, in contrast to the \( \alpha \)-model, the most constricted region of the pore equilibrated to \(~3\) Å radius and was completely hydrated throughout our unrestrained simulations of the \( \pi \)-model (Figs. S6 B and 5 A), with water density similar to bulk solvent across the entire pore. Indeed, the free-energy profile for solvation was effectively flat for the \( \pi \)-model, in contrast to barriers up to 3 kcal/mol in our previous \( \alpha \)-model simulations (Fig. 5 B).

### The \( \pi \)-model conducts Na\(^{+}\) ions

To further probe the conductive properties of our NavMs \( \pi \)-model, we computed the sodium ion permeation free-energy profiles along the bottom half of the pore axis, using the AWH method of enhanced sampling (58). The barrier to sodium conduction was over 25 kcal/mol at the activation gate for the \( \alpha \)-model, but was effectively zero for the \( \pi \)-model, indicating that sodium permeates this state readily (Fig. 6 A, S9, S10). We further tested the contribution of the conserved asparagine to this apparent open state, by running simulations with various hydrophobic residues substituted at position 212 in the \( \pi \)-model. Although substitutions to Cys, Ala, or Leu did not decrease hydration in this model (Fig. 6 B), sodium ions interacted more favorably with Asn-212 than with a substituted leucine at position 212 (Fig. 6 C). Indeed, substitution to Leu increased the barrier to sodium permeation at the gate by over 1 kcal/mol (Figs. 6 D and S11). These results highlight the importance of monitoring ion as well as water interactions in the channel pore and indicate a direct role for the conserved Asn in conduction.

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1. | \( \pi \)-helix formation (shaded blue) on the orientation of a conserved asparagine (yellow star) in S6. For orientation, S5 helices (light gray) are shown from the extracellular side, showing only S6 helices (gray). The kink is evident in the leftmost subunit, with coordinated rotation of Asn-212 toward the channel pore. Dashed line indicates orientation vector used in (E), with respect to the leftmost subunit. (C) Superimposition of the pore-facing helices in the structure (\( \alpha \)-model, dark gray) and after kink formation (light gray). (D) Sequence alignment of S5–S6 regions from six bacterial Nav channels (top), with the conserved asparagine (Asn-212 in NavMs) highlighted in yellow. Alignments of all four pseudo-homologous S6 helices in nine eukaryotic Nav channels (bottom) show a similarly conserved asparagine. (E) Orientation of the conserved asparagine in a set of experimental structures. For each asparagine, orientation is defined by a projection of \( C_\alpha–C_\gamma \) onto an \( x-y \) plane perpendicular to the pore \( (z) \) axis, where \( x \) is a vector from \( C_\alpha \) to the pore center as shown in (B). Residues in the shaded blue region orient toward the channel pore. (F) Cartoon depiction of the effect of kink or \( \pi \)-helix formation (shaded blue) on the orientation of a conserved asparagine (yellow star) in S6. In the \( \alpha \)-model (left), the asparagine faces \( S_5 \), away from the pore; in the \( \pi \)-model, it rotates into the pore. (G) Homologous regions of S6, with the channel pore at right, in structures of the representative channels NavPaS (PDB: 6A91), Nav1.7 (PDB: 6J8I), NavEe1 (PDB: 5XSY), and the NavMs \( \pi \)-model (PDB: 5HVX) and \( \alpha \)-model. Each structure shows canonical i+4–i (solid gray), disrupted i+4–i (dashed gray), and noncanonical (bold black) hydrogen bonds; i+5–i interactions, characteristic of \( \pi \)-helices (blue circles), are indicated in the middle of the pore. For clarity, only backbone atoms are shown, except for the conserved asparagine (yellow). Formation of a \( \pi \)-helix is associated with orientation of the asparagine toward the pore.

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**FIGURE 4** A helix kink and conserved asparagine at the activation gate of NavMs. (A) Representative frame from an extended simulation of the \( \alpha \)-model at \(-750\) mV, showing the S6 helices (gray) from the membrane plane. A spontaneous kink (shaded blue) is evident in a proximal S6 helix, N-terminal to Asn-212 (yellow). For orientation, S5 helices (white) are shown for the left and right subunits. (B) Representative frame as in (A), viewed from the extracellular side, showing only S6 helices (gray). The kink is evident in the leftmost subunit, with coordinated rotation of Asn-212 toward the channel pore. Dashed line indicates orientation vector used in (E), with respect to the leftmost subunit. (C) Superimposition of the pore-facing helices in the structure (\( \alpha \)-model, dark gray) and after kink formation (light gray). (D) Sequence alignment of S5–S6 regions from six bacterial Nav channels (top), with the conserved asparagine (Asn-212 in NavMs) highlighted in yellow. Alignments of all four pseudo-homologous S6 helices in nine eukaryotic Nav channels (bottom) show a similarly conserved asparagine. (E) Orientation of the conserved asparagine in a set of experimental structures. For each asparagine, orientation is defined by a projection of \( C_\alpha–C_\gamma \) onto an \( x-y \) plane perpendicular to the pore \( (z) \) axis, where \( x \) is a vector from \( C_\alpha \) to the pore center as shown in (B). Residues in the shaded blue region orient toward the channel pore. (F) Cartoon depiction of the effect of kink or \( \pi \)-helix formation (shaded blue) on the orientation of a conserved asparagine (yellow star) in S6. In the \( \alpha \)-model (left), the asparagine faces \( S_5 \), away from the pore; in the \( \pi \)-model, it rotates into the pore. (G) Homologous regions of S6, with the channel pore at right, in structures of the representative channels NavPaS (PDB: 6A91), Nav1.7 (PDB: 6J8I), NavEe1 (PDB: 5XSY), and the NavMs \( \pi \)-model (PDB: 5HVX) and \( \alpha \)-model. Each structure shows canonical i+4–i (solid gray), disrupted i+4–i (dashed gray), and noncanonical (bold black) hydrogen bonds; i+5–i interactions, characteristic of \( \pi \)-helices (blue circles), are indicated in the middle of the pore. For clarity, only backbone atoms are shown, except for the conserved asparagine (yellow). Formation of a \( \pi \)-helix is associated with orientation of the asparagine toward the pore.
interface of membrane and solvent, an interfacial region characterized by its hydrophilicity (Figs. S2 and S3). Thus, drug binding as well as ion permeation and pore hydration calculations supported the annotation of the NavMs \( \pi \)-model as a putative open state, determined at least in part by the pore orientation of the conserved asparagine.

**DISCUSSION**

The full-length NavMs structure (\( \sigma \)-model) initially appeared to be representative of an open functional state, based on the pore radius estimated at the activation gate (24). MD simulations, however, consistently report that this conformation is dehydrated, possibly reflecting the closure of a hydrophobic gate (36). This dewetting is observed in absence of substantial rearrangements of the pore backbone, stabilized by contacts between the intracellular domains and the lower parts of the VSD and S4-S5 linker, resulting in the backbone helices positioned in a splayed, open conformation (68). Instead, such a behavior can be ascribed to subtle side-chain rearrangements of hydrophobic residues at the level of the gate (Leu-211 and Ile-215). A conformational change in which the rotation of the C-terminus results from the introduction of a \( \pi \)-helix upstream of Asn-212, on the other hand, leads to full hydration of the pore (\( \pi \)-model). Ion permeation and open-pore blocker binding profiles are consistent with the assignment made on the basis of hydration: the original \( \sigma \)-model is impermeable to \( \text{Na}^+ \) and open-pore blockers, whereas the \( \pi \)-model appears to display determinants of an open state. This is consistent with previous MD simulations reporting \( \text{Na}^+ \) conduction of \( \sigma \) models only when restraining the protein or inserting mutations that promote pore hydration (36,69).

An approach we have used to assess the potential of the two models for representing open functional states has been to consider the accessibility of the inner cavity to open-pore blocker drugs. According to the guarded receptor hypothesis, open-pore blockers can only access their binding site in the channel when the activation gate is open (Fig. S12) (34). Channel opening should indeed open the activation gate, opening up an access pathway for the drug to reach its binding site in the pore. Upon inactivation, the drug is trapped in the pore as the activation gate closes. In the inactivated state, drugs cannot access the pore from the intracellular side as the pore is closed (guarding the drug receptor binding site). On top of confirming the validity of the \( \pi \)-model as an open-state model, these simulations further revealed the potential molecular determinants of interaction of these drugs and propose a role for Asn-212 in binding flecainide.

An obstructed, nonconductive pore can correspond to a closed functional state (usually coupled to deactivated VSDs) or to an inactivated or preopen functional state (coupled to activated VSDs). The latter two are difficult to

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**Open-pore blockers bind in the pore cavity of the \( \pi \)-model**

As a final test of the functional relevance of our \( \pi \)-model as a NavMs open state, we investigated its capacity for state-dependent drug binding. According to the guarded receptor hypothesis, open Nav channels must allow open-pore blockers—such as the antiarrhythmic drugs lidocaine and flecainide—to enter the pore cavity from the cytoplasm (Fig. S12) (34). We therefore calculated the free energy profiles of binding of lidocaine and flecainide along the bottom half of the pore of both the \( \sigma \)- and \( \pi \)-models. Similar to \( \text{Na}^+ \) permeation free energy profiles, the drug binding free energy profiles for charged lidocaine and flecainide featured large free energy barriers to enter the central cavity via the activation gate in the \( \sigma \)-model (barriers >20 kcal/mol) but were effectively unimpeded in the \( \pi \)-model (Figs. 7 and S13–S16). Interestingly, the binding site of flecainide/ lidocaine in the \( \sigma \)-model is similar to the one reported in NavAb (11). In contrast, in the \( \pi \)-model, flecainide engages in interactions with pore-facing Asn-212, whereas the smaller lidocaine appears to preferentially bind higher up in the central cavity, forming interactions with Thr-207 (Fig. 7). Note that its binding in a hydrophilic region of the pore is reminiscent of its preferred localization at the

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**FIGURE 5** Pore hydration in a NavMs model with an introduced \( \pi \)-helix (\( \pi \)-model). (A) Time-averaged number density of water projected along the pore axis of the \( \pi \)-model (red), with standard deviation in transparent representation, and bulk-water density as a dashed blue line. Inset at right shows an aligned view of the pore-lining S6 helices in a representative simulation frame, with proximal water molecules in blue, and the conserved asparagine in yellow. The -1 nm activation gate is clearly hydrated. (B) Free-energy profiles of pore hydration, derived from water densities, projected along the lower half of the pore axis for the \( \sigma \)-model at 0 mV (black), -750 mV (green), or +750 mV (blue), or for the \( \pi \)-model (0 mV, red). Shaded regions indicate standard deviations; overlaid cartoon shows an aligned view of two opposing S6 helices.
distinguish based on their structural features only. Indeed, structures of another bacterial channel (NavAb) with activated VSD and obstructed pore have been proposed to represent preopen (10) and inactivated states (15). This functional assignment, however, rests largely on the assumption that the slow-inactivation mechanism is conserved between BacNavs and eukaryotic channels (15,70), which remains to be firmly established. In addition, the molecular basis for slow inactivation remains unclear, even in eukaryotic channels, and may involve several functional and structural states (70,71). Toxin binding and mutations in the outer pore region, selectivity filter, pore helix, and S6 have all been shown to modify the slow inactivation phenotype of Navs (72–84). Whether the α-model of NavMs is then likely to represent one of the inactivated states is a possibility that remains to be explicitly tested.

Our extensive simulations of the α-model of NavMs under depolarized potentials showed a spontaneous conformational change, namely the disruption of α-helical h-bonding pattern and the formation of a defect reminiscent of a π-helix formation, and the consequent rotation of Leu-211 and Ile-215 away from the pore lumen coupled to the rotation of Asn-212 into it. How might this rotation be energetically allowed? At first glance, the introduction of an h-bond defect in an α-helix might seem prohibited. Nevertheless, structures of both Nav and TRP channels containing unpaired h-bonds involved in α-helices have been resolved, suggesting such a conformational change is energetically accessible (Fig. 4 G). Energetic stabilization from protein-protein contacts (Figs. S17 and S18) or protein-solvent might compensate the energetic cost associated with disrupting a canonical α-helical structure (38,85). We note in particular that part of the stabilization energy of the open pore appeared to originate from interactions between the C-terminus of S6 and the CTD domain, in particular through the central cavity environment becomes unfavorable for the hydrophobic residues lining the pore (Leu-211 and Ile-215). These thus tend to reorient to face away from the pore. Concomitantly, hydrophilic Asn-212 finds a favorable environment in a pore-facing configuration. Both these molecular-level effects presumably lead to the rotation of this section of S6 and the formation of the π-helix.

Asn-212 has previously attracted attention as the only hydrophilic residue in an otherwise hydrophobic region (86). Furthermore, this residue has an outstanding conservation in Bac Navs and eukaryotic Navs (63) and is also conserved in TRP channels, which are only distantly phylogenetically related. In TRPV1, pore wetting occurred as a consequence of asparagine residues localizing in a pore-facing conformation (65), thus demonstrating the interplay between hydration and Asn orientation. Nevertheless, contrary to our expectations, our in-silico mutagenesis study showed that a substitution of hydrophilic Asn-212 by hydrophobic residues did not affect pore hydration, demonstrating that the physicochemical properties of the residue at this position are not a crucial determinant of pore hydration. Instead, it
appears that it is rather the removal of Leu-211 and Ile-215 from the pore constriction that results from the helix rotation that are determinant for the transition from an obstructed dehydrated state to a hydrated conductive one.

Although the importance of the Asn residue is clearly substantiated by its conservation throughout evolution, its specific role in our model thus remains quite mysterious. One aspect that appears important is that it plays a role in the ion permeation process, by interacting favorably with permeating Na\(^+\) ions. Its substitution by a hydrophobic residue indeed raises slightly the permeation free energy barrier, possibly playing a role in determining single channel conductance. In addition, the conserved Asn may play a role in inactivation, though the structural basis for this phenomenon remains largely unknown. The mutation of this Asn to Asp in NaChBac leads to enhanced inactivation, whereas the mutation to any other residue is nonfunctional (86). In eukaryotic channels, the conserved Asn itself has been implicated in inactivation of Nav1.4 and Nav1.2a (79,87), and a direct interaction between the Asn on DIV-S6 (facing away from the pore lumen) and the Ile-Phe-Met particle responsible for fast inactivation is found in structures of eukaryotic Nav channels solved in inactivated states (3,4,7,8). Of particular note, in all these structures, the S6 helix of DIV is systematically α-helical, whereas the S6 of the other domains may feature h-bonding defects and formation of π-helix segments coupled to the orientation of the conserved Asn into the pore domain (Fig. 4 E and G). Thus, although we are so far unable to pinpoint precisely the role of this Asn residue, a set of clues point toward its implication in inactivation in Nav channels.

Scrutinizing TRP channel structures may provide additional clues into the stabilization of π-helices. In TRPV1 and TPC1 channels, the side chain of the conserved asparagine residue forms hydrogen bonds with the backbone of a residue four positions above it (88). Our simulation of the π-model (Fig. S17 A and B) as well as structures of sodium channels in which helical defects are observed (Fig. S17 C) also feature such hydrogen bonds between the side chain of the asparagine and the backbone of a residue located a helical turn away.

Taken together, our study also highlights the relevance of molecular modeling and MD simulations for improving our understanding of structure-function relationship in ion channels. Nevertheless, one should be mindful of the limitations of the technique, and in particular of the imprecisions of the interaction model (force field) at the basis of the algorithm, in particular when modeling noncanonical helices (89). Water models may be of specific concern in this study, given that their ability to reproduce the behavior of confined water droplets or single water molecules has been questioned (90,91). Here, we have used the...
CHARMM force field with the TIP3P water model, a common choice when simulating membrane proteins. We note that previous work on very similar systems had probed the effect of changing water models on the hydration of the NavMs pore. Simulations carried out with the AMBER force field had revealed no major discrepancies (36), giving us confidence that these findings are robust with respect to the choice of force field.

CONCLUSION

In conclusion, this work confirmed that the full-length NavMs structure reflects a nonconductive state, which is possibly reflective of an inactivated channel. In addition, based on spontaneous conformational change and scrutinizing experimental structures of eukaryotic Nav channels, we proposed a structural change that leads to the pore assuming a conductive state: introducing a \( \pi \)-helix results in the rotation of S6 in a manner compatible with pore hydration. This conformational change is coupled to the orientation of a conserved Asn residue into the pore. Since this residue is conserved throughout Nav channels, we hypothesize that such an opening model may apply to eukaryotic channels.

SUPPORTING MATERIAL

Supporting material can be found online at https://doi.org/10.1016/j.bpj.2021.12.010.

AUTHOR CONTRIBUTIONS

K.C., M.K., S.M., and L.D. designed the research. K.C., M.K., and S.M. performed the experiments and analyzed the results. All the authors interpreted the data. K.C., R.H., and L.D. wrote the manuscript.

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