Mechanism of pH-dependent activation of the sodium-proton antiporter NhaA

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Escherichia coli NhaA is a prototype sodium-proton antiporter, which has been extensively characterized by X-ray crystallography, biochemical and biophysical experiments. However, the identities of proton carriers and details of pH-regulated mechanism remain controversial. Here we report constant pH molecular dynamics data, which reveal that NhaA activation involves a net charge switch of a pH sensor at the entrance of the cytoplasmic funnel and opening of a hydrophobic gate at the end of the funnel. The latter is triggered by charging of Asp164, the first proton carrier. The second proton carrier Lys300 forms a salt bridge with Asp163 in the inactive state, and releases a proton when a sodium ion binds Asp163.

These data reconcile current models and illustrate the power of state-of-the-art molecular dynamics simulations in providing atomic details of proton-coupled transport across membrane which is challenging to elucidate by experimental techniques.

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Sodium-proton antiporters play a major role in regulating H\(^+\) and Na\(^+\) concentrations in the cell. NhaA from *Escherichia coli* is perhaps the most widely studied one, as evident from two high-resolution crystal structures and abundant biochemical and biophysical data. NhaA exchanges two protons in the periplasm for one sodium in the cytoplasm, and its activity is tightly regulated by pH (ref. 2). Below pH 6.5 NhaA appears inactive, while between pH 7 and 8.5 the sodium efflux rate increases dramatically, with a maximum around pH 8.7 (refs 3–5). The first atomic-resolution crystal structure of NhaA solved at pH 4 (ref. 6) revealed a bundle of 12 transmembrane helices (TM) forming the inward-facing conformation where the ion passage funnel opens to the cytoplasm (Fig. 1a). Remarkably, TM IV and XI are interrupted by extended chains crossing each other in the middle of the membrane, where Asp163, Asp164, Thr132 and Lys300 form the active site responsible for ion binding. Asp163 and Asp164 are highly conserved and their mutations completely abolish the activity of NhaA. Thr132, Asp133 and Lys300 are also well conserved and their mutations impair the antiporter activity. Biophysical and biochemical experiments suggested that Asp163, Asp164 and Thr132 are involved in ion binding.

Based on the first crystal structure of NhaA, Poisson–Boltzmann (PB) continuum electrostatics calculations with an effective protein dielectric constant of 4 showed that the pK\(_a\)’s of Asp163 and Asp164 are shifted above 15, thus suggesting the two residues as the proton carriers required for exchanging a sodium ion. Supported by Cys-scanning mutagenesis experiments, Padan and coworkers proposed that a ‘pH sensor’, a cluster of residues at the entrance of the cytoplasmic funnel (Fig. 1a), is responsible for the pH dependence of the NhaA activity. They further proposed that a pH signal from the cytoplasm results in deprotonation of a single pH-sensing residue, presumably Glu78 based on the PB calculations, which is transduced down to the active site triggering a conformational change and consequently the pK\(_a\) downshifts of Asp163 and Asp164, leading to the release of two protons.

The above pH activation-based allosteric mechanism was recently challenged by a new set of data. Electrophysiology experiments showed that, under symmetric, saturating sodium concentration and pH 8.5 on the periplasmic side, reverse transport persists when pH on the cytoplasmic side is lowered from 8 to 5 (ref. 4). Together with studies on related antiporters, it prompted the proposal of an alternative model, in which Na\(^+\) and H\(^+\) directly compete for binding to a common site. The identity of the residues involved in the binding site remained however speculative. Measurements of lithium binding to mutants of Lys300 suggested its positive charge and pK\(_a\) are essential for antiporter activity, possibly by affecting sodium binding.

Additional evidence for the involvement of Lys300 came from a new crystal structure of NhaA, solved at the same pH condition and diffraction resolution as the previous one, showing a salt bridge between Lys300 and Asp163 (Fig. 1b). An equivalent salt bridge is also seen in the crystal structures (solved at pH 7.8) of the homologous sodium-proton antiporter NapA from *Thermus thermophilus* in the outward- and inward-facing states. Like NhaA, NapA is electrogenic and therefore the Lys300–Asp163 salt bridge is likely of functional importance.

The competitive binding model is also supported by the recent molecular dynamics (MD) simulations based on the new crystal structure of NhaA and combinations of protonation states of Asp163/Asp164/Lys300. The simulations revealed that when Asp164 was protonated, no sodium binding occurred, and the salt bridge remained intact between charged Asp163 and Lys300; however, when Asp164 was deprotonated, sodium entered and bound to Asp163 and Asp166, which destabilized the salt bridge. Calculations using PROPKA based on the trajectory snapshots suggested that Lys300 is likely charged when it is in salt bridge with Asp163. These results, together with the recent crystal structures of NapA, as well as biochemical experiments, led to a modified competitive binding mechanism. At low pH in the inward-facing state of NhaA, protons are bound to Asp164 and Lys300; the latter forms a salt bridge with the deprotonated Asp163. Sodium entrance to the active site results in binding to Asp163 and Asp164. The former leads to the breakage of the salt bridge and release of a proton from Lys300, while the latter represents a direct competition with a proton and leads to the deprotonation of Asp164. Following the proton-sodium exchange, a conformational switch from the inward- to the outward-facing conformation of the cytoplasmic funnel is observed.
outward-facing state takes place. Thus, a major difference from the allosteric mechanism is whether the sodium-proton exchange requires a pH-induced activation step. Most recently, based on the new crystal structure, the function of NhaA was explored by Warshel and coworkers using a novel approach which combines semi-macroscopic \( pK_a \) calculations with Monte-Carlo (MC) simulations. This study supported Asp163 and Asp164 as the two proton carriers and the recent competitive binding and alternating access models. Further, it was able to reproduce and rationalize features of the observed antiporter activity, including the 2:1 stoichiometry and pH activity profile.

Over the past decade, constant pH MD techniques have been developed that can offer atomic description of pH-coupled conformational dynamics (see refs 25–28 and a recent review). A promising alternative approach that can offer kinetic information has also been developed based on time-dependent MC simulations and the Empirical Valence Bond model. Here we use the state-of-the-art continuous constant pH MD (CpHMD) simulations, which incorporates a membrane-embedded hybrid-solvent scheme and pH replica-exchange sampling protocol to explore the pH-dependent conformational dynamics of NhaA. Our study suggests that the pH sensor responds to \( pH \) and Asp164 and Lys300 are the two proton carriers. Further, deprotonation of Asp164 allows opening of the cytoplasmic gate and binding of a sodium to the core residues, including Asp163. The latter disrupts the Lys300–Asp163 salt bridge, leading to neutralization of Lys300 and a conformational change involving the region between the core and dimerization domains. Thus, our study offers an atomically detailed view of the pH activation and initial events of the sodium-proton exchange of NhaA, reconciling the current models regarding the intricate working of the antiporter.

**Results**

**Overview of simulations.** Three sets of pH replica-exchange CpHMD simulations of NhaA embedded in a POPC lipid bilayer were performed (Supplementary Table 1). The first set was initiated from the recent crystal structure (PDB ID: 4AU5), while the other two were initiated from the previous crystal structure (PDB ID: 1ZCD). During the simulations, all Asp, Glu, His and Lys sidechains were allowed to titrate. Unless otherwise noted, the discussion refers to the first set of simulations. The corresponding data from the other two sets are given in Supplementary Information and show general agreement with the first set of simulations. Convergence analyses are given in Supplementary Figs 1–4.

**pH sensor responds to the pH signal and attracts sodium.** To explore the role of the ‘pH sensor’, we examined the calculated \( pK_a \)‘s of ionizable residues located at the entrance of the cytoplasmic funnel of NhaA, including Asp11 (TM I), Glu78, Arg81, and Glu82 (TM II), His243 (loop between TM VIII and TM IX), Lys249, Arg250, Glu252, His253 and His256 (TM IX) (Table 1). The previous continuum electrostatics calculations predicted Glu78 to have a \( pK_a \) of 2.8 in the physiological pH range. Our simulations, however, showed that Glu78 is the only acidic residue in the pH sensor, with a \( pK_a \) of 2.6, which is deprotonated at a lower pH value compared to the model prediction, with a \( pK_a \) of 2.8. This is in qualitative agreement with the pH sensor’s \( pK_a \) values, consistent with the conclusions from the previous computational work. Apologies for the poor quality of our simulations. Asp164 and Lys300 are the two proton carriers. To identify the proton carriers in the sodium-proton exchange process of NhaA, we turn to the calculated \( pK_a \)‘s of the core residues (Table 1). Asp164 is absolutely conserved in the sodium-proton antiporters across all species. Based on our simulations starting from both the recent and previous crystal structures, Asp164 is the only one among the three acidic core residues that has a \( pK_a \) of 2.6, close to the activation \( pH \) for all three simulation runs. In contrast, the \( pK_a \) of Asp163 is below 4 in all three runs (2.4/3.5/3.9). Thus, Asp164 is the residue that switches protonation state near the activation \( pH \), in support of the hypothesis that Asp164 is one of the two proton carriers based on recent experiments.

| Residue | \( pK_a \) |
|---------|-----------|
| Asp11   | 2.6 (<2.5) |
| Glu78   | 3.1 (3.7/2.6) |
| Glu82   | 3.3 (3.2/2.6) |
| Glu252  | 2.6 (3.1/2.7) |
| His243  | 6.8 (6.9/7.0) |
| His253  | 6.3 (6.5/6.3) |
| His256  | 6.9 (7.2/7.0) |
| Lys249  | 11.2 (11.1/11.0) |

| Active site | \( pK_a \) |
|-------------|-----------|
| Asp133      | 4.5 (4.7/4.4) |
| Lys300      | 10.1 (10.0/10.0) |
| Asp163      | 4.6 (4.6/4.5) |
| Lys300      | 5.0 (6.6/5.8) |

*The \( pK_a \)s calculated from run 2 and 3 starting from the previous crystal structure (PDB ID: 1ZCD) are listed in parenthesis. The \( pK_a \)s are based on all configurations. When separating configurations in the presence and absence of sodium binding to Asp163, the calculated \( pK_a \)s are 8.9 and 11.6, respectively. (Fig. 3a,b). The latter is approximate as the deprotonation is incomplete at the highest simulation \( pH \) 11.5. The \( pK_a \)s of all titratable sites are given in Supplementary Table 1.
shifts can be dampened by adopting a larger $\varepsilon_p$ to implicitly account for reorganization of the interior polar groups and water penetration, the improvement is limited$^{39}$, as a single dielectric constant is insufficient to accurately capture both self energy and charge–charge interactions$^{38}$. On the other hand, the CpHMD-estimated $pK_a$ of Asp164 is likely too low (by up to 2 units$^{39}$), as the underlying GBSW model$^{40}$ underestimates desolvation$^{33,41}$, due to the use of van der Waals surface reflecting the inherent difficulty in treating solute–solvent dielectric transition by GB models$^{35}$. However, the extent of the error is fortuitously cancelled by the overestimation of desolvation due to inadequate structural relaxation$^{33,41}$. As a result, the calculated $pK_a$’s of internal groups from the hybrid-solvent CpHMD simulations show surprisingly small deviations from experiment$^{33,39}$. The second limitation is related to the incomplete sampling in CpHMD simulations, which manifests itself in the dependence of the calculated $pK_a$’s on the initial configuration. As a result, the calculated $pK_a$’s of Asp164 from the simulations initiated from the earlier crystal structure are 0.8/1.6 units higher than the $pK_a$ from the simulation based on the recent crystal structure, because of the significant deviation between the two structures in this region (Fig. 1b). Given sufficient sampling (not within current computational resources), however, identical $pK_a$’s should be obtained regardless of the starting structure.

The identity of the second proton carrier has been controversial. Computational studies based on the earlier crystal structure pointed to Asp163 (refs 12,36), which is also supported by the recent semi-macroscopic simulations based on the new crystal structure$^{24}$. However, the recent MD simulation based on the new crystal structure suggested Lys300 (ref. 20), consistent with the experimental evidence that the $pK_a$ of Lys300 is critical for the antiporter activity$^{11}$. Our simulations initiated from both the recent and earlier crystal structures gave a macroscopic $pK_a$ of around 10 for Lys300, which is only slightly below the model $pK_a$ of 10.4 (model lysine in solution). The small $pK_a$ shift is a result of the balance between the desolvation effect which decreases the $pK_a$ and the electrostatic attraction with Asp163 which increases the $pK_a$. Importantly, the latter is significantly influenced by ion binding. The simulation initiated from the recent crystal structure which contains the Lys300–Asp163 salt bridge showed that, in the presence of sodium binding to Asp163, the distance between Asp163 and Lys300 is significantly increased relative to configurations without sodium binding (Fig. 3a; Supplementary Fig. 6). Thus, sodium binding leads to the disruption of the Lys300–Asp163 salt bridge. Consequently, deprotonation of Lys300 is shifted to a lower pH range, and the (microscopic) $pK_a$ in the presence of ion binding is reduced to 8.9, nearly three units from the $pK_a$ of 11.6, calculated using configurations in the absence of sodium binding to Asp163 (Fig. 3b). Interestingly, experiment showed that the peak current increases at pH above 7 and reaches a maximum around pH 8.7 (refs 4,5). Thus, our data suggest that within the active pH range Lys300 releases a proton, lending support to the most recent hypothesis that Lys300 is the second proton carrier$^{20}$. We note that, while the calculated $pK_a$ in the presence of sodium binding reports on the change in conformational environment, it does not account for the explicit ion interactions in the electrostatic calculations for propagating titration coordinates$^{33,32}$, for example, between sodium and Lys300, which would destabilize the charged state of Lys300, thereby lowering its $pK_a$. However, since the average distance between sodium and Lys300 is about 8 Å when sodium is bound to Asp163 (Supplementary Fig. 7), the $pK_a$ decrease due to the latter effect is expected to be small.

Our calculated $pK_a$ (11.6) in the absence of sodium binding shows an upshift from the model value, which is in qualitative agreement with the $pK_a$ (12.81) obtained by the semi-macroscopic calculations$^{24}$ (see Supplementary Information for detailed comparison), and suggests that Lys300 is charged in the inactive state of NhaA. To further validate the protonation state of Lys300, we examined its interactions with the partial negative dipoles of the C termini of TMs XIc and IVp. When Lys300 is charged, the interactions are intact; however, when Lys300 is

Figure 2 | Net charge of the pH sensor switches sign at activation pH.

Calculated total net charge of the residues in the pH sensor, Asp11, Glu78, Arg81, Glu82, His243, Lys249, Arg250, Glu252, His253 and His256. The error bars indicate the root-mean-squared fluctuations in the simulation. A horizontal line at net charge zero is drawn to guide the eye. Except for arginines which were kept in the charged state, all of the above residues were allowed to titrate in the simulation.

Figure 3 | Sodium binding disrupts the Lys300–Asp163 salt bridge and lowers the $pK_a$ of Lys300. (a) Probability distribution of the distance between the amine nitrogen of Lys300 and nearest carboxylate oxygen of Asp163 in the presence (red) and absence (blue) of sodium binding. Data from all pH conditions were used. (b) Fraction of deprotonated Lys300 at different pH in the presence (red) and absence (blue) of sodium binding. Solid curves are the best fits to the generalized Henderson–Hassebalch equation. The estimated $pK_a$’s are indicated. Sodium ion is considered bound if the distance to the nearest carboxylate oxygen of Asp163 is below 3 Å.
neutralized, the interactions are disrupted (Supplementary Fig. 8). Thus, Lys300 is protonated in the inactive structure of NhaA. We note that, due to the limited timescale of MD simulations, the calculation of sodium flux was not attempted in this work. However, the study by Warshel and coworker based on MC simulations\textsuperscript{24} was able to provide the pH profile of sodium flux as well as the free energy profile of sodium binding, consistent with experiment\textsuperscript{4}.

**Deprotonation of Asp164 triggers gate to open.** Having established that Asp164 is one of the proton carriers, and considering that it is the first core residue located immediately below the cytoplasmic funnel, we tested whether its deprotonation induces a conformational change and entrance of water. Opening of hydrophobic cavity and water penetration due to ionization of internal groups has been previously observed in both experimental and computational studies of Staphylococcal nuclease\textsuperscript{39,43,44}. We zoom in on the end of the cytoplasmic funnel of NhaA, which narrows to a passage lined by five hydrophobic sidechains, Val75 (TM II), Ile134 (TM IV), Met157, Ala160 and Ile161 (TM V), before reaching the active site (Fig. 4a). These hydrophobic sidechains will be referred to as the cytoplasmic gate, since they control the access of water and ion from the cytoplasm. As Asp164 is located immediately below the gate with Asp163 next to it (Fig. 4a), we examined the titration behaviour of both residues (Fig. 4b; Supplementary Fig. 9) and the possible correlation with the passage opening and hydration of the core region.

To characterize the passage opening and hydration of the core, we calculated the radius of gyration based on the gate residues and the hydration number of Asp163 and Asp164 (number of water in the first solvation shell) as well as water density map (Fig. 4c–e; Supplementary Figs 10 and 11). At pH 2, Asp163 is partially and Asp164 is fully protonated; the radius of gyration is about 4.6 Å, similar to the crystal structure values, 4.2 Å of the earlier and 4.5 Å of the recent structure; there is one water near Asp163/Asp164. However, no ion is present in the core region (see later discussion). As pH is increased to 4, Asp163 becomes completely deprotonated (charged) and Asp164 remains protonated (neutral); the radius of gyration increases to about 5 Å. Interestingly, the hydration number of Asp164 increases to 2, whereas that of Asp163 remains below 1, and ions remain absent in the core region (later discussion). These data show that, charging of Asp163 leads to slight opening of the gate; however, the passage is not wide enough to accommodate a possibly hydrated sodium ion. Thus, at low pH the cytoplasmic funnel is closed, consistent with experiments showing NhaA is inactive either under a forward proton gradient with an inside pH below 6.5 (ref. 3) or under a sodium gradient with symmetric pH below 6.5 (ref. 4).

In the pH range 4–7, Asp163 remained deprotonated, while Asp164 titrates to become fully charged; there is a steep increase in both the radius of gyration and the hydration number of Asp164. At pH 7, the radius of gyration is about 5.5 Å, and the hydration number of Asp164 reaches 5, indicating that the gate is open and water enters, similar to the rapid wetting transition observed in hydrophobic nanopores of comparable size\textsuperscript{5,46}. The pH-induced gate opening and water entrance into the core region is also visible from the water density map (Fig. 4c). In contrast, the hydration number of Asp163 remains very low. This is because Asp164 is immediately below the hydrophobic gate readily accessible to the cavity, whereas Asp163 is buried (Fig. 4c) and forms a salt bridge interaction with Lys300. Finally, as pH further increases above 7, there is little change in the radius of gyration or the hydration number of Asp164, which is readily understood since the deprotonation of Asp164 is complete at pH 7. In contrast, the hydration number of Asp163 sharply increases at pH above 8, which can be attributed to the disruption of the salt bridge with Lys300 following its deprotonation (Supplementary Fig. 12). Together, this set of data suggests that, while the deprotonation of Asp163 initiates local relaxation of the gate region, it is the deprotonation of Asp164 that triggers the gate to open so that water can enter the core region.

**Sodium binding to Asp163/Asp164/Thr132.** Along with the pH-induced gate dynamics and water penetration, our simulations reveal that a single sodium ion enters the cytoplasmic funnel and binds to the core residues in a pH-dependent manner. To identify the sodium binding sites, we calculated the probability of sodium binding based on a distance cutoff (Fig. 5a;
Asp164 are charged 24. that sodium binding is most favourable when both Asp163 and accord with the semi-macroscopic calculations which suggested entered and bound to Asp163 and Asp164 (ref. 20). It is also in simulations showing that when Asp164 is deprotonated, sodium consistent with the conventional fixed-protonation-state

Supplementary Fig. 13). Below pH 4, the probability of sodium binding to the core region is zero, in accord with the closed cytoplasmic gate and very limited water accessibility. Between pH 4 and 7, sodium occasionally coordinates to Asp164 and Thr132 through the carboxylate and backbone carbonyl groups, respectively, consistent with the opening of the cytoplasmic gate and water entrance. However, above pH 7, unlike the radius of gyration of the cytoplasmic gate or the hydration number of Asp164, which plateaus once the deprotonation of Asp164 is completed, the ion binding probability of Asp164 (and also Thr132) sharply increases. Moreover, Asp163 starts to bind sodium, resulting in the coordination to all three residues, Asp163, Asp164 and Thr132. These data suggest that the participation of Asp163 in ion binding stabilizes the ion residence in the core, facilitating the activation of NhaA above pH 7, consistent with experiments 3,4. We note that the CpHMD data is flexible. Remarkably, as pH is further increased such that Lys300 becomes deprotonated, the distribution broadens to the range 5–22° (cutoff based on data shown in Supplementary Fig. 15). Data used all pH conditions (2.5–11.5).

The release of a second proton from Lys300 and the accompanying sodium binding of Asp163 lead to a significantly increased distance between the two (Fig. 6b,d). These data suggest that the release of a second proton from Lys300 and the accompanying sodium binding of Asp163 lead to the bending of TM V. We note that, although TM V bending was not observed in the other two sets of CpHMD simulations, likely due to the limited sampling time, it was also seen in the conventional simulation initiated from the recent crystal structure and with Asp163/Asp164/Lys300 fixed in the deprotonated state (Supplementary Fig. 15). To further understand the origin of TM V bending, we examined the dynamics of TM V and its interactions with the environment. We found that the bending is correlated with the breakage of a hydrogen bond between the carboxylate of Asp163 and the backbone amide of Thr132 located on the adjacent helix. When TM V is straight, the hydrogen bond is intact; however, when TM V bends, the hydrogen bond is disrupted (Supplementary Fig. 16). Because the bend is located at Asp163, the Thr132–Asp163 hydrogen bond appears to hold the helix in place.

**Figure 5 | pH-dependent sodium binding to the core residues.** (a) Probability of sodium binding to Thr132 (blue), Asp164 (magenta) and Thr132/Asp163/Asp164 (cyan) as a function of pH. (b) A snapshot showing a sodium ion bound to Thr132/Asp163/Asp164. Sodium is considered bound if the distance to the nearest carboxylate oxygen of Asp163/Asp164 or the backbone carbonyl oxygen of T132 is below 3 Å. The probability was calculated by counting the number of frames. We note that the residence time of sodium is very long. Once it is bound, it does not leave in the CpHMD simulations. In the conventional simulations, the residence time is greater than 400 ns (ref. 20).

**Figure 6 | Deprotonation of Lys300 is correlated with bending of TM V.** (a) Snapshot of NhaA with a straight TM V. (b) Snapshot of NhaA with a bent TM V. (c) Occupancy of the TM V-bent state versus fraction of the deprotonated Lys300. A configuration is considered as in the TM V-bent state if the TM V bending angle is greater than 28° (cutoff based on data shown in Supplementary Fig. 15). Data used all pH conditions (2.5–11.5). (d) Probability distribution of the minimum distance between the carboxylate oxygen of Asp163 and the amine nitrogen of Lys300 when TM V is bent (magenta) and straight (cyan). The data are from pH conditions 9–11.5 where TM V bending was observed. In all panels, the simulation run 2 starting from the previous crystal structure (PDB ID: 1ZCD) was used.
Agreement between CpHMD and conventional simulations. A major limitation of the current CpHMD implementation is the relatively short timescale (on the order of 10 ns per replica), which may result in the incomplete sampling of conformational states of protein and solvent despite the use of the pH replica-exchange enhanced sampling protocol. To assess convergence, we compare key quantities with the conventional simulations for different combinations of protonation states (1–3 μs each). Agreement is observed for the hydration levels for Asp163, and Asp164, the probability of sodium binding to Asp163, Asp164 and Thr132, and the behaviour of Asp163–Lys300 interaction (Supplementary Note 2; Supplementary Figs 17–20). As to the latter, the new NhaA crystal structure solved at pH 3.5 shows a salt bridge, which remains stable in the conventional simulations S1 and S2 with charged Asp163 and Lys300. Consistent with these data, the CpHMD simulations now clearly explain this observation as a consequence of the low pKₐ of Asp163. The consistency between low pH crystal structure and both sets of simulations also corroborates the hypothesis that Asp163 is not one of the proton carriers because it remains charged at all physiologically accessible pH conditions.

Discussion

A key question regarding the mechanism of NhaA is related to the identities of specific residues responsible for proton uptake and release in the transport cycle. For a number of years, Asp163 and Asp164 have been considered as the two proton carriers. However, recent molecular dynamics simulations based on the new crystal structure of NhaA suggested Lys300 but not Asp163 is the second proton carrier, consistent with biochemical data. Our results support the latter model. The CpHMD simulations showed that, when Lys300 forms a salt bridge with Asp163, its pKₐ is high (11.6) due to stabilization of the charged state by the attractive Coulomb interaction; however, when a sodium ion binds to Asp163, Lys300 rotates away leading to disruption of the salt bridge and lowering of the Lys300 pKₐ by almost 3 units to 8.9. We note that, the latter pKₐ is likely somewhat overestimated due to a known limitation of the hybrid-solvent CpHMD method which neglects the explicit interactions with ions, which would destabilize the charged state of Lys300, lowering its pKₐ further. Thus, it is very likely that as pH reaches above 7–8, the release of the second proton from Lys300 could occur simultaneously with sodium binding to Asp163.

A second hypothesis our study aimed to test is related to a single residue at the entrance of the cytoplasmic funnel (pH sensor), which is thought to sense the pH signal and regulate the activation of NhaA allosterically. An earlier study based on the continuum electrostatics calculations suggested that Glu78 has a pKₐ near the physiological range and can therefore trigger a conformational change required for activation. Our simulations do not support this conjecture, as the calculated pKₐ’s of all acidic residues including Glu78 in the pH sensor are far below the physiological pH. Instead, three histidines in the pH sensor have pKₐ’s in the physiological range, and the switch of their protonation states together changes the net charge of the pH sensor from positive to negative at around pH 7, the same pH range in which sizable transporter activity could be experimentally measured. Thus, we propose that the pH sensor residues collectively respond to the pH signal and attract sodium ions when pH rises above the activation value. This hypothesis is consistent with the observations that mutations of all the pH sensor residues strongly influence the pH-dependent activity of NhaA.

Until now two models have been put forth to explain the pH regulation of NhaA. In the allosteric model proposed by Padan and coworkers, a pH sensor residue (located at the cytoplasmic funnel entrance but far from the core region) senses the pH signal and induces a conformational change that activates NhaA. In the competitive binding model, a sodium ion and two protons compete for binding to the active site. Substrate binding lowers the energy barrier between two conformational states, resulting in the transition from one state to the other. Our data showed that, above the activation pH the deprotonation of Asp164 triggers the opening of the cytoplasmic gate formed by several hydrophobic residues lining the bottom of the cytoplasmic funnel and entrance of water as well as ion to the core region in a pH-dependent manner. Thus, access to the binding site is controlled via a pH-dependent hydrophobic gating mechanism. Further, our simulations suggested that, sodium binding to Asp163 can trigger the release of a proton from Lys300, which in turn can induce binding of TM V located between the core and dimerization domains. Although such movement is not seen in the outward-facing state of NapA and MnNhaP from archaea, we hypothesize that, since our simulations only capture the initial stage of the transport cycle, TM V bending may represent a kinetic intermediate accompanying the large conformational transition to the outward-facing state. Interestingly, it is consistent with the electrophysiology data which showed that mutation A167P on helix V markedly slows the rate of conformational transition but does not affect the optimum pH of NhaA. To directly verify TM V bending, we suggest to test the mutation Thr132 to Val132, which eliminates the hydrogen bond with Asp163, would possibly facilitate TM V bending and accelerate the conformational switch. Thus, our data lend support to the competitive binding model for NhaA, modulated with the two pH-dependent effects of electrostatic funneling due to the overall charge of the pH sensor and the hydrophobic gate controlling access to the binding site.

Taken together, our work suggests the following mechanism for the antiport activity of NhaA. As pH is increased to the activation pH, the net charge of the pH sensor residues decreases to a negative value, attracting a sodium ion into the funnel. At the same time, Asp164 releases the first proton which induces opening of the cytoplasmic hydrophobic gate. The latter allows the entrance of water and a sodium ion, which is first captured by Asp164, the residue immediately below the cytoplasmic gate, and subsequently shared with Asp163 and Thr132. Sodium binding to Asp163 disrupts the salt bridge with Lys300, destabilizing its charged state and leading to the release of the second proton. The latter triggers a conformational change, possibly involving bending of TM V, which may precede a large conformational transition to the outward-facing state of NhaA. Because of the short simulation timescale, the above mechanism describes only the early events of the transport cycle. Nonetheless, our work reconciles the current models and provides atomic details of the pH-dependent activation and sodium-proton antiport of NhaA. Finally, we note that the CpHMD methodology is general and can be applied to illuminate other proton-coupled conformational processes in biology that are difficult to delineate by current experimental techniques.

Methods

System preparation. Two crystal structures of NhaA (PDB IDs: 1ZCD and 4AU5) were employed for this study. CpHMD run 1 was initiated from the subunit A of the previous crystal structure which is a monomer (PDB ID: 1ZCD, sequence 10–383), while run 2 and 3 were initiated from the subunit B of the new crystal structure which is a monomer (PDB ID: 4AU5, sequence 10–383). Hydrogen atoms were added to the protein using the HBUILD facility in CHARMM. The protein were then inserted into a preassembled lipid bilayer with 135 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (DPPC).
phospholipid (POPC) lipids using Membrane Builder in CHARMM-GUI (http://www.charmm-gui.org)49. The resulting number of lipids is 63 for the phosphocholine (POPC) lipids using Membrane Builder in CHARMM-GUI and 72 for the periplasm-facing leaflet. A water layer of 15 Å thickness was added to both sides of the lipid bilayer. Twenty-one sodium and 30 chloride ions for 4AU5 or 20 sodium and 29 chloride ions for 1ZCD were added to neutralize the system. For the continuous constant pH molecular dynamics (CpHMD) simulations dummy hydrogen atoms were added to the carbonyl groups of acidic residues following the documentation of the PHMD module31,32 in CHARMM35. The final systems contain about 50,000 atoms.

CpHMD simulations with pH replica exchange. The work employed the membrane-embedded hybrid-solvent CpHMD method23,24,49, with pH replica-exchange sampling protocol33, Here conformational sampling of transmembrane proteins is performed in explicit lipid bilayer, as in the conventional all-atom MD. The generalized Born GBSW membrane model40,52 is used to efficiently calculate electrostatics, with a real-space cutoff of 12 Å and a sixth-order interpolation with parameters taken from Chen40,52. The GBSW input radii for the protein were taken from Chen et al.40,52. The system was first equilibrated at pH 4 (near the crystallization pH condition) for 2-fs timestep while constraining all bonds including hydrogen atoms. The temperature was maintained using a modified Hoover thermostat method59, with a real-space cutoff of 12 Å. The particle mesh Ewald method61 was used to calculate long-range electrostatics, with a real-space cutoff of 12 Å and a sixth-order interpolation with parameters taken from Chen40,52. The system was first equilibrated at pH 4 (near the crystallization pH condition) for 2.4–5.4 ns using the default multi-step protocol in CHARMM-GUI49,58. Following equilibration, the production simulation was performed using hybrid-solvent CpHMD with the pH replica-exchange protocol33, whereby each replica was simulated under constant pH conditions for 20 ns per replica. The specific pH conditions for run 1 were 1.25, 2.5, 2.75, 3.0, 3.25, 3.5, 3.75, 4.0, 4.25, 4.5, 4.75, 5.0, 5.5, 6.5, 7.5, 7.8, 8.5, 9.0, 9.5, 10.0, 10.25, 10.5, 10.75, 11.0 and 11.5. An exchange between adjacent pH conditions was attempted every 500 MD steps (or 1ps) with an acceptance ratio of about 30% for all replicas. The production simulation lasted 10–14 ns per replica, resulting in an aggregate sampling time of 280–381 ns for each set of CpHMD simulations. Data from the last 4 ns per replica for simulation runs 1 and 3, and the last 5.6 ns per replica for simulation run 2, were used for analysis. First, we noted the number of hydrogen bonds per time unit, the pK_a values of relevant residues were calculated. Although longer simulation time may be desirable, we were constrained by the current speed of the CpHMD implementation in CHARMM and the available hardware resources.

Conventional fixed-protonation-state simulations. Simulations with fixed protonation states were taken from previous work by Lee et al.23,24. In brief, all-atom explicit solvent MD simulations were run with GROMACS62,63, with the OPLS-AA force field for protein and ions, and the TIP4P model for water10. The force field for POPC lipids was taken from Ullsmee and Ullsmee48. The orthorhombic simulation cell contained a NaHaA dimer and about 112,000 atoms. Simulations were performed in the NPT ensemble with PME electrostatics and a 2-fs timestep while constraining all bonds including hydrogen atoms. The simulation conditions were as detailed in Supplementary Table 1; other residues were set to default values (see ref. 20 for further details). Simulations S1, S2, S4 were repeated three times and analysed in aggregate (totalising between 1.4 and 3 ns for each charge configuration); for S3 only a single 1-μs simulation was analysed (Supplementary Table 1). Trajectories were analysed at 1 ns intervals.

To check for a significant dependence on lipids, we performed a second, smaller set of simulations using a 4:1 POPE:POPG membrane which approximates the composition of a native E. coli inner membrane (see Table 1 for further simulation details). The simulations utilized the CHARMM36 protein46 and CHARMM lipid force fields49, the default CHARMM sodium ion parameters32 and CHARMM TIP3P water model50. The simulations were performed with the same protocol that was previously employed for the simulations of Naph22.

Analysis. The pK_a values were calculated by fitting the unprotonated fractions of specified residue at simulated pH conditions to the generalized Henderson–Hasselbalch equation, \( p_{K_a} = \frac{1}{1 + 10^{-pH}} \), where \( p_{K_a} \) is the unprotonated fraction. The C2-atom-based principal component analysis was performed using the VIBRAN module in CHARMM35. The analysis was carried out for the trajectory at pH 11.5 from simulation run 2 in which bending of TM V was observed (see main text). The average coordinates were used as the reference. The first principal component has a larger eigenvalue than others, which accounts for 32% of the total variance.

Data availability. Molecular dynamics parameters, inputs and initial coordinates are available for download at http://drum.lib.umd.edu/handle/1903/18477. All other data are available from the corresponding author upon reasonable request.

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