Simulating the function of sodium/proton antiporters

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The molecular basis of the function of transporters is a problem of significant importance, and the emerging structural information has not yet been converted to a full understanding of the corresponding function. This work explores the molecular origin of the function of the bacterial Na^+/H^+ antiporter NhaA by evaluating the energetics of the Na^+ and H^+ movement and then using the resulting landscape in Monte Carlo simulations that examine two transport models and explore which model can reproduce the relevant experimental results. The simulations reproduce the observed transport features by a relatively simple model that relates the protein structure to its transporting function. Focusing on the two key aspartic acid residues of NhaA, D163 and D164, shows that the fully charged state acts as an Na^+ trap and that the fully protonated one poses an energetic barrier that blocks the transport of Na^+ . By alternating between the former and latter states, mediated by the partially protonated protein, protons, and Na^+ can be exchanged across the membrane at 2:1 stoichiometry. Our study provides a numerical validation of the need of large conformational changes for effective transport. Furthermore, we also yield a reasonable explanation for the observation that some mammalian transporters have 1:1 stoichiometry. The present coarse-grained model can provide a general way for exploring the function of transporters on a molecular level.

transport | computational biology | membrane protein

Active transport lies at the heart of a plethora of important functions such as muscle contraction and neuronal activity. It is the hallmark of bioenergetics in cells and is both ancient and universal. Secondary active transporters are a subgroup of active transporters in which two different substrates are transported by the same system, with the electrochemical potential of one substrate, moving down its gradient, driving the active pumping of the second substrate. Most secondary active transporters consume the gradient of either protons or sodium ions.

Na^+/H^+ antiporters exchanging protons for sodium ions, are of great interest as prototypic secondary active transporters, serving as arguably the simplest case of secondary active transport. Na^+/H^+ antiporting is present in all domains of life and is essential in many diverse processes ranging from basic survival in bacteria (1) through salt tolerance in plants (2) to complex roles in hypertension and ischemia in mammals (3, 4).

The first high-resolution X-ray structure determined for any Na^+/H^+ antiporter was the structure of NhaA from *Escherichia coli* (5), and to date only bacterial structures have been solved (6, 7). The role of NhaA in bacteria is the removal of excess Na^+ , which could have detrimental effects on the bacterium, from the interior of the cell while maintaining H^+ homeostasis. Not surprisingly, NhaA's activity is highly pH dependent and is diminished at both high and low pH values (8). The presence of a "pH sensor" has been postulated and sought but with no decisive results. Recent evidence supports an alternative hypothesis: That there is no pH sensor per se and that pH dependence is caused by competition between substrate ions and protons (9, 10).

NhaA has been extensively studied biochemically, revealing that both the residues D163 and D164 are essential for activity (11), most likely serving as the binding site for both protons and Na^+ ions. Additionally, the stoichiometry of NhaA has been determined to be 2:1, so two protons are consumed in the pumping of one sodium ion against its electrochemical gradient (12). The

transporter also has been shown to function in reverse, allowing a sodium gradient to pump protons actively, if needed (13).

The basic mode of action of NhaA, as for most other active transporters, is assumed to be alternating access (14). (General considerations that support the alternating access model in the case of LacY are discussed in ref. 15.) However, despite the very reasonable assumption that an alternating access model with large conformational changes is essential for the function of transporters, we are not aware of kinetic or landscape indications that prove other gating mechanisms are impossible (*SI Text*). In the case of NhaA, although there is no definite evidence to prove the assumption of a large conformational change, the presence of pseudosymmetry in the structure (5, 7), which is characteristic of transporters (16), and the revelation of the structure of the Na^+/H^+ antiporter from *Thermus thermophilus* (NapA), which is considered to be in the alternative conformation (6), seem to support the model of alternating access mediated by large conformational changes. Furthermore, recent structural analyses of the related Na^+/H^+ antiporter from *Methanocaldococcus jannaschii* (MjNhaP1) (17) found two conformers that probably are the two alternating configurations. Despite this reasonable picture, it is useful to approach the transport problem without assuming a priori that the alternating conformation model is essential. Therefore, we initially explored the possibility that, as in some chloride-proton antiporters in the chloride channel family (18), NhaA might be a transporter that does not adhere to the canonical paradigm of alternating access via large conformational changes.

Computational studies that were conducted after the resolution of the structure of NhaA examined the architecture (5), selectivity (19), mechanism of action (20), and homologs of *E. coli* NhaA (21). Molecular dynamics (MD) simulations have been conducted on NhaA, and a mechanism of action suggesting roles for residues D163 and D164 has been proposed, (20). A recent MD study (7) tried to explore the role of ion pairing in the action of NhaA. Furthermore, a recent extensive electrophysiological study shed

Significance

Understanding the molecular nature of the function of transporters is of significant interest in view of their biological importance. However, the actual structure-function relationship of transporters is far from being clear. To advance this understanding, we simulated the activity of the Na^+/H^+ antiporter NhaA by a Monte Carlo approach, based on the calculated coarse-grained electrostatic landscape of the system. The simulations reproduce the observed trend and provide a powerful insight into the origin of the transporting function and the molecular basis of its control. We also show that a large conformational alternation is essential for the transporter function. The approach used should be useful for general studies of other transporters.

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new light on the pH response of the protein, supported by a minimal kinetic model (22).

Although the above insightful studies have provided important advances in the understanding of the mechanism of NhaA, we still lack structure-based free-energy surfaces that are crucial for understanding the key role of the proton transport (PT) and the modulation of the pK_a of aspartic acid residues by the protein electrostatics (which include water penetration and reorganization of the protein residues). Thus, it is essential to relate the overall transporter structure to the transporter function through non-phenomenological analysis. Of course, MD atomistic simulations may lead to advances in this direction, but such approaches cannot yet provide a complete picture of the Na^+ transport process with its coupling to large conformational changes and PT. Thus, in this work we try to advance the molecular understanding of transporters by using a strategy that allows us to investigate the energy–structure relationship of NhaA, with the aim of gaining a realistic description of the transport cycle as well as the pH response. This approach reflects our belief that the challenge of understanding the directional transport should be addressed by simulations that generate the actual relevant structure–transport relationship. Doing so requires the ability to explore the long-time transport process as a function of the pH and $[\text{Na}^+]$ gradients. In the present work we address this challenge, focusing on the NhaA system as a case study.

The strategy adopted here combines several approaches. We started by exploring semi-macroscopically the energetics of Na^+ and proton binding to D163 and D164 of NhaA and obtain distinct Na^+ -binding profiles that are pH dependent. We then used the resulting energetics in Monte Carlo (MC) simulations that examine the performance of two transport models. We found that we can reproduce the observed transport features and in particular the 2:1 stoichiometry.

Results and Discussion

Charting the Landscape. To explore the function of Na^+/H^+ antiporters, we consider the NhaA system (presented in Fig. 1). The basic simulation system was constructed by starting with the X-ray structure of NhaA (believed to be in the inward-open state) and embedding it in a simplified membrane model (23). The protein plus membrane system was then solvated in a water sphere by the standard MOLARIS protocol (24), minimized (with small time steps), and run in short relaxation runs with a small restraint on the X-ray structure using MOLARIS. Several such locally relaxed structures were used for all subsequent calculations and were found to give stable results.

The first step of our study involved calculations of the pK_a s of key acidic residues in NhaA, using the semi-macroscopic protein-dipole–Langevin-dipole linear response approximation (PDLD/S-LRA) protocol (*SI Text* and ref. 25). The pK_a values of D163 and D164 were computed, in each case with the other residue being either protonated or charged (without the transported Na^+). The initial calculations were performed while the rest of the protein

ionizable groups were kept at their neutral state, given the effect of distant residues macroscopically with a large dielectric constant (ϵ_p) (25). The results obtained are summarized in *Table S1*. The pK_a values for D163 and D164 were fairly similar, at ~ 7 . Next, we focused on the roles of the adjacent residues D133 and K300, which have been suggested to be important for NhaA function (5, 11), and computed pK_a values of -2.15 and 12.81 , respectively, indicating that both are definitely found in their charged form in the protein. Taking this result into account, we repeated the pK_a calculations of D163 and D164 with D133 and K300 explicitly charged; the corresponding results also are summarized in *Table S1*. Encouragingly, the results of the second computation provided a significant improvement in the agreement with the experimentally observed pH dependence (as described below). Therefore we used the pK_a values of 9.3 for the first protonation and 7 for the second; these values reflect the average of the calculated results obtained for the two different aspartic acid side chains (*Table S1*). The pK_a relation to the Na^+ binding is discussed further in *SI Text*.

Reliable pK_a calculations are quite crucial for modeling the function of transporters, and it is very hard to accomplish such a task with empirical approaches such as the method applied in the popular PROPKA server (26) used by Lee et al. (7). This method is not based on the actual electrostatic free energy in protein interiors, nor has it been validated with relevant experimental results in protein interiors (the validation issue is discussed in ref. 27). The issue is of equal importance when one considers the proposal of ion pairing (e.g., with K300), which is based on PROPKA-type calculations. Exploring such a crucial issue requires proper modeling of the protein compensating dielectric, which includes water penetration and reorientation of polar groups and which cannot be obtained without proper sampling and LRA or extensive free-energy perturbation studies (e.g., see the discussion in ref. 27). Of course, pK_a calculations should be judged by their ability to reproduce the observed pH profile, as presented below. One may try to use Poisson–Boltzmann (PB) calculations, but only if such calculations reflect the proper ϵ_p and include an LRA or equivalent treatment (*SI Text*).

Next we examined the binding of substrates by using the PDLD/S-LRA method to calculate the energetics of Na^+ binding by evaluating the solvation energy of bringing an Na^+ ion from the bulk to several points within the protein's cavities along a vector from the cytoplasmic or periplasmic bulk regions to the binding site. The binding site was chosen to be a point in the vicinity of D163 and D164. Although this point does not necessarily represent the exact physiological binding location (which has not yet been determined by structural means), it is likely to be very close, and the MD relaxation is expected to lead the ion into a position that represents the actual binding position (also see *SI Text*).

The energy curves were calculated for four different protonation states: both D163 and D164 in the protonated form (HH); only one of them in the protonated form [(H–) and (–H), henceforth marked as (H–) for simplicity]; and both D163 and D164 in the charged form (–)(–). The results, shown in Fig. 2, express three discrete types of energy curve. One is the Na^+ -repulsion curve, seen for HH, in which the energies rise steeply as the Na^+ ion is brought into the protein cavities. Such an energy profile creates a very high barrier for the Na^+ ion, and this barrier plays a major role in helping understand the transport cycle (see below). This result is a logical one, because when no residue is charged, there is no electrostatic attraction between an Na^+ ion and the binding site. Another type of binding curve is the electrostatic trap, seen in (–)(–), with moderate barriers but a deep trough, binding strongly and potentially trapping the ion. This result also is reasonable, because the charge of two adjacent charged aspartic acid residues would apply a strong electrostatic force on an Na^+ ion. The third curve is for intermediates, seen for both H(–) states, which show similar but less extreme cases of the HH and (–)(–) curves. An important characteristic of all the curves, as discussed in *SI Text*, is the relative symmetry with respect to the z axis. It is encouraging that the

Fig. 1. Presentation of the simulation system. The protein appears in orange, and the membrane particle grid is shown as cyan spheres. The membrane has been truncated so it does not obscure the protein. Residues D133, D163, D164, and K300 are shown in a licorice presentation.

Fig. 4. The pH response in the MC simulations. Normalized net flux of Na^+ in the MC simulations (red trace) compared with the experimental results of Mager et al. (13) (blue trace). Insets show the energy profiles of the MC (using the same colors as in Fig. 2) as a function of the pH in that point on the chart. For clarity, only some of the states are depicted by a schematic square representing NhaA. The profiles are translated vertically to account for the differences in energies caused by the protonation state, based on the averaged pK_a values and the corresponding pH. The results are shown for model 2, but similar results were obtained with model 1. Note that the x axis has different values for each curve.

Reproducing and Understanding the Transport Function. After obtaining the observed trend for the case without a pH gradient with both models 1 and 2, we moved to the more challenging test, examining which model could account for the antiport function, i.e., the transport of Na^+ in the opposite direction of a proton gradient. MC simulations using model 1 were conducted with a proton gradient but no Na^+ gradient. No correlation was found between the proton and the Na^+ fluxes (Fig. 5). Careful analysis of the system revealed the likely cause for this problem: With a transporter that is open to both sides of the membrane simultaneously, the protonation state of the transporter is dictated by the lower pH of the two adjacent spaces, so the higher pH on the other side has little to no effect. This conclusion is very important because it indicates that the alternating access model is essential. (Although the current paradigm for transporters incorporates the alternating access model, we thought it was instructive to consider a model that did not include this feature to determine the requirement for alternating access without initially assuming it.)

The MC simulations using model 2, with a proton gradient and no Na^+ gradient, resulted in a much lower proton flux than seen with model 1 and in a biased flux of Na^+ in the opposite direction (Fig. 5). The lower flux for protons reflects the closed position of the transporter in some of the MC steps, resulting in less proton movement and thus in less flux. Repeating the calculations with a pH gradient in the opposite direction or with an Na^+ gradient and no proton gradient produced the fluxes shown in Fig. 5 and Fig. S5, respectively. The active Na^+ flux is a result of the energetics as described by our PDL/D-S-LRA calculations. In brief, when the transporter is exposed to the more acidic side, the HH state becomes predominant and promotes Na^+ unbinding; when exposed to the other side, it assumes the $(-)(-)$ state and promotes Na^+ binding. These states effectively give NhaA different affinities for Na^+ on both sides of the membrane, an efficient scheme for transport (34). Over sufficiently long simulations, a net antiport outcome with a stoichiometry of $\sim 2:1$ is expected. These conditions are expected only under a fairly narrow range of pH values, within which, indeed, NhaA functions (albeit with a pH shift; see above). However, the stoichiometry that was obtained was higher than $2:1$ (Fig. 5), probably because the global minimum on each side is not

occupied 100% of the time (Movie S1). However, in physiological conditions some extent of substrate leakage is probably inevitable (34).

To clarify the origin of the above results, we plotted our free-energy landscape, considering proton and Na^+ binding as the two reaction coordinates (Fig. 6). The surfaces reflect the alternating access model by having a high barrier on only one of the sides at any given time. Examination of the surfaces immediately reveals the mechanism explained above. When the transporter is open to the acidic side, two protons will bind, and an Na^+ ion (if present) will unbind. When the transporter is open to the basic side, the two protons will unbind, and an Na^+ ion will bind instead. The cycle can be summarized as $2\text{H}^+:1\text{Na}^+$ antiporting. In our model a deviation from the global minima would result in a deviation from the perfect $2:1$ stoichiometry, but we do not expect this deviation to have a substantial effect when averaged over large numbers of cycles.

Our study provides an interesting hint regarding the stoichiometry of mammalian Na^+/H^+ exchangers (for a review, see ref. 35), which, in contrast to bacterial NhaA, is $1:1$. We note that the extremely conserved DD motif in NhaA (D163 and D164) appears as ND in many Na^+/H^+ exchanger (NHE) members (36). Thus, following our model it is reasonable to expect that the stoichiometry of $2:1$ seen when two aspartic acid residues participate may translate to $1:1$ when only one aspartic acid residue is present. This notion has been supported by our MC simulations in which we removed one of the aspartic acid residues but kept the other binding curves of NhaA unchanged (Fig. S6). However, a full verification requires the still unavailable high-resolution structure for NHE and the corresponding calculated binding profiles, as well as accounting for the different pH-activity profile of NHE-type transporters. In this case we would need a binding site that provides a strong binding for Na^+ without having a second ionized acid, as well as a pK_a for a single aspartic acid that would allow exchange in the observed pH profile of NHE. Interestingly, the recently reported structure of the MjNhaP1 transporter (17) offers an opportunity to explore this issue, because this protein probably also supports a $1:1$ stoichiometry and has a binding site with ND instead of DD. This point is considered in SI Text and should be explored further in the future.

When we consider the sequence of binding of either the aspartic acid residue or the Na^+ ion, our pK_a values suggest that D163 is the first residue to lose a proton or the last to bind one (Table S1) and that an Na^+ ion would be expected to bind only after D164 becomes deprotonated (Fig. 2). Therefore, the energetically logical sequence of events starting from the HH state would be deprotonation of D163 and then an exchange between the proton of D164 and an Na^+ ion, a sequence very similar to that suggested by Arkin et al. (20).

The pathway of a perfect transport cycle is presented in Fig. 6B. We note that this model is based essentially on direct competition

Fig. 5. The results of the MC simulations of the two models, showing the net calculated flux of the Na^+ and H^+ ions. 0 represents no pH gradient; + and – represent a pH gradient created by a difference of 2 pH units in opposite directions. Each arrow represents the average of 25 simulations, proportionally scaled down for visual clarity. The dashed line emphasizes the experimental stoichiometry.

Fig. 6. The effective landscapes for the transporting function. (A) A 3D surface of model 2. For simplicity the proton axis depicts two sequential proton bindings (see *Results and Discussion, Reproducing and Understanding the Transport Function*), as shown by the squares representing NhaA. The left and right surfaces represent the inward-facing and outward-facing states, respectively. (B) The same surfaces shown in 2D color. For visual clarity energies above 10 kcal/mol were truncated to black color. Squares representing NhaA are shown to represent only some of the states. Dashed circles highlight the global minima. The arrows depict the path of a perfect transport cycle.

between the Na^+ ions and the protons, where protonation of D163 and D164 results in Na^+ repulsion, and deprotonation results in Na^+ attraction. Competition between substrates has been suggested and shown biochemically for NhaA (9, 37).

Overall Considerations of the Mechanism of Action. The present simulation study appears to provide a working model for the transport cycle of NhaA and perhaps other transporters that function in a similar way. With the presence of a pH gradient across the membrane, the transporter alternates and is exposed to a different pH at different times. When facing the more acidic side, the transporter undergoes protonation of both its aspartic acid residues, resulting in the unbinding of the Na^+ ion (or no binding if no Na^+ was bound initially). Because this is the lowest energy state, it is expected to be maintained (on average) until the transporter undergoes a conformational change to face the more basic side of the membrane. Then, the transporter will release the two protons, a process that could be enhanced by Na^+ binding. Energetically, the most stable state is fully deprotonated with a bound Na^+ ion. Being stable, this state is maintained (on average) until another conformational change ensues, repeating the cycle at a 2:1 stoichiometry. Both binding of Na^+ to the double-protonation state and releasing of Na^+ from the double-charged state would lead to leak and loss of energy. These states, however, are unfavorable. The overall cycle is presented in Fig. 7.

Concluding Remarks

The present simulations using structure-based models reproduced the observed transport features of the NhaA system. It was found that the fully charged protein acts as an Na^+ trap, whereas the fully protonated protein poses an energetic barrier too high for the ion to cross. It is through the alternation between these states, mediated by the partially protonated protein, that Na^+ and protons can be exchanged across a pH gradient at 2:1 stoichiometry. Interestingly, our study provides very strong support for the reasonable but unproven alternating access model for NhaA.

A key feature that has been strongly supported by the current work is the requirement of conformational changes. This effect

appears to be essential for reproducing the observed transport properties. Furthermore, we established the origin of the difficulties with models that do not include conformational changes. Obviously it would be important to explore the details of the conformational change, as was attempted by Lee et al. (6), and to produce a detailed structure-based energy landscape. In this respect our progress has been somewhat limited. We found strong support for the conformational model by the overcharging calculations (*SI Text* and Fig. S3). However, a more direct landscape calculation would require the generation of the path between the two conformations and exploring the Na^+ and H^+ profile along this path. Interestingly, the equivalent of the overcharging effect may play some role in coupling the conformational change to the flow of Na^+ and H^+ , but this effect is not expected to be a major one as long as the conformational barrier without the coupling is sufficiently high.

The stoichiometry obtained in our study deviates from 2:1 as a function of pH and is pK_a dependent. This result actually might hold true for NhaA and has not been tested thoroughly enough. That is, the original work on NhaA (12) measured ratios of nearly 2:1 within a fairly narrow range of pH values; the pH range was restricted for technical reasons in play at the time of that investigation, and to the best of our knowledge no work has tested stoichiometry at pH values beyond that range. Therefore, further work testing varying pH values on both sides of the membrane is required to validate or disprove this prediction.

Our model attributes a passive role to the Na^+ ions. That is, although an Na^+ gradient alone creates a biased inverted proton flux (*SI Text* and Fig. S5), PT still takes place in the absence of Na^+ . This trend seems to be in contrast to the corresponding experimental behavior of NhaA. One optional explanation is that the conformational changes discussed above are coupled to the Na^+ and its solvation, and protons by themselves (H_3O^+) cannot perform this action efficiently. For example, it is possible that the conformational barrier is different when the protein is found in the $(-)(-)$ configuration or in the (HH) configuration. This option has been supported by our MC simulations (Fig. S7), but the interesting issue of electrostatic coupling has not yet been explored by structural studies.

In relating our simulation studies to other current strategies, one should realize that using powerful MD simulations to explore ion penetration and ion-pairing effects (e.g., ref. 7) does not necessarily provide information about the action of complex systems such as transporters. The analysis of functions must be based on calculating free-energy surfaces and free-energy profiles, which are very hard to obtain even with simulation runs in the microsecond range. Without a free-energy surface, we cannot explore the proposal in ref. 7 and thus cannot confirm or disprove it.

The progress presented in this work in achieving a detailed understanding of the control of the NhaA transport function provides more than just an intellectual insight. The Na^+/H^+ antiporter itself is of high importance in medicine. Bacterial Na^+/H^+ antiporters potentially could be used as a drug target in cases in

Fig. 7. A schematic presentation of the working model suggested in this work. NhaA is depicted as two banana-shaped domains, alternating access through an inversion process. The circles represent particles and binding sites.

which NhaA is essential for the pathogenicity of bacteria (38). Perhaps more importantly, the human equivalent of NhaA, NHE, is associated with a growing number of human pathologies, such as cancer, hypertension, and reperfusion damage, among others (for a review, see ref. 3), so the study of NhaA has potential pharmaceutical significance.

Methods and Systems

The NhaA simulation system was constructed by taking the protein from the Protein Data Bank (PDB ID code: 4AU5) (7). A single monomer was aligned so that the z axis corresponds roughly to the membrane normal, and all atoms and molecules except for the protein itself were removed from the system. A 3D grid of nonpolar particles, using a spacing of 3 Å, was added around the protein, representing a simplified membrane with dimensions of 66 × 66 × 30 Å. The system was first minimized using MD with small time steps and low temperature and was then relaxed with several 20-ps runs subjected to small restraint on the X-ray structure. The several resulting

structures were used as starting points for the subsequent PDLD/S-LRA calculations (described in *SI Text*). The calculations were performed with the MOLARIS program (24).

To explore the possible role of conformational changes, we adopted the overcharging (OC) idea (33), comparing NhaA and NapA. First, we constructed a simulation system for NapA (PDB ID code: 4BWZ) (6) as described above. Then, we took the same starting structures used for the binding calculations, placing an Na atom in different points along the binding path and changing the atom's charge from 0 to +2 using the adiabatic charging method of the MOLARIS package (*SI Text* and ref. 24).

A key element of the present work has been a time-dependent MC simulation similar to the one used in our previous studies of PT (for details, see *SI Text*) (39). The construction of the energy surfaces is detailed in *SI Text*.

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