Published in final edited form as:

J Phys Chem B. 2016 October 27; 120(42): 10951–10958. doi:10.1021/acs.jpcb.6b08126.

Simulating the Function of the MjNhaP1 Transporter

Raphael Alhadeff and Arie Warshel*

Department of Chemistry, University of Southern California, SGM 418, 3620 McClintock Avenue, Los Angeles, California 90089, United States

Abstract

The structures of transport proteins have been steadily revealed in the last few decades, and yet the conversion of this information into molecular-level understanding of their function is still lagging behind. In this study, we try to elucidate how the action of the archaeal sodium/proton antiporter MjNhaP1 depends on its structure-energy relationship. To this end, we calculate the binding energies of its substrates and evaluate the conformational change barrier, focusing on the rotation of the catalytic residue D161. We find that sodium ions and protons compete against a common binding site and that the accessibility of this binding site is restricted to either the inside or outside of the cell. We suggest that the rotation of D161 χ_1 angle correlates with the conformational change and is energetically unfavorable when D161 does not bind any substrate. This restriction ensures coupling between the sodium ions and the protons, allowing MjNhaP1 and probably other similar transporters to exchange substrates with minimal leak. Using Monte Carlo simulations we demonstrate the feasibility of our model. Overall we present a complete picture that reproduces the electroneutral (at 1:1 substrate ratio) and coupled transport activity of MjNhaP1 including the energetic basis for the criteria provided by Jardetzky half a century ago.

Graphical Abstract

*Corresponding Author: warshel@usc.edu; Telephone: +1 213 740 4114.

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcb.6b08126.

Detailed assessment of the barrier for the conformational change (PDF)

Free energy curves for Na⁺ binding using WHAM superimposed on the PDLS/S-LRA (PDF)

Cartoon illustrations of the different conformation changing models (PDF)

Free energy profile for the dihedral angle χ_1 of D161, expansion of Figure 3 (PDF)

MC energy interpolation of the PDLD/S-LRA results (PDF)

Overlay of binding energies from different stoichiometries of Na⁺/H⁺ antiporters (PDF)

I. INTRODUCTION

The movement of ions across cell membranes is mediated by transport proteins. Such transport can be passive (through channels) or active (through pumps), which can in turn be coupled to different sources of energy. One common form of energy that allows active transport is the coupling of two (or more) substrates, and their electrochemical gradients, so that one substrate is providing the driving force to actively pump the second one. Such co-transport can be in the same direction (symporting), or in opposite directions (antiporting or exchange).

Here, we focus on sodium/proton antiporting. Such antiporters appear in prokaryotes, eukaryotes, and archaea, and they are involved in a range of processes, such as survival in bacteria,¹ salt tolerance in plants,² and have potential pharmacological implications in hypertension.^{3,4} Sodium/proton antiporters belong to the cation/proton antiporter (CPA) family, which can be divided into two subfamilies; the CPA1 subfamily which includes the medically important mammalian NHE exchangers,⁵ and the CPA2 subfamily, which includes many bacterial antiporters (and only two human exchangers: NHA1-2⁶). The archaeal NhaP antiporters share sequence homology with mammalian NHE antiporters, and are classified in the CPA1 subfamily.^{7,8} One of the functional differences between the sodium/proton transporters from the two subfamilies are stoichiometry, where to the best of our knowledge, CPA1 members that were tested seem to be electroneutral,⁵ suggesting a 1:1 substrate ratio, whereas CPA2 members are electrogenic,⁵ with a measured ratio of 2:1 for some members.⁹ Physiologically, the driving force of several NhaA homologues, members of the CPA2 subfamily, is the proton motive force (PMF; see ref 10), and the purpose is removal of excess Na⁺, whereas the driving force for NhaP and mammalian NHE members is the sodium gradient,¹¹ and the function is removal of protons.¹²

The structure of the sodium/proton antiporter from the single-celled organism *Methanocaldococcus jannaschii* (MjNhaP1) was determined recently, in the first study that has presented two high resolution conformations for the same sodium/proton antiporter.¹³ The transporter belongs to the CPA1 superfamily and it is pH dependent, becoming inactive at high and low pH values. One study found that MjNhaP1 is active at pH 6 and inactive at a pH as low as 7.5.⁷ Another found it inactive below pH 4 or above pH 9 with maximal activity at pH 7.5.¹³ To put these numbers in perspective, the natural habitat of *M.jannaschii*, an archaeon isolated from a submarine hydrothermal vent, has a pH of 4–6.¹⁴ Recent studies argued that this pH dependence is thought to be achieved by competition between sodium ions and protons.^{15–18} Additionally, a study on MjNhaP1 conformational dynamics revealed that there are no pH-induced changes in the transporter,⁷ but rather changes are Na⁺-induced.

In the CPA2 family, *Escherichia coli*'s NhaA (EcNhaA) is probably the most studied sodium/proton antiporter. In EcNhaA it was revealed that residues D163 and D164 are both essential for activity,¹⁹ acting as binding site for sodium ions and protons. This DD motif is extremely conserved (in virtually 100% of the sequences on Pfam²⁰). In contrast, in the CPA1 family this motif appears to be replaced by ND, also thought to be responsible for substrate binding,²¹ and the stoichiometry is found to be 1:1, making MjNhaP1

electroneutral. Nonetheless, the structures of CPA1 and CPA2 members are strikingly similar. However, and surprisingly, mutating the DD motif to ND or vice versa does not seem to alter the mutants' stoichiometry, and is often detrimental to activity, indicating that there are structural or energetic differences beyond these two residues, that have not yet been fully characterized.

Like most other active transporters, MjNhaP1 is thought to function through alternating access.²² The relevant structures of both the inward-facing (IF) and outward-facing (OF) conformations have been determined, albeit using different methods and conditions.¹³ These structures reveal relatively small differences, where some of the helices are barely shifted at all, but others, and notably the ion binding residues, are more shifted (see refs 11 and 13). In contrast, the IF and OF structures of NapA, a CPA2 member, that were more recently revealed, both using X-ray crystallography, show a much larger conformational change.²³ Interestingly, visual inspection of these two cases and constructing an animated morph for each pair implies that the nature of the movement is similar. Taken together, it is possible that the published OF conformation of MjNhaP1 is in fact an intermediate conformation, "partway"-outward, and that the "fully"-outward conformation remains unknown, and would entail larger movements, along the same dynamic "path". Notwithstanding, this work is based on the MjNhaP1 structures as they were published.

MjNhaP1, like EcNhaA, forms dimers in the membrane,^{7,14} with a fairly large dimerization interface.⁷ The dimerization interface is reported to change very little between the two conformations,¹³ making it reasonable to assume that, similar to Glt_{Ph},²⁴ the dimerization domain remains mostly static while the so-called core domain performs most of the movement, to alternate between the IF and OF conformations (see discussion in ref 23).

Our recent energetic analysis on the action of EcNhaA¹⁷ accounts for all the properties of that system, including the stoichiometry. That work found that the pK_a values of the DD motif were crucial for the transporter to function. More so, our work predicted the requirements for the model to function for an electroneutral case as well. In the present work we provide structure based free energy landscapes that pave the way to understanding the major steps in the transport cycle of MjNhaP1 and probably many Na⁺/H⁺ transporters. Our study takes into consideration the two major conformations (IF and OF), substrate binding, and the rotation of the catalytic residue D161 (the equivalent of D164 in EcNhaA). We provide a self-consistent energy scheme that explains the transport cycle, its substrate ratio (stoichiometry) and energy coupling, the latter being a major leap from our previous study on the related system. To put all our energies to the test we employ very long MC simulations under physiological conditions for *M.jannaschii*.

Our approach for simulating the transporter function involves several stages. We start by exploring semimacroscopically the energetics of proton binding to D161, and proceed to calculating the sodium binding as a function of the protonation state of D161. This is repeated for both conformations of MjNhaP1. Following that, we attempt to characterize the dynamics and energetics of the conformational change. Finally, the results are used in Monte Carlo (MC) simulations that are aimed at reproducing the observed trend. We thoroughly investigate which traits that are attributed to transporters in the literature are crucial for the

model to function, and provide structural and energetic accountability for them. To summarize we explore a model that accounts for MjNhaP1 activity and energy coupling.

METHODS AND SYSTEMS

The simulation systems were built by inserting one subunit of MjNhaP1 (PDB ID: 4czb and 4d0a¹³) in a 3D grid of nonpolar particles, at 3 Å spacing, representing a membrane, and surrounded by a water sphere. The membrane was constructed perpendicular to the *z*-axis. Then the systems' energy was minimized using the steepest descent algorithm, followed by the 20 ps production run with a small constraint on the original structure. All simulations and calculations were done using MOLARIS.²⁵

For the calculations of binding energies of the Na⁺ ion or protons (p*K*_a) we used the scaled semimacroscopic protein dipoles Langevin dipoles approach (PDLD/S) of the MOLARIS software.^{25,26} The scaled version assumes that the protein has a dielectric constant ϵ_p which is obtained consistently with the linear response approximation (LRA) that evaluates the average binding energy of the charged and the uncharged states of the protein, and at several configurations extracted from a molecular dynamics (MD) simulation (PDLD/S-LRA²⁷).

For the p*K*_a calculations we had to correct the values obtained by adding 1 to the p*K*_a value in order to compensate for the inconsistency arising from the usage of a spherical model with a limited radius (with a bulk dielectric of water outside the sphere) while the actual system includes the membrane region (see refs 17 and 28).

In using the overcharging (OC) approach,²⁹ we changed the atom's charge from of +1 to +3 using the adiabatic charging method of the MOLARIS package.²⁵

The data collection for the WHAM analysis was performed by applying a harmonic potential on the *z*-axis for Na⁺ or on the dihedral angle χ_1 of D161, at different starting values ("windows") at 10 kcal·mol⁻¹·nm⁻¹ and unbiasing was done using WHAM.³⁰

The Monte Carlo (MC) simulations were performed as previously described,¹⁷ using the metropolis acceptance criteria.³¹

RESULTS AND DISCUSSION

Binding of Substrates

Our study considered the MjNhaP1 system (presented in Figure 1) as a case study for investigating the function of Na⁺/H⁺ antiporters. Initially, we inserted each of the two structures of MjNhaP1¹³ into a membrane model composed of a particle grid.³² A water sphere was then used to solvate each system, using the standard MOLARIS protocol.²⁵ The composite systems were minimized (using the steepest decent algorithm), and subjected to short MD runs with a small restraint on the original structure, to allow the systems to relax. We then used the relaxed structures to perform our calculations and analyses. As a control, we conducted one longer simulation (100 ns for the IF structure) and repeated the same analyses presented below, and obtained identical results (within the statistical error).

In considering the substrates we start with H^+ , where we focused on the pK_a values of the catalytic residue of MjNhaP1, namely D161, using the PDL/D/S-LRA protocol.²⁶ Prior to the pK_a calculation of D161, we explored the possible contribution of the adjacent residues D132 and R320 to the close-range electrostatic field. Both residues were found to be important for function,^{18,21} (equivalents of D133 and K300 in EcNhaA^{19,33}). Our calculations yielded apparent pK_a values of ~ -3 and ~ 0.5 for D132 and ~ 21 and ~ 16 for R320, for the IF and OF conformations, respectively, pointing out that both are predominantly found in their charged state in the protein, in either conformation. With this finding in mind we could proceed to perform the pK_a calculations of D161 having D132 and R320 explicitly charged and all other residues kept at their neutral state (considering their effect macroscopically with a large dielectric constant²⁶). Our calculations provided pK_a values of ~ 9.5 and ~ 5.5 for the IF and OF states, respectively. These results are in line with a predicted pK_a of 6.8 previously reported.¹⁸

The next substrate explored was Na^+ , where we used yet again the PDL/D/S-LRA method to assess its binding profile using the z -axis (membrane normal) as a reaction coordinate. The initial binding site used was the position of the Tl^+ ion in the structure of PaNhaP1,³⁴ a close relative of MjNhaP1, proposed to bind in the same position as substrate Na^+ would, including the putative binding residue D161. From that position, the Na^+ ion was translated and locally relaxed all along the z -axis and out into the bulk (in both directions; at 1 Å intervals).

The energy curves were calculated for the charged and protonated states of D161, in the IF and OF conformations. The results, seen in Figure 2a, show that in both conformations Na^+ binds in the charged state, and that it is repulsed in the neutral state. The high barrier for Na^+ , seen in the latter, prevents sodium from crossing to the other side of the membrane. In the former, on the other hand, there is a minimum point, suggesting binding of Na^+ , flanked by a moderate barrier on the open side and a higher barrier on the closed side. However, the closed-side barrier in the OF curve does not seem to be high enough to prevent occasional release of Na^+ , allowing decoupled substrate leak. Returning to the arguments in the introduction, we suggest that the structure is not fully OF and this might lead to energies that are not entirely realistic. To try and overcome this, we used an overcharging (OC) treatment²⁹ on both structures. Briefly, we place an ion in the vicinity of the binding site, on the open side, and gradually increase the charge of the ion to +3. This exaggerated charge recruits water molecules to hydrate the ion, which in turn presumably promotes further opening of the transporter on that side, concomitant with closing on the other side. After the treatment, the ion is removed and the structure is relaxed and used for a repeated PDL/D/S-LRA analysis. This treatment yielded the curves presented in Figure 2b. As can be seen, the general trend is maintained, but the barriers on the closed sides are higher, while the binding minima are not as deep as in the untreated structures. We proceed with using an average of the energies from Figure 2. An important characteristic of the curves is the asymmetry with respect to the coordinate. One might expect a transporter with alternating access to exhibit some form of functional asymmetry with mirror images for the different conformations (IF vs OF) and we indeed clearly see a mirror image trend in these curves. Lastly, we note that the binding for Na^+ is tighter for the IF structure, which is in line with the observation that Na^+ promotes conversion to the IF conformation and that the resting state is mentioned to be

IF and Na⁺ binding.¹³ Comparison of the calculated K_d for the computed energy minima (~ -5 kcal/mol at pH ~ 7 , or 0.24 mM) to recent experimental apparent K_M results (0.84 mM¹³) shows a decent agreement.

To reinforce our findings, we performed WHAM analyses for the Na⁺ binding. The results correlate very well qualitatively (see Supporting Figure S1) but are quantitatively higher. The reason for the higher values obtained is probably related to convergence problems. That is, as we have demonstrated in many of our works (e.g., refs 26 and 35) it is very hard to obtain converging results in microscopic free energy calculations of charges in protein interiors. Now, while it may seem that running longer and longer simulations for the mapping windows provides the same results, giving the impression of convergence, actually the electrostatic effects and the compensation of the protein's dipoles, and water penetration is not being taken into account properly, from our experience, and the resulting energies are higher. In Supporting Figure S1 the WHAM results are scaled down by a factor of 2 for easier visual comparison. On the other hand, the PDLD/S-LRA method takes into account implicitly missing compensation effects and frequently gives more accurate electrostatic energies in complex slowly relaxing environments.

Following our previous work on EcNhaA,¹⁷ we applied the same model to MjNhaP1, while trying to put more effort into understanding the nature of the conformational change. In this respect, MjNhaP1 offered a great opportunity since it has structures reported for both IF and OF conformations. The transporter was said to alternate its accessibility by means of a "rocking bundle"¹³ (see refs 36 and 37 and Supporting Figure S2), where at any time only one side of the membrane can access the transporter's binding side (also see the works in refs 38 and 39). Recently this view has been challenged and the "elevator model" has been suggested instead²³ (see Supporting Figure S2). However, for most considerations in this work (as well as in our previous one¹⁷) this distinction has no consequences. Regardless, it is fairly likely that the conformational change is much slower than binding and unbinding of the substrates, and therefore serves as the rate-determining step. Thus, based on the observed rate of ion transfer of 0.94 ions/second at pH 6 and 1.68 ions/second at pH 8 (made at 0 °C for technical reasons¹³) we can project a barrier of ~ 18 kcal/mol for the conformational change (more on this calculation in the Supporting Information and in ref 17). However, using a barrier of 18 kcal/mol would not allow a simulation to complete in a reasonable amount of time. Therefore, we scaled down the barriers (to 8, 8.5, 9, 9.5, 10, 10.5, and 11 kcal/mol) and verified that the ratio and direction of transport do not change, and that the rate of transport behaves according to thermodynamic laws. Once we found that this treatment is consistent, we performed our production MC simulations with a barrier of 8 kcal/mol.

This value (i.e., 18 kcal/mol) highlights an inaccuracy in the energies presented in Figure 2 that needs to be addressed. The PDLD/S calculations yielded barriers of ~ 10 kcal/mol on the closed sides for Na⁺ binding, but clearly the actual barriers have to be higher than the barrier for the conformational change, or else by the time the transporter switches its conformation, ions would leak between both sides of the membrane. Using other methods to assess these barriers (such as WHAM, see Supporting Figure S1) we were able to get higher barriers, in the range of ~ 20 kcal/mol. Note, however, that microscopic methods can suffer from major

convergence problems. Thus, for purposes of binding affinity calculations, we feel that the PDLD/S-LRA method provides a more accurate depiction of the actual energetic picture, as argued above. With this in mind, we choose to use the PDLD/S-LRA results bearing in mind that it underestimates the very high barriers.

Tentative Examination of the Barriers for Local and Global Conformational Changes

Although it is not clear if the available structures represent the full conformational changes, we attempted to gauge the conformational barrier by employing WHAM, using as a generalized reaction coordinates a selected vector of the distance between certain residues in the two structures (which either come together or grow apart as the conformation switches). However, the WHAM calculations were not able to produce consistent energy profiles, perhaps because of the convergence problems mentioned above. Nevertheless, zooming in on the catalytic residue D161 it is clear from the structures that, whereas the helix on which it is situated moves fairly little, D161 itself is facing almost opposite direction (see Figure 1). This observation is perfectly expected, since D161 binds the substrates and needs to be accessible to the different vestibules of the transporter, as a function of the conformation. Therefore, we calculated the free-energy profile as a function of the dihedral angle of D161 (χ_1) while keeping the rest of the protein in a single conformation. Clearly though, the protein conformational change plays a part as well, and thus we repeated this simplified analysis using different protein conformation scaffolds. We used both IF and OF structures, as well as 3 selected points from each targeted MD (TMD) result of going from one to the other (in both directions) for a total of 8 different starting structures. We averaged structures that correspond to the same “point” along the conformational change process (e.g., the first step of the IF to OF TMD corresponds to the last step of the OF to IF TMD)

The results show two interesting trends. The first observation is that the transition from the “IF-rotamer” (gauche⁻) to the “OF-rotamer” (trans) becomes more energetically favorable as the structure assumes the OF conformation (Figure 3a and Supporting Figure S3). This might be obvious, but this coupling between the conformational change and the rotation of D161 is important for drawing conclusions based solely on the rotamer angle energy (as we attempt below). The second observation, and probably the more interesting one, is that the energy barrier when going from IF to OF conformations is lower when the residue is protonated, compared to the charged form (Figure 3b and Supporting Figure S3). This suggests that D161 rotation is energetically more likely when it is protonated. When we repeated this analysis with a bound Na⁺ we see that the barrier is actually higher, but considering the fact that it is not possible for the protein to relax properly in our computers’ performance, this barrier is probably much lower in reality. Nonetheless, the conformational change is less likely to occur when the transporter is found in the apo form because at the presence of protons and Na⁺ it is energetically more stable when it binds one of them. Taken together, we conclude that the transition from IF to OF conformations in the charged state for D161 is energetically unfavorable.

Overall, our analysis of D161 suggests that the conformational change is concerted with the rotation of the residue, and the rotation of the residue is more likely when D161 is bound to a substrate, and if we take this one step further, we suggest that when conformational

changes happen, D161 is preferentially bound to a substrate. It is important to note that for all of the above calculations we removed from the computations all the energy barriers of the dihedral potentials from D161 χ_1 and the energies are compared to the same process in water (isolated aspartic acid residue). In Figure 3 we added a standard periodic dihedral potential to the WHAM energies calculated.

We are aware of the major caveat here, where we do not have information on the conformational change itself, but considering the fairly consistent gap between the curves, using several points along this trajectory (Figure 3b and Supporting Figure S3) we feel that this result can be considered as a reasonable approximation. Also, it is hard to conceive that the conformational effects will favor the charged form (where a charge has to cross a generally hydrophobic surrounding) so that any effect we neglect here will increase, if anything, this difference in barriers. To conclude, we suggest that the energy barrier for the conformational change in the charged state of D161 should be somewhat higher than in the bound state. We proceed using a minimal difference of 1.5 kcal/mol based on the energies presented in Figure 3b. We keep in mind that the difference is most likely higher, but increasing this difference can only further improve our results (e.g., at 65 °C with an inward Na^+ gradient the leak rate goes down from $49.7 \pm 21.8\%$ to $4.4 \pm 1.5\%$ when we increase this barrier difference from 1.5 to 3 kcal/mol; data not shown). We note again that the conformational barrier was determined based on the assumption that it is the rate-determining step (see above).

Simulating the Function of MjNhaP1

The main aim of our work is to shed light on the anitport function of MjNhaP1 by structure based simulations. To move in this direction we used the same MC model used previously for EcNhaA.¹⁷ Briefly, we simplified the system to discrete sites: bulk on each side, a binding site, and barriers on both sides (see Methods and Systems Section and ref 17 for more details). The energies used were taken from Figure 2. A comparison of the energies from Figure 2 and the interpolated energies used in the MC simulations is presented in Supporting Figure S4. The treatment of the barriers for the conformational changes, which are less certain, will be discussed below.

In our previous work we ignored any mechanistic coupling between Na^+ and H^+ . That model was still instructive, and functioned well under many conditions (including some of the experimental conditions tested in the works cited), but our new results enable us to improve it. The natural addition to the model is a restrictive conformational changes, i.e., the barrier to cross between IF and OF conformations is higher under certain circumstances (such as no bound substrates). Whereas many may take this for granted, our belief is that a model should assume as little as necessary to reproduce the function, and only when proving that some element is crucial, should it be carefully considered and added. Upon addition, one should provide some structural explanation to how it is accounted for. Hence, our improved MC model incorporates an energy barrier which is 1.5 kcal/mol higher when switching conformations at the D161 charged state (i.e., no substrates bound).

Next we performed long MC simulations ($n = 32$ per condition) using conditions that are close to what *M.jannaschii* encounter in their natural habitat. That is, an inward-facing Na^+

gradient of $\sim 2\text{--}3$ kcal/mol and an inward facing proton gradient of $1\text{--}2$ kcal/mol. Overall the gradients drive a Na^+_{in} -dependent H^+_{out} -active pumping. These gradients allow a margin of ~ 0.5 kcal/mol driving force. As a control, we changed the gradients so that this margin is in favor of either Na^+ or H^+ , and in either direction to ensure there are no directional or substrate-dependent biases assuming that MjNhaP1, like Ec-NhaA, can function in any direction and with any gradient.¹⁰ The results of the MC simulations, at natural $\sim 65^\circ\text{C}$ and at room temperature are presented in Figure 4. As can be seen, all conditions show statistically significant antiporting, at ratios close to 1:1, with coupling between Na^+ and H^+ transport, regardless of the driving force and its direction. Clearly, the rate at 65°C is higher, because more energy is needed to overcome the barrier between the conformations, but the rate of leak is also increased (overcoming the closed-side barrier for substrates), thus the deviation from perfect 1:1 ratio (also note that these are scaled rates, see discussion on the height of the barrier used above). Overall, the model works well under these very limiting conditions (small driving force to push particles up their gradients) and it was somewhat surprising (at least to us) that a fairly small energy gap of 1.5 kcal/mol was mostly enough for coupling. However, this can be explained simply because the substrate-less transporter is less stable in energy (compared to bound transporter) making the conformational change at this state unlikely to happen, even when the barriers are similar in height (see Figure 5). It might be helpful to conceptualize the transporter's mode of function as a selective pendulum, where the transporter conformations switch only when a substrate is bound, thus allowing coupled exchange or the occasional "futile" swing where it transports the same substrate back and forth.

We present in Figure 6 energy surfaces for the system, using the energies of the MC model. To iterate, our MC model energies (rendered as Gaussians) are based on the PDL/D/S-LRA results (average of the original structures and the structures after the OC treatment) having the barriers on the closed side corrected by increasing them, based on our WHAM results (qualitatively) and considerations discussed above. The energy surfaces are insightful because they allow visualization of the coupling between the substrates.

To summarize, we present in Figure 5 a complete and simplified scheme for MjNhaP1 transport cycle, starting for convenience with an apo transporter (which in effect is expected to be quite rare).

IV. CONCLUDING REMARKS

In this study we analyzed the structures of the IF and OF conformations of archaeal MjNhaP1 transporter. We calculated energy for substrate binding as well as the energy of the rotation of the catalytic residue D161 from the IF rotamer to the OF one. We find that charged D161 binds Na^+ , while the protonated form entails a barrier which is too high for the ion to cross. Also, each conformation prevents crossing of either substrate to the other side. By rotating D161 and exposing the binding site to one side of the membrane at a time, antiporting can be achieved down the free energy path. Subsequently, by allowing this rotation to occur only when either substrate is bound we can achieve coupled exchange at any gradient and any conditions. In this regard though, we were unable to reproduce the actual conformational change energy barrier, and can only point out on the trends and

qualitative nature of it. Considering the possibility that the structures (specifically OF) might not represent the exact structure at the resting state of an active transporter, for that conformation, which could have an effect on the calculations we present, we feel that our results still provide very valuable insight on the action of MjNhaP1 and probably other transporters as well. Overall we were able to report binding energies and pK_a 's that correspond to experimental results reasonably well, as well as allow coupled transport at the expected ratio.

Beyond the insight gained on the function of MjNhaP1 itself, we note that pharmaceutical opportunities might be gleaned as well. NHE exchangers are the human equivalents of MjNhaP1 and their implication in human diseases is being revealed constantly (e.g., hypertension, reperfusion damage, cancer; for review see ref 3). Thus, we hope that our study here might serve as a helpful base for other more medically relevant studies on NHE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the NIH grant GM40283, the National Science Foundation Grant MCB-1243719. We acknowledge the University of Southern California's High-Performance Computing and Communications Center for computer time.

References

1. Herz K, Vimont S, Padan E, Berche P. Roles of NhaA, NhaB, and NhaD Na⁺/H⁺ antiporters in survival of *Vibrio cholerae* in a saline environment. *J Bacteriol.* 2003; 185:1236–1244. [PubMed: 12562793]
2. Shi H, Ishitani M, Kim C, Zhu JK. The *Arabidopsis thaliana* salt tolerance gene SOS1 encodes a putative Na⁺/H⁺ antiporter. *Proc Natl Acad Sci U S A.* 2000; 97:6896–6901. [PubMed: 10823923]
3. Hendus-Altenburger R, Kragelund BB, Pedersen SF. Structural dynamics and regulation of the mammalian SLC9A family of Na⁺/H⁺ exchangers. *Curr Top Membr.* 2014; 73:69–148. [PubMed: 24745981]
4. Jia Y, Jia G. Role of intestinal Na⁺/H⁺ exchanger inhibition in the prevention of cardiovascular and kidney disease. *Ann Transl Med.* 2015; 3:91. [PubMed: 26015933]
5. Brett CL, Donowitz M, Rao R. Evolutionary origins of eukaryotic sodium/proton exchangers. *Am J Physiol Cell Physiol.* 2005; 288:C223–239. [PubMed: 15643048]
6. Donowitz M, Ming Tse C, Fuster D. SLC9/NHE gene family, a plasma membrane and organellar family of Na⁺/H⁺ exchangers. *Mol Aspects Med.* 2013; 34:236–251. [PubMed: 23506868]
7. Goswami P, Paulino C, Hizlan D, Vonck J, Yildiz O, Kuhlbrandt W. Structure of the archaeal Na⁺/H⁺ antiporter NhaP1 and functional role of transmembrane helix 1. *EMBO J.* 2011; 30:439–449. [PubMed: 21151096]
8. Lee C, Yashiro S, Dotson DL, Uzdavinyas P, Iwata S, Sansom MS, von Ballmoos C, Beckstein O, Drew D, Cameron AD. Crystal structure of the sodium-proton antiporter NhaA dimer and new mechanistic insights. *J Gen Physiol.* 2014; 144:529–544. [PubMed: 25422503]
9. Taglicht D, Padan E, Schuldiner S. Proton-sodium stoichiometry of NhaA, an electrogenic antiporter from *Escherichia coli*. *J Biol Chem.* 1993; 268:5382–5387. [PubMed: 8383669]
10. Calinescu O, Fendler K. A universal mechanism for transport and regulation of CPA sodium proton exchangers. *Biol Chem.* 2015; 396:1091–1096. [PubMed: 25719314]

11. Paulino C, Kuhlbrandt W. pH- and sodium-induced changes in a sodium/proton antiporter. *eLife*. 2014; 3:e01412. [PubMed: 24473071]
12. Thauer RK, Kaster AK, Seedorf H, Buckel W, Hedderich R. Methanogenic archaea: ecologically relevant differences in energy conservation. *Nat Rev Microbiol*. 2008; 6:579–591. [PubMed: 18587410]
13. Paulino C, Wohler D, Kapotova E, Yildiz O, Kuhlbrandt W. Structure and transport mechanism of the sodium/proton antiporter MjNhaP1. *eLife*. 2014; 3:e03583. [PubMed: 25426803]
14. Vinothkumar KR, Smits SH, Kuhlbrandt W. pH-induced structural change in a sodium/proton antiporter from *Methanococcus jannaschii*. *EMBO J*. 2005; 24:2720–2729. [PubMed: 16015376]
15. Schuldiner S. Competition as a way of life for H(+)-coupled antiporters. *J Mol Biol*. 2014; 426:2539–2546. [PubMed: 24862284]
16. Calinescu O, Danner E, Bohm M, Hunte C, Fendler K. Species differences in bacterial NhaA Na⁺/H⁺ exchangers. *FEBS Lett*. 2014; 588:3111–3116. [PubMed: 25017436]
17. Alhadeff R, Warshel A. Simulating the function of sodium/proton antiporters. *Proc Natl Acad Sci U S A*. 2015; 112:12378–12383. [PubMed: 26392528]
18. Calinescu O, Paulino C, Kuhlbrandt W, Fendler K. Keeping it simple, transport mechanism and pH regulation in Na⁺/H⁺ exchangers. *J Biol Chem*. 2014; 289:13168–13176. [PubMed: 24644283]
19. Inoue H, Noumi T, Tsuchiya T, Kanazawa H. Essential aspartic acid residues, Asp-133, Asp-163 and Asp-164, in the transmembrane helices of a Na⁺/H⁺ antiporter (NhaA) from *Escherichia coli*. *FEBS Lett*. 1995; 363:264–268. [PubMed: 7737413]
20. Finn RD, Coghill P, Eberhardt RY, Eddy SR, Mistry J, Mitchell AL, Potter SC, Punta M, Qureshi M, Sangrador-Vegas A, et al. The Pfam protein families database: towards a more sustainable future. *Nucleic Acids Res*. 2016; 44:D279–285. [PubMed: 26673716]
21. Hellmer J, Teubner A, Zeilinger C. Conserved arginine and aspartate residues are critical for function of MjNhaP1, a Na⁺/H⁺ antiporter of *M. jannaschii*. *FEBS Lett*. 2003; 542:32–36. [PubMed: 12729893]
22. Jardetzky O. Simple allosteric model for membrane pumps. *Nature*. 1966; 211:969–970. [PubMed: 5968307]
23. Coincon M, Uzdavinyas P, Nji E, Dotson DL, Winkelmann I, Abdul-Hussein S, Cameron AD, Beckstein O, Drew D. Crystal structures reveal the molecular basis of ion translocation in sodium/proton antiporters. *Nat Struct Mol Biol*. 2016; 23:248–255. [PubMed: 26828964]
24. Reyes N, Ginter C, Boudker O. Transport mechanism of a bacterial homologue of glutamate transporters. *Nature*. 2009; 462:880–885. [PubMed: 19924125]
25. Lee FS, Chu ZT, Warshel A. Microscopic and semimicroscopic calculations of electrostatic energies in proteins by the Polaris and Enzymix programs. *J Comput Chem*. 1993; 14:161–185.
26. Warshel A, Sharma PK, Kato M, Parson WW. Modeling electrostatic effects in proteins. *Biochim Biophys Acta, Proteins Proteomics*. 2006; 1764:1647–1676.
27. Warshel A, Sharma PK, Kato M, Xiang Y, Liu H, Olsson MH. Electrostatic basis for enzyme catalysis. *Chem Rev*. 2006; 106:3210–3235. [PubMed: 16895325]
28. Alden RG, Parson WW, Chu ZT, Warshel A. Calculations of electrostatic energies in photosynthetic reaction centers. *J Am Chem Soc*. 1995; 117:12284–12298.
29. Kato M, Warshel A. Using a charging coordinate in studies of ionization induced partial unfolding. *J Phys Chem B*. 2006; 110:11566–11570. [PubMed: 16771433]
30. Grossfield, A. WHAM: the weighted histogram analysis method. membrane.urmc.rochester.edu/content/wham
31. Metropolis N, Rosenbluth AW, Rosenbluth MN, Teller AH, Teller E. Equation of state calculations by fast computing machines. *J Chem Phys*. 1953; 21:1087–1092.
32. Rychkova A, Vicatos S, Warshel A. On the energetics of translocon-assisted insertion of charged transmembrane helices into membranes. *Proc Natl Acad Sci U S A*. 2010; 107:17598–17603. [PubMed: 20876127]
33. Hunte C, Screpanti E, Venturi M, Rimon A, Padan E, Michel H. Structure of a Na⁺/H⁺ antiporter and insights into mechanism of action and regulation by pH. *Nature*. 2005; 435:1197–1202. [PubMed: 15988517]

34. Wohlerl D, Kuhlbrandt W, Yildiz O. Structure and substrate ion binding in the sodium/proton antiporter PaNhaP. *eLife*. 2014; 3:e03579. [PubMed: 25426802]
35. Chakrabarty S, Warshel A. Capturing the energetics of water insertion in biological systems: the water flooding approach. *Proteins: Struct, Funct Genet*. 2013; 81:93–106. [PubMed: 22911614]
36. Lee C, Kang HJ, von Ballmoos C, Newstead S, Uzdavinyas P, Dotson DL, Iwata S, Beckstein O, Cameron AD, Drew D. A two-domain elevator mechanism for sodium/proton antiport. *Nature*. 2013; 501:573–577. [PubMed: 23995679]
37. Forrest LR, Rudnick G. The rocking bundle: a mechanism for ion-coupled solute flux by symmetrical transporters. *Physiology*. 2009; 24:377–386. [PubMed: 19996368]
38. Li J, Wen PC, Moradi M, Tajkhorshid E. Computational characterization of structural dynamics underlying function in active membrane transporters. *Curr Opin Struct Biol*. 2015; 31:96–105. [PubMed: 25913536]
39. Adelman JL, Dale AL, Zwier MC, Bhatt D, Chong LT, Zuckerman DM, Grabe M. Simulations of the alternating access mechanism of the sodium symporter *Mhp1*. *Biophys J*. 2011; 101:2399–2407. [PubMed: 22098738]

Figure 1.

Side view of MjNhaP1 structures in both conformations. The protein is shown as gray ribbons; the membrane boundaries are marked by black lines, and residues N160 and D161 are shown explicitly. A zoom-in view of residue D161 is shown in a dashed circle.

Figure 2. PDL/D-S-LRA binding curves for Na⁺ for different pronation states of D161 and conformations of MjNhaP1 (see legend). The faded contour around each curve represents the standard deviation ($n = 10$). Results are shown for the (a) relaxed structures and (b) structures after the OC treatment.

Figure 3.

Free energy profiles for the dihedral angle χ_1 of D161. IF and OF rotamers are marked by yellow columns accompanied by a cartoon. Each color represents a structure taken from the TMD simulations, such that red is IF, blue is OF, and green and cyan are intermediates closer in the conformational space to the IF and OF, respectively. (a) Comparison of different starting structures, using the charged D161 form as a representative example. (b) Comparison of the state of D161, such that the full line is for charged D161, dotted line is for protonated D161, and the dashed line is for Na⁺ bound. Only IF and OF conformations are shown as representative examples. The curves were vertically translated so that they begin at the same energy, for visualization purposes. For the entire set of results see Supporting Figure S3.

Figure 4.

MC simulation results. At the end of each MC simulation, we count the net number of Na^+ and H^+ particles transferred from one side of the membrane to the other. Each simulation is depicted by a circle on the chart and the lines show the average with error bars ($n = 32$). The colors define which substrate has the larger gradient, and is therefore the driving force. By convention, we assigned one side of the membrane to be the reference for counting, and “for” and “rev” in the legend describe driving forces in opposite directions, with respect to this reference. The gray diagonal line represents perfect electroneutrality (1:1 ratio).

Figure 5.

A summary of the overall cycle. Cartoon representations of the protein at different conformations (IF, OF, and high energy transition state) and binding states are presented at various heights qualitatively corresponding the relative free energy. For visualization purposes, exaggerated opposing gradients for Na⁺ and H⁺ are presented.

Figure 6.

2D energy surfaces of the MjNhaP1 system. The total energy of the system, as a function of the Na^+ position and the H^+ position, with respect to the binding site, is depicted. Energies were taken from the MC simulations and rendered as Gaussian (for visual smoothness). The color code represents the energy as shown in the scale (in kcal/mol; note that the deep red represents energies outside the scale, at >15 kcal/mol). The two axes represent the position of Na^+ or H^+ from one bulk to the other, through the transporter's binding site, as shown in the cartoon figures (blue and white circles are Na^+ and H^+ , respectively). Each surface depicts one conformation (based on one structure). The white line follows one optimal exchange cycle.