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Molecular Modeling and Inhibitor Docking Analysis of the Na⁺/H⁺ Exchanger Isoform One

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Running Title, Molecular Model of the Na⁺/H⁺ Exchanger

Abstract

Na^+/H^+ exchanger isoform one (NHE1) is a mammalian plasma membrane protein that removes intracellular protons, thereby elevating intracellular pH (pH_i). NHE1 uses the energy of allowing an extracellular sodium down its gradient into cells, to remove 1 intracellular proton. The ubiquitous protein has several important physiological and pathological influences on mammalian cells as a result of its activity. The three dimensional structure of human NHE1 (*hNHE1*) is not known. Here, we modeled NHE1 based on the structure of *MjNhaP1* of *Methanocaldococcus jannaschii* in combination with biochemical surface accessibility data. *hNHE1* contained 12 transmembrane segments including a characteristic Na^+/H^+ antiporter fold of two transmembrane (TM) segments with a helix-extended region-helix conformation, crossing each other within the membrane. Amino acids 363-410 mapped principally to the extracellular surface as an extracellular loop (EL5). A large preponderance of amino acids shown to be surface accessible by biochemical experiments, mapped near to, or on, the extracellular surface. Docking of Na^+/H^+ exchanger inhibitors to the extracellular surface suggested that inhibitor binding on an extracellular site is made up from several amino acids of different regions of the protein. The results present a novel testable, three-dimensional model illustrating NHE1 structure and accounting for experimental biochemical data.

Key Words; Molecular Modeling, *MjNhaP1*, Na^+/H^+ exchanger, NhaA, NHE1,

Introduction

Membranes are key to cellular function. They separate cells from the extracellular environment and allow cells to maintain intracellular compartments of specific content. The plasma membrane of eukaryotic cells provides a barrier to ions permitting, for example, a relatively low intracellular sodium level and a high intracellular potassium relative to the extracellular milieu of mammalian cells. However, at the same time the plasma membrane restricts the removal of cations such as protons, which accumulate as a result of cellular metabolism. One protein that removes intracellular protons, and thereby elevates intracellular pH (pH_i) is the mammalian Na^+/H^+ exchanger isoform one (NHE1). NHE1 is an 815 amino acid plasma membrane protein ubiquitous in mammalian cells. It consists of a membrane domain of about 500 amino acids and an internal cytosolic regulatory domain (Fig. 1A). NHE1 allows an extracellular sodium down its gradient into the cell and removes 1 intracellular proton. It thus maintains and elevates pH_i while protecting cells from metabolism-induced acidification. NHE1 also responds actively to osmotic stress, thereby aiding in regulation of cell volume (Fliegel 2005; Hendus-Altenburger et al. 2014). There are ten isoforms of Na^+/H^+ exchangers that are different gene products. Isoform one, the ubiquitous NHE1, has several important physiological and pathological influences on mammalian cells as a result of its activity (Fliegel 1999; Fliegel et al. 1993; Fliegel et al. 1991; Lee et al. 2013a; Lee et al. 2008; Orłowski 2004; Orłowski et al. 1992; Takaichi et al. 1992). In myocardial disease, elevated NHE1 activity acts as a trigger in both heart hypertrophy and ischemia reperfusion damage (Karmazyn 2013; Karmazyn et al. 1999). NHE1 is also important in breast cancer, where

elevated activity can trigger metastasis (Amith and Fliegel 2016, 2017; Reshkin et al. 2014). In animal models NHE1 inhibitors have demonstrated cardioprotective properties during ischemia reperfusion treatment episodes in the myocardium (Karmazyn 2013). In addition, NHE1 inhibition has been shown to suppress metastasis of breast cancer cells (Amith et al. 2016). There has therefore been a great deal of interest in the development of clinically useful NHE inhibitors and though the concept of NHE inhibitors as a therapeutic agent remains sound, off target actions of one type of inhibitor prevented successful outcomes in a clinical trial (Karmazyn 2013). The *h*NHE1 protein is 815 amino acids long. The N-terminal 500 amino acids form the membrane transport domain while the C-terminal balance, forms a regulatory domain that modulates activity of the membrane domain (Lee et al. 2013a).

Given that NHE1 plays an important role in human pathology and physiology, there has been a great deal of interest in determining the structure of the protein, both to determine its mechanism of transport, and to facilitate the development of clinically useful inhibitors. This manuscript describes developments in characterization of the structure of the NHE1 protein, from initial topology models to more recent modeling. We also present a new model of the mammalian *h*NHE1 protein, based on a combination of biochemical experiments and molecular modeling, and based on the recently deduced crystal structure of the *Methanococcus* Na⁺/H⁺ exchanger. The three-dimensional model takes into account recent biochemical accessibility experiments.

The Na⁺/H⁺ exchanger family - recent advances in structure determination.

Transmembrane exchange of protons for sodium ions is ubiquitous across all phyla and kingdoms and thus NHEs play an important role in a multitude of species. The evolutionary aspects of NHEs were last reviewed by Brett et al. in 2005 (Brett et al. 2005). They divided up the monovalent cation proton antiporter (CPA) superfamily into CPA1, CPA2 and NaT-DC (Na-transporting carboxylic acid decarboxylase) families, which each had unique bacterial ancestors. The CPA1 family transports Na^+ , Li^+ , K^+ or Rb^+ in electroneutral exchange for a proton and includes mammalian NHE1-9. CPA2 family can catalyze electrogenic activity, includes Na^+ , K^+/H^+ exchangers and includes the well-known antiporter *E. coli* NhaA. It also includes the fungal antiporters and the mammalian NHA1 and NHA2 proteins that are electroneutral. The NaT-DC transporters mediate the transmembrane export of 1–2 Na^+ in exchange for an extracellular H^+ as part of a multiprotein complex catalyzing the decarboxylation of oxaloacetate, malonyl-CoA, or glutaconyl-CoA.

Not surprisingly, the greatest progress has been made on elucidation of Na^+/H^+ exchanger structures of the bacterial transporters that have proven easier to overproduce and crystallize. We (Dutta and Fliegel 2018) and others (Hendus-Altenburger et al. 2014) reviewed progress on the structure of bacterial plasma membrane antiporters, which is summarized here. The structures of four plasma membrane Na^+/H^+ antiporters, *E. coli*, NhaA, (Hunte et al. 2005), NapA of *Thermus thermophilus* (Lee et al. 2013b), MjNhaP1 of *Methanocaldococcus jannaschii* (Paulino and Kuhlbrandt 2014) and PaNhaP of *Pyrococcus abyssi* (Wohlert et al. 2014) are known. The initial structure solved, that of

E. coli NhaA, led to the idea that Na^+/H^+ antiporters have a novel fold that consists of two transmembrane segments with a helix-extended region-helix conformation, TM4 and TM11. These cross to make the NhaA fold. *EcNhaA* was also found to be a dimer of two 12 transmembrane helices per protomer with both N- and C-terminal cytoplasmic tails. It had a dimerization or scaffolding subdomain and a 6-helix bundle cylindrical transport subdomain (Hunte et al. 2005; Padan 2014). The “*EcNhaA* fold” (Padan 2014) contributes to the cation binding site and allows for rapid conformational changes that occur in transport. This catalytic region is in the middle of membrane. The antiparallel unfolded regions lie mid membrane between the helical regions. Where these unfolded regions cross each other, the helices surrounding the unfolded regions have a charge imparted from the helix dipoles mid membrane. This charge is neutralized by a charged polar amino acid within the membrane. The unfolded region of TM4 undergoes conformational changes with varying physiological pH and several of the conserved and functionally important residues of TM4 have been suggested to line the cationic passage channel, according to results of mutagenesis experiments (Rimon et al. 2012). *EcNhaA* is active at alkaline pH (>7.5) to extrude one Na^+ in exchange for two H^+ 's. Clusters of titratable groups near the cytoplasmic funnel entrance of the antiporter may be part of the pH sensor (Olkhova et al. 2006) altering activity in response to pH. Another suggestion for the mechanisms of NhaA activation is that it 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 (Huang et al. 2016).

The NhaA fold is present in *Tth*NapA (Lee et al. 2013b), *Mj*NhaP1 (Goswami et al. 2011), and *Pa*NhaP (Wohlert et al. 2014). They have an additional TM helix N-terminal to the fold but have discontinuous helices 5 and 12, which correspond to 4 and 11 of *Ec*NhaA (Wohlert et al. 2014). *Ec*NhaA is a dimer. There are dimer contacts with extracellular beta strands and TM 1, TM 9, TM 6 and TM 7. Helices 6 and 7 form an alpha hairpin at the dimer interface (Padan et al. 2015). In *Ec*NhaA, a beta sheet on the periplasmic side of the membrane is also important in dimerization (Rimon et al. 2007). *Mj*NhaP1 is a dimer like NhaA but the dimer contacts are different. The two peptides are held together by tight helix–helix interactions across an extensive, probably hydrophobic, contact surface at the dimer interface (Goswami et al. 2011).

Topology of Human NHE1

The topology of the *h*NHE1 isoform of the Na^+/H^+ exchanger has been the subject of some debate. The first comprehensive model based on biochemical analysis was that of Wakabayashi (Wakabayashi et al. 2000). They did a thorough analysis using cysteine-scanning accessibility and suggested a 12 transmembrane segment model. Of note, TM1 was amino acids 15-36 with an N-terminal that was cytosolic. It was followed by a large extracellular loop of about 70 amino acids. Additionally, amino acids 363-410 were thought to form a membrane associated segment, extracellular loop (EL) 5 which was preceded by TM9 (amino acids 341-362) and followed by TM10 (aa 411-432). TM 4 and TM11 were amino acids 154-176 and 449-471 respectively.

Later an alternative 3D model was proposed which was based on homology modeling with *EcNhaA* (Landau et al. 2007). This model was similar to that of Wakabayashi and had 12 transmembrane regions but it was suggested that amino acids 1-125 were removed by cleavage. TM9 of (Wakabayashi et al. 2000) was reassigned as two short helices denoted TM7 and TM8 and re-entrant segment EL5 was reassigned as TM9 (amino acids 374-398). The last three transmembrane segments were the same in both models.

Our own (Liu et al. 2015) follow up work to these two studies suggested that the essential features of the Wakabayashi model were correct. We examined the cell surface accessibility of amino acids in the proposed first extracellular loop (amino acids 36-106). A fully active cysteine free NHE1 protein was made and we used cysteine scanning mutagenesis and MTSET labeling combined with cell surface biotinylation of this protein to determine accessibility of residues mutated to cysteine. Results showed that amino acids within this region occurred in the mature protein that was present on the extracellular surface. Additionally, by using a combination of site-specific mutagenesis and cell surface labeling, we showed that NHE1 protein with the Asn75 glycosylation site, was present on the cell surface. Using the same techniques we also examined surface accessibility of the downstream amino acids of the contentious sequence amino acids 363-410. We (Liu et al. 2015) found amino acids 366, 369, 377, 401 and 402 are accessible from the extracellular surface, which agreed with the earlier results which used similar techniques and showed that 10 other amino acids in this segment are accessible extracellularly (Wakabayashi et al. 2000). Thus a total of 15 amino acids within this segment are accessible to extracellular labeling when changed to Cys. Another study,

(Jinadasa et al. 2015) also examined amino acids of this region and their results were more supportive of the Wakabayashi model. Thus, empirically obtained biochemical data from three different laboratories, support the idea that amino acids 363-410 are EL5 with amino acids 341-362 preceding it as TM9. Nevertheless this left only a two-dimensional model of the *h*NHE1 protein.

Another study (Nygaard et al. 2011) attempted to make a three dimensional model of *h*NHE1 threaded on the structure of NhaA. They attempted to assign comparable roles for Asp172 and Arg425 with the important functional residues of Nha, Asp133 and Lys300. Recently however, another report (Calinescu et al. 2017) showed that mutation of Lys300 of *Ec*NhaA to several amino acids did not prevent electrogenic transport. And while it was suggested that mutation of these human residues should cause severe disruption of catalytic activity, mutation of Asp172 to Cys decreased activity mostly due to effects on reduced protein expression and targeting (Slepkov et al. 2005) and mutation of Asp172 to Asn only had a minor effect on NHE1 activity (Slepkov et al. 2007). This does not agree with the proposed role of Asp172 in screening a helix dipole. Their model did, however, predict that amino acids 363-410 are on the extracellular surface of the protein.

The development of new and improved inhibitors against *h*NHE1 can be facilitated by knowledge of the details of the atomic structure of the protein. However, obtaining the atomic structure of the entire mammalian NHE1 protein remains a challenge as yet unmet. On the other hand, computationally predicted models can still provide useful

information when the atomic structure is not available (Franca 2015). While this approach has validity, we suggest that structural modeling should take into account empirically determined biochemical data, when available, rather than being based solely on computer modeling that are untested. *E. coli* NhaA is only 15% identical and 31% similar to *hNHE1* and among other new Na^+/H^+ exchanger structures elucidated, the sequence of *Methanococcus jannaschii* (Goswami et al. 2011) is more similar to *hNHE1* with 20% identity and 38% similarity. Additionally, both *hNHE1* and *MjNhaP1* show similar pH profiles for activity being activated by decreases in pH_i (Hellmer et al. 2002) while that of *E. coli* is quite different from *hNHE1* (Dutta and Fliegel 2018). Recently, we predicted the structure of the plasma membrane Na^+/H^+ antiporter for plant and yeast proteins, using a procedure that is based on an inclusive sequence alignment (Dutta and Fliegel 2018; Dutta et al. 2017; Ullah et al. 2016). Here we take a similar approach to build a *hNHE1* structure based on *MjNhaP1*.

Methods

Building a human NHE1 model

Alignment

Human NHE1 sequence shows 20% sequence identity with *MjNhaP1* in the membrane domain, higher than that of *E. coli* NhaA that is 15% identical. Therefore, *MjNhaP1*

(PDB 4CZB) was used as a template to build the *hNHE1* model. Multiple and pairwise sequence alignment was done as described earlier (Dutta and Fliegel 2018) and is shown in Fig. 1B. Briefly, the MAFFT server (Kato and Standley 2013) was used for multiple and pairwise sequence alignment. Secondary structure prediction was done using the PSIPRED server (Buchan et al. 2013). Transmembrane segment assignment was performed using TMHMM (Moller et al. 2001) and the TMpred server (Hofmann and Stoffel 1993). In the alignment, we included only amino acids 96 to 520 of *hNHE1* for a pairwise sequence alignment with amino acids 1-415 *MjNhaP1*. These residues contain the entire membrane domain of *hNHE1* except for the first TM segment (TM1) and the first extracellular loop (EL1). TM segment predictions of *hNHE1* were compared with the known secondary structure elements of *MjNhaP1* structure (Paulino and Kuhlbrandt 2014). The alignment of TM9 and the EL5 region was manually edited based on their predicted secondary structure, NMR structural data (Reddy et al. 2008) and amino acid accessibility data (Liu et al. 2015; Wakabayashi et al. 2000).

Three Dimensional Modeling

To obtain a three-dimensional structure of the *hNHE1* protein a model was built using MODELLER 9.v2 (Sali and Blundell 1993). The NhaA fold has been shown to be present in several types of Na^+/H^+ exchanger including NhaA of *E. coli*, (Hunte et al. 2005), NapA of *Thermus thermophilus* (Lee et al. 2013b), *MjNhaP1* of *Methanocaldococcus jannaschii* (Paulino and Kuhlbrandt 2014) and PaNhaP of

Pyrococcus abyssi (Wohlert et al. 2014). Therefore, the first round of modeling was performed with default parameters of MODELLER 9v2 with an assumption that relative positions of the transmembrane helices of human NHE1 would be similar to those of *MjNhaP1*. The initial 50 structures of *hNHE1* contained TM2 through TM12 (residues 96-250). From these structures one that closely matched the predicted TM spans, was chosen for further structural editing using COOT (Emsley et al. 2010). The C-terminal region of TM9 (349 to 365) was built from the NMR structure (PDB 2K3C) (Reddy et al. 2008). Other structural editing of the reentrant loop containing the residues 366 to 415 was built *ab initio* (Emsley et al. 2010) based on cysteine scanning mutagenesis studies for surface accessibility data (Jinadasa et al. 2015; Liu et al. 2015; Reddy et al. 2008; Slepko et al. 2005; Tzeng et al. 2010; Wakabayashi et al. 2000).

Because there is no known structural template for EL1 (amino acids 35-95) and TM1, we incorporated TM1 (amino acids 12-34) separately into the model. The structure of TM1 (12-34) was obtained from a previously published *hNHE1* model (Nygaard et al. 2011) and submitted to the HADDOCK server (van Zundert et al., 2016) which contains protein-protein docking software. As the NHE1 protein is known to be a dimer (Fafournoux et al. 1994; Hisamitsu et al. 2006; Moncoq et al. 2008), the relative position of TM1 on the dimer interface of the NHE1 model was also predicted using HADDOCK (van Zundert et al. 2016) with the assumption made that TM1 should be present at the dimerization interface. TM1 has earlier been shown to be present at the dimerization interface in all Na^+/H^+ antiporter structures reported (Hunte et al. 2005; Lee et al. 2013b; Paulino and Kuhlbrandt 2014; Wohlert et al. 2014). The second assumption was that

TM1 should be in proximity to TM2. Which is also typical of the previously reported NHE structures. With these two assumptions hydrophobic residues from TM1 (Leu20, Ala24, Val31) and TM2 (Leu107, Leu114) were defined as “active residues” that can take part in TM1-TM2 interactions. Several potential interacting clusters were obtained from the HADDOCK analysis. However, the docking output of TM1 on the dimer interface was chosen based on two criteria. One, was the orientation of the helix; the N-terminal of the first helix should be located on the same, cytosolic side as the C-terminal of the protein. The second criterion was the selection of the populated cluster of TM1 at the dimer interface involving the most hydrophobic interactions, with buried surface area. Between two potential positions the one with the highest HADDOCK score (-76 ± 4.2) was used further. The entire TM1-docked NHE1 structure was checked manually for any potential clashes and the Ramachandran plot improved using COOT (Emsley et al. 2010). The structure was refined using the ModRefiner server (Xu and Zhang 2011). Finally, the quality of the model was checked using the software RAMPAGE (Lovell et al. 2003). The final model contains 93.7% favored regions, 5.6% allowed region, and 0.7% disallowed region in Ramachandran Plot.

Docking of NHE1 Inhibitors

To explore the *h*NHE1 inhibitor binding site we docked *h*NHE1 inhibitors computationally to the NHE1 model structure. The coordinates of the ligand EIPA (5-(N-ethyl-N-isopropyl)amiloride) (and other ligands 5-(N,N-Hexamethylene)amiloride

(HMA), HOE694, and amiloride) were obtained from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) and format converted using UCSF Chimera (Pettersen et al. 2004). For docking studies, both ligand and protein were prepared using AutoDock Tools v1.5.6 (Morris et al. 2009). The ligand was added with all hydrogens to calculate Gasteiger charges and then non-polar hydrogens were merged. Macromolecules were prepared by adding polar hydrogen and then the Kollman United Atom charges were assigned. AutoDock Tools was also used to prepare a grid box for blind docking that included the entire protein surface open to either side of the membrane. Any flexible bonds on the ligand were selected using AutoDock Tools. Docking of the inhibitor was performed using Autodock Vina 1.1.2 (Trott and Olson 2010). A default exhaustiveness level was chosen to perform the docking run. Similar protocols were used to dock other *h*NHE1 inhibitors: amiloride, cariporide, HOE694, and HMA [5-(N,N-hexamethylene)-amiloride]. RMSD was determined using C α superposition in Pymol.

Results and Discussion

NHE1 and *Mj*NhaP1 Alignment

Here we aligned amino acids 96-520 of *h*NHE1 with amino acids 1-415 of *Mj*NhaP1 as described above. Human NHE1 sequence shows more (20%) sequence identity with *Mj*NhaP1 in the membrane domain, in comparison with *E. coli* NhaA (15% sequence identity). Residues 96-520 of *h*NHE1 do not contain TM1 and EL1. In the multiple sequence alignment (Fig. 1B), the first TM segment of *Mj*NhaP1 is aligned with the

second TM segment of *hNHE1*. The orientations of these helices are in agreement. The first TM segment of *MjNhaP1* is oriented from the outside to inside (Paulino and Kuhlbrandt 2014), which is the same orientation as the second TM segment of the *hNHE1*. A notable disparity between the sequences is observed in reentrant loop EL5 (349-415) of human NHE1.

Several motifs of Na^+/H^+ exchangers have been shown to be critical in activity (Dutta and Fliegel 2018). In TMXI of Na^+/H^+ exchangers there is a GLRG motif in the extended non-helical region of the protein. An ND motif is found in the CPA1 antiporter family and its mutation compromises activity. A motif, TDPV, contains a critical hydroxyl on the Thr (which is sometimes a Ser) and a critical Pro. In the pair alignment it is evident that most of these motifs critical in activity of the Na^+/H^+ antiporters are conserved. For example, the TDPV motifs of TM6 (TM5 of *MjNhaP1*), the “ND” motifs of TM7 (TM8 of *MjNhaP1*) and “GLRG” motifs of TM11 (TM-12 of *MjNhaP1*)(Dutta and Fliegel 2018) are aligned and mostly conserved. In fact, overall, the only poor alignment was found across TM-9, which is followed by the membrane reentrant loop EL5 (Liu et al. 2015; Wakabayashi et al. 2000).

Model Description

For three dimensional modeling of *hNHE1* protein a model was built using MODELLER 9.v2 (Sali and Blundell 1993). MODELLER has been earlier used to model membrane proteins based on related templates. For example, a similar approach was used earlier

with this software to generate models of fungal H⁺-ATPases based on a distantly related Ca²⁺-ATPase that shares a conserve structural fold. The resulting model closely matched the cryoEM structure of the protein (Kuhlbrandt et al. 2002). Our final three-dimensional model (Fig. 2) of the NHE1 membrane domain contains twelve transmembrane helices with both cytoplasmic N- and C-termini. The twelve TM spans are Arg14-Arg33 (TM1), Val99-Val121 (TM2), Glu131 to Leu141 (TM3), Phe155 to Phe182 (TM4), Leu186 to Cys212 (TM5), Asn227 to Glu248 (TM6), Glu253 to Val272 (TM7), Gly287 to Gly313 (TM8), Leu336 to Met354 (TM9), Ser416 to Thr433 (TM10), Lys447 to Leu468 (TM11), and finally Phe480 to Met497 (TM12). Fig. 2A, B, D illustrate lateral views of the model and Fig. 2C a view from the cytoplasmic side.

TM1 and extracellular loop 1 (EL1)

The process above so far describes *h*NHE1 modeling of TM2-12. The N-terminal of TM1 of *h*NHE1 is cytoplasmic. TM1, although predicated to be a signal sequence, remains attached to the mature protein (Fafournoux et al. 1994; Liu et al. 2015). As noted above, TM1 modeling was from Nygaard et al. 2011 (Nygaard et al. 2011) and docked independently. Therefore it was docked separately using the HADDOCK protein-protein docking server. Although, HADDOCK was designed for aqueous phase proteins, it has been used for membrane proteins with the primary prerequisite being the definition the binding site (Kaczor et al. 2013; Rashid and Kuyucak 2012). EL1 joins TM1 with TM2 and possess one N-linked site and possibly O-linked glycosylation sites (Counillon et al. 1994; Haworth et al. 1993). TM1 and EL1 could not be modeled based

on *Mj*NhaP1 as it does not align with the protein (Fig. 1). TM1 showed mostly hydrophobic interactions with TM2 (Fig. 2C).

TM4

Several residues of TM4 of NHE1: Phe162, Leu163, Phe167, and Gly164 are regarded as critical for inhibitor sensitivity (Counillon et al. 1997; Counillon et al. 1993b; Pedersen et al. 2007; Touret et al. 2001). The NMR structure of a peptide of TM4 showed only a partly helical conformation (Slepkov et al. 2005). Our results here suggest that TM4 is a helix followed by an unwound region, that is followed by a helix (Fig. 2C, E). The unwound region consists of the sequence 165LLPPIIL171. In comparison with *Mj*NhaP1 (Fig. 1) *h*NHE1 has an extra Pro residue (Pro168) in this segment which can account for the extended conformation (Slepkov et al. 2007). TM4 is associated with the TM6 and TM11. Spin labeling Ala173Cys of TM4 and Ile461Cys of TM11 suggested an approximate distance of 15Å between these two residues (Nygaard et al. 2011). In our model we found a distance of 9.5Å between two Ca the differences could be due to a bias created by using *Mj*NhaP1 as a template or different conformations of the protein. The template *Mj*NhaP1 structure was determined at pH 8, that may be responsible for the conformation that is reflected in our model. The predicted structure of TM4 that we found is similar to that obtained by examining the structure of a peptide of TM4 (Slepkov et al. 2005) (Fig. 3A).

TM6 and TM7

In the model, TM7 is a continuous helix whereas TM6 is a discontinuous helix and both are packed closely in presence of other helical segments (Fig. 2C). This model structure closely resembles an NMR structure of a peptide of TM6-TM7 published earlier (Alves et al. 2014). Analysis of these two TM segments structure (Alves et al. 2014) also showed that there was a close interaction between Val242 and Phe260 which is also found in our structure (7.7Å C_α distance). The RMSD between the modeled TM6-TM7 and the NMR structure (PDB 2MDF) of TM6-TM7 is 4.7Å over 318 atoms. Plasma membrane Na⁺/H⁺ antiporters have a “TDPV” [(V/T)DPV] motif present that has a role in activity of the protein (Dutta and Fliegel 2018). In TM6, this is present in the discontinuous region in the model structure (Fig. 2E). A similar discontinuity is present in the equivalent helix of *SpNHE1* (Ullah et al. 2013). A peptide of TM6-7 was examined earlier by NMR. It has the same general structure, with TM6 having a discontinuous region (Alves et al. 2014) (Fig. 3B).

TM9

Leu336 to Met354 make up TM9 in the model as a continuous helix (Fig. 2D). Assignment of these residues as a helix has been contentious in the past with one model assign them as a helix (Wakabayashi et al. 2000) and another suggesting that they form two separate TM segments (TM7 and TM8) (Landau et al. 2007). This region is weakly conserved among Na⁺/H⁺ antiporters (Dutta and Fliegel 2018) and the corresponding region in NhaA of *E. coli* is located at the dimer interface (Padan et al. 2015). When mutated to Cys, amino acid 351 of this region is accessible to extracellular sulfhydryl labeling. Also, interestingly, a peptide of this region shows a kink around Ser351 that

may indicate some mobility in this region (Reddy et al. 2008) (Fig. 3C). Mutations to TM9 can affect sensitivity towards mammalian NHE1 inhibitors (Jinadasa et al. 2015; Khadilkar et al. 2001).

TM11

In our model, TM11 is a discontinuous helix (Fig. 2C-E). An NMR structure of a peptide of *h*NHE1 TM11 (Fig. 3D) was also a discontinuous helix (Lee et al. 2009). In other Na^+/H^+ exchanger isoforms, TM11 forms part of the NhaA fold and is believed to participate in cation transport including in NhaA and in *Sp*NHE1 of *S. pombe* (Dutta et al. 2017; Hunte et al. 2005). In the present model, the unwound region of *h*NHE1 TM11 contains the motif ⁴⁵⁶GLRG⁴⁵⁹. This unwound region was also found in the structure of a peptide of this region, deduced by NMR (Lee et al. 2009). The presence of an arginine (Arg458) at the center of segment is intriguing. In mutagenesis experiments, mutation of this Arg458 to Cys caused intracellular localization of the protein and compromised activity. Mutation of Gly459 also greatly compromised activity (Lee et al. 2009). The corresponding sequence is GPRG (Fig. 1) in *Mj*NhaP1 and in *Mj*NhaP1, *Pa*NhaP, and *Tth*NapA this Arg side chain correlates with a kink in the TM segment with a peptide backbone interaction (Goswami et al. 2011; Lee et al. 2013b; Wohlert et al. 2014). The structure of TM11 predicted in the model is similar to the structure of a peptide of TM11 (Fig. 2D) obtained by NMR (Lee et al. 2009).

Extracellular loop 5

In the model the C-terminal (355-360) of TM9 continues as EL5 on the extracellular surface (Fig. 2C-D). From amino acid 361, it extends extracellularly initially including approximately amino acids 361-380. EL5 then reenters the protein core to enter the membrane twice (381-391 and 391-399). The rest of EL5 is exposed extracellularly (401-415). The modeling software suggests that residues 394 to 402 are helical and residues 372-389 are an extended sheet, though other secondary structure predication software (not shown) suggested that amino acids 394 to 402 may also be an extended sheet.

TM Segment Organization

Na^+/H^+ antiporters have been shown to consist of two distinct domains: a dimerization or scaffolding domain and a helix bundle or transportation domain (Hendus-Altenburger et al. 2014; Padan et al. 2015). The NHE1 model possesses an inverted topology of transmembrane helices as reported (Hendus-Altenburger et al. 2014; Padan et al. 2015) for other Na^+/H^+ exchangers. It is arranged such that TM2-TM3-TM4 and TM8-TM-9 are intertwined to make the dimerization domain whereas TM5-TM6-TM-7 and TM10-TM11-TM12 form the metal transportation domain (Fig. 2C,D). TM5-TM6-TM-7 and TM10-TM11-TM12 are inverted relative to each other.

Other Considerations

There are several striking differences in this model from the existing models of *h*NHE1 (Landau et al. 2007; Nygaard et al. 2011). First, is the position of TM1 in the dimerization domain. This position is based on protein-protein docking though it is

noteworthy that in bacterial Na^+/H^+ antiporters structures, the first TM segment is also an integral part of the dimerization domain (Lee et al. 2013b; Paulino and Kuhlbrandt 2014; Wohlert et al. 2014). The second difference is the position of re-entrant loop EL5 after TM9. As TM9 (Leu336 to Met354) is positioned at the dimer interface and TM10 (Ser416 to Thr433) is positioned in the transportation domain, it is possible that the 60 amino acids situated between these two helices helps span the two domains. As noted above, a large preponderance of biochemical data has placed this segment on the extracellular surface (Liu et al. 2015; Wakabayashi et al. 2000). To demonstrate this on the model, Fig. 2F illustrates the amino acids in the model that have been shown earlier in previous publications (Liu et al. 2015; Reddy et al. 2008; Slepko et al. 2005; Tzeng et al. 2010; Wakabayashi et al. 2000) to be surface accessible and active, when mutated to Cys. These experiments were done in earlier publications by biotinylating cell surface proteins and examining the percent of immunoprecipitated NHE1 protein on the cell surface. Surface accessible residues of NHE1 were almost invariably positioned on the extracellular face of our model on what is predicted to be extracellular loops of the protein (Fig. 2F). A few, such as Ser351, Phe161 and Ile233 are near the extracellular side of transmembrane helices, or possibly are accessible because they are within the extracellular accessible funnel of the protein (Slepko et al. 2005). Four surface accessible residues, T183, E184, S324 and H325 (Wakabayashi et al. 2000) are positioned on intracellular loops. This occurs even though nearby residues are not surface accessible. It was earlier (Wakabayashi et al. 2000) suggested that these residues are part of the structure of the NHE1 pore making them accessible from the extracellular surface. For amino acids 183 and 184 this seems to be supported by the model structure

as they are immediately following TM4 and could be in alignment with the pathway of cations. Amino acids 324 and 325 are not as apparently involved in the cation transport pathway, but reside on a long unstructured intracellular loop and could adopt a conformation near the intracellular side of the transport pathway. Some mutations of this intracellular loop compromise activity of the protein (Liu et al. 2015).

Another difference between this model and other ones is in the transmembrane segments making up the Na^+/H^+ exchanger fold. Others have reported that TM4 and TM11 make up the NhaA transportation fold (Hunte et al. 2005; Lee et al. 2013b; Paulino and Kuhlbrandt 2014; Wohlert et al. 2014). Here we show that while there is such a fold (Fig. 2E) interestingly, it is made up of TM6 (227-248) and TM11 (Lys447 to Leu468). TM4 (155 to 182) is also a discontinuous helix, but does not interact with TM11 to make a typical fold. TM4, while still being a discontinuous helix, is near the NhaA fold but the N-terminal half of the helix extends upward and possibly out of the membrane domain. The suggestion that TM6 is part of the critical fold is supported by results of sequence alignment (Dutta and Fliegel 2018). Additionally, we earlier showed that TM6 is a discontinuous pore lining helix (Tzeng et al. 2011).

Docking of Na^+/H^+ Exchanger Inhibitors

Docking results identified three putative EIPA interacting sites with relatively close affinities. Position 1 (Fig. 1S, Table 1S) was the most favorable position with an affinity

of -6.4 kcal/mol. It was located in close proximity of the residues of EL5 (Trp386, Ser387, Ser388, Val389), TM9 (Leu336, Tyr339, Met340, Leu343) and TM8 (Gly305, Val306, Gly309, and Tyr312). At position 2 (Fig. 2S) (affinity -6.1 kcal/mol) EIPA is within interacting distance of TM2 (Lys116), TM4 (Pro178), EL5 (Phe395, Leu398, Gly399), TM6 (Asp238, Val240, Leu243), and the residues of N-terminal of TM3 (Val129, Pro130, Glu131). The third EIPA interacting position (Fig. 3, -5.9 kcal/mol) involves the residues from TM6 (Leu228, Leu229), EL4 (Val272, Leu273, Tyr274, His275), and EL5 (Lys379, Ala380, Tyr381), Leu465 (TM11) and Pro475 (EL6)). In all cases EL5 was involved in the interaction between EIPA and NHE1. (Similar results were obtained with amiloride, HMA and HOE694 (Supplementary Fig. 3-5.) Table 1S lists the amino acids in close proximity with the various compounds.

Position1 and position 2 are at the dimeric scaffolding domain whereas position 3 is located at the extracellular side of the interface between scaffolding domain and the transportation domain. Because NHE1 inhibitors can cause complete inhibition of the protein, we reasoned that position 3 is the mostly likely physiological binding site, despite have a slightly lower affinity than that of position one and two (Fig. 4). Supporting this idea, is the suggestion that amiloride based inhibitors act through inhibition of the extracellular accessible Na^+ binding site because the tri-hydrated Na^+ ion is nearly identical in structure to the guanidinium ion of amiloride (Lang 2003).

When mutated, residues of TM4 and His349 of TM9 affect the efficacy of NHE1 inhibitors (Counillon et al. 1993a; Counillon et al. 1997; Lee et al. 2009; Wang et al.

1995; Yun et al. 1993). We did not see any interaction of EIPA with the TM4 segment ¹⁶⁰VFFLFLLPPIIL¹⁷¹ or with His349 of TM9. The closest distance from TM4 was with Pro168 which was 6.5 Å from EIPA. However, it has not been shown that EIPA directly binds to this region. In fact, mutations in a quite a large number of residues in varied transmembrane segments affect inhibitor potency (Ding et al. 2006; Jinadasa et al. 2015). Some improve and some decrease inhibitor efficacy. It therefore seems likely that these mutations may have subtle effects on the conformation of the protein, thus altering the inhibitor-binding site.

Summary

We present a three-dimensional model of *hNHE1*. The model takes into account empirically determined biochemical data on surface accessibility of the protein in combination with molecular modeling and is based on the structure of *MjNhaP1*, that is more similar to *hNHE1* than *NhaA*. In this model, TM6 and TM11 form a Na⁺/H⁺ antiporter fold. Residues 363-410 form EL5, in agreement with Cys accessibility studies that place this segment on the cell surface. A large preponderance of amino acids shown to be accessible on the cell surface are present on the extracellular surface, or nearby. Structures of transmembrane segments in the model are similar to the structures of peptides of *hNHE1* deduced in NMR studies. Preliminary studies examined docking of the Na⁺/H⁺ exchanger inhibitor EIPA (5-(N-Ethyl-N-isopropyl)amiloride) to *hNHE1* and at one site, it is on the extracellular surface in association with several amino acids of different TM regions. Studies are presently underway to determine the three dimensional

structure of inhibitor bound NHE1 at atomic resolution. Additionally, experiments are underway to examine intramolecular distances with a spin label using the purified cysteineless NHE1 protein with a single or double cysteine introduced.

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Literature Cited

- Alves, C., Lee, B.L., Sykes, B.D., and Fliegel, L. 2014. Structural and functional analysis of the transmembrane segment pair VI and VII of the NHE1 isoform of the Na^+/H^+ exchanger. *Biochemistry* 53: 3658-3670. 10.1021/bi500392y.
- Amith, S.R., and Fliegel, L. 2016. The Na^+/H^+ exchanger in metastasis. *Aging (Albany NY)* 8: 1291. 10.18632/aging.101002.
- Amith, S.R., and Fliegel, L. 2017. Na^+/H^+ exchanger-mediated hydrogen ion extrusion as a carcinogenic signal in triple-negative breast cancer etiopathogenesis and prospects for its inhibition in therapeutics. *Semin Cancer Biol* 43: 35-41. 10.1016/j.semcancer.2017.01.004.
- Amith, S.R., Wilkinson, J.M., and Fliegel, L. 2016. KR-33028, a potent inhibitor of the Na^+/H^+ exchanger NHE1, suppresses metastatic potential of triple-negative breast cancer cells. *Biochem Pharmacol* 118: 31-39. 10.1016/j.bcp.2016.08.010.
- Brett, C.L., Donowitz, M., and Rao, R. 2005. Evolutionary origins of eukaryotic sodium/proton exchangers. *Am J Physiol Cell Physiol* 288: C223-239.
- Buchan, D.W., Minneci, F., Nugent, T.C., Bryson, K., and Jones, D.T. 2013. Scalable web services for the PSIPRED Protein Analysis Workbench. *Nucleic Acids Res* 41: W349-357. 10.1093/nar/gkt381.
- Calinescu, O., Dwivedi, M., Patino-Ruiz, M., Padan, E., and Fendler, K. 2017. Lysine 300 is essential for stability but not for electrogenic transport of the Escherichia coli NhaA Na^+/H^+ antiporter. *J Biol Chem* 292: 7932-7941. 10.1074/jbc.M117.778175.

- Counillon, L., Franchi, A., and Pouyssegur, J. 1993a. A point mutation of the Na^+/H^+ exchanger gene (NHE1) and amplification of the mutated allele confer amiloride resistance upon chronic acidosis. *Proc Natl Acad Sci USA* 90: 4508-4512.
- Counillon, L., Noel, J., Reithmeier, R.A.F., and Pouyssegur, J. 1997. Random mutagenesis reveals a novel site involved in inhibitor interaction within the fourth transmembrane segment of the Na^+/H^+ exchanger-1. *Biochemistry* 36: 2951-2959.
- Counillon, L., Pouyssegur, J., and Reithmeier, R.A. 1994. The Na^+/H^+ exchanger NHE-1 possesses N- and O-linked glycosylation restricted to the first N-terminal extracellular domain. *Biochemistry* 33: 10463-10469.
- Counillon, L., Scholz, W., Lang, H.L., and Pouyssegur, J. 1993b. Pharmacological characterization of stably transfected Na^+/H^+ antiporter isoforms using amiloride analogs and a new inhibitor exhibiting anti-ischemic properties. *Mol Pharmacol* 44: 1041-1045.
- Ding, J., Rainey, J.K., Xu, C., Sykes, B.D., and Fliegel, L. 2006. Structural and functional characterization of transmembrane segment VII of the Na^+/H^+ exchanger isoform 1. *J Biol Chem* 281: 29817-29829.
- Dutta, D., and Fliegel, L. 2018. Structure and function of yeast and fungal Na^+/H^+ antiporters. *IUBMB Life* 70: 23-31. 10.1002/iub.1701.
- Dutta, D., Shin, K., Rainey, J.K., and Fliegel, L. 2017. Transmembrane segment XI of the Na^+/H^+ antiporter of *S. pombe* is a critical part of the ion translocation pore. *Sci Rep* 7: 12793. 10.1038/s41598-017-12701-z.
- Emsley, P., Lohkamp, B., Scott, W.G., and Cowtan, K. 2010. Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* 66: 486-501. 10.1107/S0907444910007493.

- Fafournoux, P., Noel, J., and Pouysségur, J. 1994. Evidence that Na^+/H^+ exchanger isoforms NHE1 and NHE3 exist as stable dimers in membranes with a high degree of specificity for homodimers. *J Biol Chem* 269: 2589-2596.
- Fliegel, L. 1999. Functional and cellular regulation of the myocardial Na^+/H^+ exchanger. *J Thrombosis Thrombolysis* 8: 9-14.
- Fliegel, L. 2005. The Na^+/H^+ exchanger isoform 1. *Int J Biochem Cell Biol* 37: 33-37. 10.1016/j.biocel.2004.02.006.
- Fliegel, L., Dyck, J.R.B., Wang, H., Fong, C., and Haworth, R.S. 1993. Cloning and analysis of the human myocardial Na^+/H^+ exchanger. *Mol Cell Biochem* 125: 137-143.
- Fliegel, L., Sardet, C., Pouysségur, J., and Barr, A. 1991. Identification of the protein and cDNA of the cardiac Na^+/H^+ exchanger. *FEBS Lett* 279: 25-29.
- Franca, T.C. 2015. Homology modeling: an important tool for the drug discovery. *J Biomol Struct Dyn* 33: 1780-1793. 10.1080/07391102.2014.971429.
- Goswami, P., Paulino, C., Hizlan, D., Vonck, J., Yildiz, O., and Kuhlbrandt, W. 2011. Structure of the archaeal Na^+/H^+ antiporter NhaP1 and functional role of transmembrane helix 1. *Embo J* 30: 439-449. 10.1038/emboj.2010.321.
- Haworth, R.S., Frohlich, O., and Fliegel, L. 1993. Multiple carbohydrate moieties on the Na^+/H^+ exchanger. *Biochem J* 289: 637-640.
- Hellmer, J., Patzold, R., and Zeilinger, C. 2002. Identification of a pH regulated $\text{Na}(+)/\text{H}(+)$ antiporter of *Methanococcus jannaschii*. *FEBS Lett* 527: 245-249.
- Hendus-Altenburger, R., Kragelund, B.B., and Pedersen, S.F. 2014. Structural dynamics and regulation of the mammalian SLC9A family of $\text{Na}(+)/\text{H}(+)$ exchangers. *Curr Top Membr* 73: 69-148. 10.1016/B978-0-12-800223-0.00002-5.

- Hisamitsu, T., Ben Ammar, Y., Nakamura, T.Y., and Wakabayashi, S. 2006. Dimerization is crucial for the function of the Na^+/H^+ exchanger NHE1. *Biochemistry* 45: 13346-13355.
- Hofmann, K., and Stoffel, W. 1993. TMBASE - A database of membrane spanning protein segments. *Biol Chem Hoppe-Seyler* 374: 166
- Huang, Y., Chen, W., Dotson, D.L., Beckstein, O., and Shen, J. 2016. Mechanism of pH-dependent activation of the sodium-proton antiporter NhaA. *Nat Commun* 7: 12940. 10.1038/ncomms12940.
- Hunte, C., Screpanti, E., Venturi, M., Rimon, A., Padan, E., and Michel, H. 2005. Structure of a Na^+/H^+ antiporter and insights into mechanism of action and regulation by pH. *Nature* 435: 1197-1202.
- Jinadasa, T., Josephson, C.B., Boucher, A., and Orlowski, J. 2015. Determinants of Cation Permeation and Drug Sensitivity in Predicted Transmembrane Helix 9 and Adjoining Exofacial Re-entrant Loop 5 of Na^+/H^+ Exchanger NHE1. *J Biol Chem* 290: 18173-18186. 10.1074/jbc.M115.642199.
- Kaczor, A.A., Selent, J., Sanz, F., and Pastor, M. 2013. Modeling Complexes of Transmembrane Proteins: Systematic Analysis of ProteinProtein Docking Tools. *Mol Inform* 32: 717-733. 10.1002/minf.201200150.
- Karmazyn, M. 2013. NHE-1: still a viable therapeutic target. *J Mol Cell Cardiol* 61: 77-82. 10.1016/j.yjmcc.2013.02.006.
- Karmazyn, M., Gan, T., Humphreys, R.A., Yoshida, H., and Kusumoto, K. 1999. The myocardial Na^+/H^+ exchange. Structure, regulation, and its role in heart disease. *Circ Res* 85: 777-786.

- Katoh, K., and Standley, D.M. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* 30: 772-780. 10.1093/molbev/mst010.
- Khadilkar, A., Iannuzzi, P., and Orlowski, J. 2001. Identification of sites in the second exomembrane loop and ninth transmembrane helix of the mammalian Na^+/H^+ exchanger important for drug recognition and cation translocation. *J Biol Chem* 276: 43792-43800.
- Kuhlbrandt, W., Zeelen, J., and Dietrich, J. 2002. Structure, mechanism, and regulation of the *Neurospora* plasma membrane H^+ -ATPase. *Science* 297: 1692-1696. 10.1126/science.1072574.
- Landau, M., Herz, K., Padan, E., and Ben-Tal, N. 2007. Model structure of the Na^+/H^+ exchanger 1 (NHE1): functional and clinical implications. *J Biol Chem* 282: 37854-37863.
- Lang, H.J. 2003. Chemistry of NHE inhibitors. p 239-253, in *The Na^+/H^+ Exchanger, From Molecular to Its Role in Disease*, edited by M Karmazyn, M Avkiran and L Fliegel, Kluwer academic Publishers, Boston/Dordrecht/London, 318p.
- Lee, B.L., Li, X., Liu, Y., Sykes, B.D., and Fliegel, L. 2009. Structural and functional analysis of transmembrane XI of the NHE1 isoform of the Na^+/H^+ exchanger. *J Biol Chem* 284: 11546-11556. M809201200 [pii] 10.1074/jbc.M809201200.
- Lee, B.L., Sykes, B.D., and Fliegel, L. 2013a. Structural and functional insights into the cardiac $\text{Na}(+)/\text{H}(+)$ exchanger. *J Mol Cell Cardiol* 61: 60-67. 10.1016/j.yjmcc.2012.11.019.

- Lee, C., Kang, H.J., von Ballmoos, C., Newstead, S., Uzdavinys, P., Dotson, D.L., Iwata, S., Beckstein, O., Cameron, A.D., and Drew, D. 2013b. A two-domain elevator mechanism for sodium/proton antiport. *Nature* 501: 573-577. 10.1038/nature12484.
- Lee, S.H., Kim, T., Park, E.S., Yang, S., Jeong, D., Choi, Y., and Rho, J. 2008. NHE10, an osteoclast-specific member of the Na^+/H^+ exchanger family, regulates osteoclast differentiation and survival [corrected]. *Biochem Biophys Res Commun* 369: 320-326. S0006-291X(08)00237-4 [pii] 10.1016/j.bbrc.2008.01.168.
- Liu, Y., Basu, A., Li, X., and Fliegel, L. 2015. Topological analysis of the Na/H exchanger. *Biochim Biophys Acta* 1848: 2385-2393. 10.1016/j.bbamem.2015.07.011.
- Lovell, S.C., Davis, I.W., Arendall, W.B., 3rd, de Bakker, P.I., Word, J.M., Prisant, M.G., Richardson, J.S., and Richardson, D.C. 2003. Structure validation by Calpha geometry: phi,psi and Cbeta deviation. *Proteins* 50: 437-450. 10.1002/prot.10286.
- Moller, S., Croning, M.D., and Apweiler, R. 2001. Evaluation of methods for the prediction of membrane spanning regions. *Bioinformatics* 17: 646-653.
- Moncoq, K., Kemp, G., Li, X., Fliegel, L., and Young, H.S. 2008. Dimeric structure of human Na^+/H^+ exchanger isoform 1 overproduced in *Saccharomyces cerevisiae*. *J Biol Chem* 283: 4145-4154.
- Morris, G.M., Huey, R., Lindstrom, W., Sanner, M.F., Belew, R.K., Goodsell, D.S., and Olson, A.J. 2009. AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *J Comput Chem* 30: 2785-2791. 10.1002/jcc.21256.
- Nygaard, E.B., Lagerstedt, J.O., Bjerre, G., Shi, B., Budamagunta, M., Poulsen, K.A., Meinild, S., Rigor, R.R., Voss, J.C., Cala, P.M., *et al.* 2011. Structural Modeling and

Electron Paramagnetic Resonance Spectroscopy of the Human Na^+/H^+ Exchanger Isoform 1, NHE1. *J Biol Chem* 286: 634-648. M110.159202 [pii]

10.1074/jbc.M110.159202.

Olkhova, E., Hunte, C., Screpanti, E., Padan, E., and Michel, H. 2006. Multiconformation continuum electrostatics analysis of the NhaA Na^+/H^+ antiporter of *Escherichia coli* with functional implications. *Proc Natl Acad Sci U S A* 103: 2629-2634.

10.1073/pnas.0510914103.

Orlowski, J., Grinstein, S. 2004. Diversity of the mammalian sodium/proton exchanger SLC9 gene family. *Pflugers Arch* 447: 549-565.

Orlowski, J., Kandasamy, R.A., and Shull, G.E. 1992. Molecular cloning of putative members of the Na^+/H^+ exchanger gene family. *J Biol Chem* 267: 9331-9339.

Padan, E. 2014. Functional and structural dynamics of NhaA, a prototype for $\text{Na}(+)$ and $\text{H}(+)$ antiporters, which are responsible for $\text{Na}(+)$ and $\text{H}(+)$ homeostasis in cells. *Biochim Biophys Acta* 1837: 1047-1062. 10.1016/j.bbabbio.2013.12.007.

Padan, E., Danieli, T., Keren, Y., Alkoby, D., Masrati, G., Haliloglu, T., Ben-Tal, N., and Rimon, A. 2015. NhaA antiporter functions using 10 helices, and an additional 2 contribute to assembly/stability. *Proc Natl Acad Sci U S A* 112: E5575-5582.

10.1073/pnas.1510964112.

Paulino, C., and Kuhlbrandt, W. 2014. pH- and sodium-induced changes in a sodium/proton antiporter. *Elife* 3: e01412. 10.7554/eLife.01412.

Pedersen, S.F., King, S.A., Nygaard, E.B., Rigor, R.R., and Cala, P.M. 2007. NHE1 inhibition by amiloride- and benzoylguanidine-type compounds. Inhibitor binding loci

deduced from chimeras of NHE1 homologues with endogenous differences in inhibitor sensitivity. *J Biol Chem* 282: 19716-19727.

Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., and Ferrin, T.E. 2004. UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem* 25: 1605-1612. 10.1002/jcc.20084.

Rashid, M.H., and Kuyucak, S. 2012. Affinity and selectivity of ShK toxin for the Kv1 potassium channels from free energy simulations. *J Phys Chem B* 116: 4812-4822. 10.1021/jp300639x.

Reddy, T., Ding, J., Li, X., Sykes, B.D., Rainey, J.K., and Fliegel, L. 2008. Structural and functional characterization of transmembrane segment IX of the NHE1 isoform of the Na⁺/H⁺ exchanger. *J Biol Chem* 283: 22018-22030. M803447200 [pii]

10.1074/jbc.M803447200.

Reshkin, S.J., Greco, M.R., and Cardone, R.A. 2014. Role of pHi, and proton transporters in oncogene-driven neoplastic transformation. *Philos Trans R Soc Lond B Biol Sci* 369: 20130100. 10.1098/rstb.2013.0100.

Rimon, A., Kozachkov-Magrisso, L., and Padan, E. 2012. The unwound portion dividing helix IV of NhaA undergoes a conformational change at physiological pH and lines the cation passage. *Biochemistry* 51: 9560-9569. 10.1021/bi301030x.

Rimon, A., Tzuber, T., and Padan, E. 2007. Monomers of the NhaA Na⁺/H⁺ antiporter of *Escherichia coli* are fully functional yet dimers are beneficial under extreme stress conditions at alkaline pH in the presence of Na⁺ or Li⁺. *J Biol Chem* 282: 26810-26821. 10.1074/jbc.M704469200.

- Sali, A., and Blundell, T.L. 1993. Comparative protein modelling by satisfaction of spatial restraints. *J Mol Biol* 234: 779-815. 10.1006/jmbi.1993.1626.
- Slepko, E., Ding, J., Han, J., and Fliegel, L. 2007. Mutational analysis of potential pore-lining amino acids in TM IV of the Na⁽⁺⁾/H⁽⁺⁾ exchanger. *Biochim Biophys Acta* 1768: 2882-2889.
- Slepko, E.R., Rainey, J.K., Li, X., Liu, Y., Cheng, F.J., Lindhout, D.A., Sykes, B.D., and Fliegel, L. 2005. Structural and functional characterization of transmembrane segment IV of the NHE1 isoform of the Na⁺/H⁺ exchanger. *J Biol Chem* 280: 17863-17872.
- Takaichi, K., Wang, D., Balkovetz, D.F., and Warnock, D.G. 1992. Cloning, sequencing, and expression of Na⁺/H⁺ antiporter cDNAs from human tissues. *Am J Physiol* 262: C1069-C1076.
- Touret, N., Poujeol, P., and Counillon, L. 2001. Second-site revertants of a low-sodium-affinity mutant of the Na⁺/H⁺ exchanger reveal the participation of TM4 into a highly constrained sodium-binding site. *Biochemistry* 40: 5095-5101.
- Trott, O., and Olson, A.J. 2010. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem* 31: 455-461. 10.1002/jcc.21334.
- Tzeng, J., Lee, B.L., Sykes, B.D., and Fliegel, L. 2010. Structural and functional analysis of transmembrane segment VI of the NHE1 isoform of the Na⁺/H⁺ exchanger. *J Biol Chem* 285: 36656-36665. M110.161471 [pii] 10.1074/jbc.M110.161471.

- Tzeng, J., Lee, B.L., Sykes, B.D., and Fliegel, L. 2011. Structural and functional analysis of critical amino acids in TMVI of the NHE1 isoform of the Na^+/H^+ exchanger. *Biochim Biophys Acta* 1808: 2327-2335. 10.1016/j.bbamem.2011.05.004.
- Ullah, A., Dutta, D., and Fliegel, L. 2016. Expression and characterization of the SOS1 Arabidopsis salt tolerance protein. *Mol Cell Biochem* 415: 133-143. 10.1007/s11010-016-2685-2.
- Ullah, A., Kemp, G., Lee, B., Alves, C., Young, H., Sykes, B.D., and Fliegel, L. 2013. Structural and Functional Analysis of Transmembrane Segment IV of the Salt Tolerance Protein Sod2. *J Biol Chem* 288: 24609-24624. 10.1074/jbc.M113.483065.
- van Zundert, G.C.P., Rodrigues, J., Trellet, M., Schmitz, C., Kastitis, P.L., Karaca, E., Melquiond, A.S.J., van Dijk, M., de Vries, S.J., and Bonvin, A. 2016. The HADDOCK2.2 Web Server: User-Friendly Integrative Modeling of Biomolecular Complexes. *J Mol Biol* 428: 720-725. 10.1016/j.jmb.2015.09.014.
- Wakabayashi, S., Pang, T., Su, X., and Shigekawa, M. 2000. A novel topology model of the human Na^+/H^+ exchanger isoform 1. *J Biol Chem* 275: 7942-7949.
- Wang, D., Balkovetz, D.F., and Warnock, D.G. 1995. Mutational analysis of transmembrane histidines in the amiloride-sensitive Na^+/H^+ exchanger. *Am J Physiol* 269: C392-C402.
- Wohlert, D., Kuhlbrandt, W., and Yildiz, O. 2014. Structure and substrate ion binding in the sodium/proton antiporter PaNhaP. *Elife* 3: e03579. 10.7554/eLife.03579.
- Xu, D., and Zhang, Y. 2011. Improving the physical realism and structural accuracy of protein models by a two-step atomic-level energy minimization. *Biophys J* 101: 2525-2534. 10.1016/j.bpj.2011.10.024.

Yun, C.H., Little, P.J., Nath, S.K., Levine, S.A., Pouyssegur, J., Tse, C.M., and Donowitz, M. 1993. Leu143 in the putative fourth membrane spanning domain is critical for amiloride inhibition of an epithelial Na⁺/H⁺ exchanger isoform (NHE-2). *Biochem Biophys Res Commun* 193: 532-539.

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Figure Legends

Figure 1. Schematic model of NHE1 and NHE1 sequence alignment. **A**, Simplified schematic model of NHE1 illustrating general transport domain and intracellular regulatory domain. **B**, Sequence alignment of *h*NHE1 and *Mj*NhaP1. NHE1_SS denotes the secondary structure prediction where red denotes alpha helix and yellow denotes beta sheet; NHE1_TM indicates the position of predicted transmembrane helices (gray). Secondary structure helical elements on the *Mj*NhaP1 structure are denoted using red. Final transmembrane segments in the predicted *h*NHE1 structure are denoted using bold numbers. “GLRG” indicates position of this motif. “DP” indicates position of the A(V/T)DPV” motif. “R” indicates the positively charged residue of TM10 (equivalent to the Lys300 of *Ec*NhaA), “ND” indicates ND motifs of TM-7.

Figure 2. Molecular model of human NHE1. Amino acids 12-512 are illustrated (minus EL1, amino acids 34-97) **A**, lateral view of the model. Pro12 is shown in cyan. TM4 is shown in yellow, amino acids 155-182, TM6 (amino acids 227-248) are shown in gray, and TM11 is shown in magenta (amino acids 447-468). EL5, amino acids 363-410, is shown in orange. **B**, as in **A** but with amino acids 480-499 and 416-434 removed for clarity **C**, **D**, *h*NHE1 model structure: viewed perpendicular (**C**) to the membrane from the cytoplasmic side, and lateral (**D**) to the membrane. N- and C-terminals are shown. TM1 (magenta) and EL5 (black) TM2 (lime green)-TM3 (pale green)-TM4 (smudge) and TM-8 (green), TM9 (limon) together with EL5 forms the dimerization scaffolding domain. TM5 (red)-TM6 (fire brick)-TM7 (raspberry) and TM10 (deep salmon)-TM11 (warm pink), TM12 (chocolate) form the metal transporting helix bundle. The helices across the discontinuous helix regions of TM6 and TM11 are named TM6a (N-terminal)/TM6b (C-terminal) and TM11a (N-terminal)/TM11b (C-terminal) respectively. TM segments are labeled. **E**, lateral view of TM4 (green), TM6 (magenta), and TM11 (yellow). **F**, surface accessible residues. Lateral and transparent view of *h*NHE1, *h*NHE1 TM4 is magenta, amino acids 155-182, and TM11 is shown in yellow (amino acids 447-468). EL5, amino acids 363-410, are shown in grey. Amino acids that have been demonstrated to be accessible on the cell surface when altered to Cys (and with the protein still active) are colored red and side chains are shown. Selected amino acids are labeled. Surface accessible amino acids from previous reports (Liu et al. 2015; Reddy et al. 2008; Slepko et al. 2005; Tzeng et al. 2010; Wakabayashi et al. 2000) are E151, T152, P153, F161, T183, E184, E217, Q218, I219, N220, N221, N227, I233, F277, E279, F280, A281, Y283, E284, H285, S324, H325, S351, Y366, V367, E368, A369, I371, H373, K374, S375, H376, T377, S401, T402, H407, H408 and W409.

Figure 3. Comparison of the model structure and peptide structure of isolated segments of *h*NHE1. **A**, green, TM4 structure from NMR of peptide (Slepko et al. 2005), red, molecular model of TM4. **B**, green TM6-7 structure from NMR of peptide (Alves et al. 2014), red, molecular model of TM6-7. **C**, TM9, green, structure from NMR (Reddy et

al. 2008), red, molecular model of TM6-7. **D**, TM11, green, structure from NMR (Lee et al. 2009), red, molecular model of TM6-7.

Figure 4. View of EIPA binding site position 3, from the extracellular surface. Amino acids Leu228, Leu229 (TM6), Val272, Leu273, Tyr274, His275 (EL4), Lys379, Ala380, Tyr381 (EL5), Leu465 (TM11) and Pro475 (EL6) are shown in cyan. **A**, with entire protein shown. **B**, Close up.

Draft

Fig 1

103x167mm (300 x 300 DPI)

Fig 2

213x257mm (300 x 300 DPI)

Figure 3

228x177mm (300 x 300 DPI)

Figure 4

109x174mm (300 x 300 DPI)