

Structural analysis of the Na⁺/H⁺ exchanger isoform 1 (NHE1) using the divide and conquer approach¹

Brian L. Lee, Brian D. Sykes, and Larry Fliegel

Abstract: The sodium/proton exchanger isoform 1 (NHE1) is an ubiquitous plasma membrane protein that regulates intracellular pH by removing excess intracellular acid. NHE1 is important in heart disease and cancer, making it an attractive therapeutic target. Although much is known about the function of NHE1, current structural knowledge of NHE1 is limited to two conflicting topology models: a low-resolution molecular envelope from electron microscopy, and comparison with a crystal structure of a bacterial homologue, NhaA. Our laboratory has used high-resolution nuclear magnetic resonance (NMR) spectroscopy to investigate the structures of individual transmembrane helices of NHE1 — a divide and conquer approach to the study of this membrane protein. In this review, we discuss the structural and functional insights obtained from this approach in combination with functional data obtained from mutagenesis experiments on the protein. We also compare the known structure of NHE1 transmembrane segments with the structural and functional insights obtained from a bacterial sodium/proton exchanger homologue, NhaA. The structures of regions of the NHE1 protein that have been determined have both similarities and specific differences to the crystal structure of the NhaA protein. These have allowed insights into both the topology and the function of the NHE1 protein.

Key words: membrane protein, membrane protein topology, Na⁺/H⁺ exchanger, NhaA.

Résumé : L'isoforme 1 de l'échangeur sodium/proton (NHE1) est une protéine de la membrane plasmique ubiquiste qui règle le pH intracellulaire en supprimant l'excès d'acide intracellulaire. NHE1 est important dans les maladies cardiaques et le cancer, ce qui en fait une cible thérapeutique intéressante. Même si la fonction de NHE1 est bien connue, les connaissances actuelles de la structure de NHE1 sont limitées à deux modèles topologiques contradictoires, une détermination en microscopie électronique à faible résolution de l'enveloppe moléculaire, et une comparaison avec la structure cristalline d'un homologue bactérien NhaA. Nos laboratoires ont utilisé la résonance magnétique nucléaire (RMN) à haute résolution pour examiner les structures des hélices individuelles de NHE1, une approche du type « diviser pour régner » afin d'étudier cette protéine membranaire. Dans cette synthèse, nous discutons des données structurales et fonctionnelles obtenues grâce à cette approche combinée aux données fonctionnelles obtenues d'études de mutagenèse de la protéine. Nous comparons aussi la structure connue des segments transmembranaires de NHE1 avec les données fonctionnelles et structurales obtenues de l'échangeur sodium/proton bactérien homologue, NhaA. Les structures des régions de NHE1 qui ont été déterminées ont des similarités et des différences spécifiques avec la structure cristalline de NhaA. Ceci a permis d'avoir un aperçu de la topologie et de la fonction de NHE1.

Mots-clés : protéine membranaire, topologie d'une protéine membranaire, échangeur Na⁺/H⁺, NhaA.

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Abbreviations: NHE1, Na⁺/H⁺ exchanger type 1 isoform; NMR, nuclear magnetic resonance spectroscopy; TM, transmembrane.

B.L. Lee, B.D. Sykes, and L. Fliegel.² Department of Biochemistry, School of Molecular and Systems Medicine, University of Alberta, Edmonton, AB T6G 2H7, Canada.

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²Corresponding author (e-mail: lfliegel@ualberta.ca).

Introduction

Na⁺/H⁺ exchangers (NHEs) are a family of membrane proteins that catalyze the transmembrane exchange of sodium ions for protons. They are widely distributed across phyla and kingdoms. Three general cation proton antiporter families have been described: CPA1, CPA2, and a Na⁺-transporting carboxylic acid decarboxylase family. The CPA1 family includes many well-studied examples of NHEs from fungi, plants, and mammals. It includes the eukaryotic NHE clade with several subgroups (Brett et al. 2005). Nine isoforms of mammalian sodium/proton exchangers (NHE1–NHE9) have been identified in this family and are thought to be electroneutral (Brett et al. 2005; Malo and Fliegel 2006; Lee et al. 2008). The CPA2 family consists of several clades and its members include the well-studied *E. coli* NhaA protein and the human NHA1 and NHA2 proteins, which have not been well characterized (Brett et al. 2005; Xiang et al. 2007).

The sodium/hydrogen exchanger isoform 1 (NHE1) is the best-characterized eukaryotic member of the NHEs and has multiple critical roles in humans (and is therefore the subject of this review). NHE1 is ubiquitously expressed in all tissues and localizes to the plasma membrane, where it plays a “housekeeping” role in cells. NHE1 is a mammalian integral membrane protein that exchanges one extracellular sodium ion for one intracellular proton. Through its control of intracellular sodium and proton concentrations, NHE1 plays an important role in the regulation of intracellular pH and cell volume and has been shown to have roles in stimulating cell growth, proliferation, differentiation, and apoptosis, as well as cell migration (Malo and Fliegel 2006). A splice variant of NHE1 has been described (Zerbini et al. 2003) and may have an important role in Na–Li countertransport (reviewed in Kemp et al. 2008). NHE1 shows little activity under normal resting conditions and intracellular pH; however, a decrease in intracellular pH allosterically increases its activity through an intracellular “pH sensing region” in the transmembrane (TM) domain (Grinstein 1988). The activity of NHE1 can be further stimulated through signalling cascades from numerous hormones and growth factors that affect its phosphorylation state and through the direct binding of regulatory proteins to its cytosolic regulatory domain (Malo and Fliegel 2006).

In addition to its normal physiological roles, NHE1 has a number of pathological roles in cells. NHE1 is involved in tumour metastasis by helping to create the alkaline intracellular and acidic extracellular environment found in tumours. It also promotes tumour invasiveness through the regulation of pseudopodia formation (Cardone et al. 2005). NHE1 is also important in ischemic heart disease (Fliegel 2009), where intracellular acidification due to ischemia results in activation of NHE1 and, in combination with the sodium/calcium exchanger, ultimately leads to a buildup of intracellular calcium that can lead to cell death and tissue damage. NHE1 is also implicated in cardiac hypertrophy, during which it is activated and plays a permissive role in the disease. Inhibition of NHE1 activity has been shown to prevent hypertrophy in several models (Karmazyn et al. 2008). The roles of NHE1 in these diseases have made it an attractive candidate for drug inhibitors. While results in animal models

have proved promising (Karmazyn 1988; Liu et al. 2009), results from clinical trials using NHE1 inhibitors to treat heart disease have been mixed. This may be due to inappropriate use of the inhibitors of NHE1 within the stages of ischemic heart disease as well as problems with the specificity of the inhibitors (Murphy and Allen 2009). A greater understanding of the mechanisms of NHE1 transport and its structure could certainly lead to improved effectiveness in control of its activity plus possibly improved design of NHE1 inhibitors.

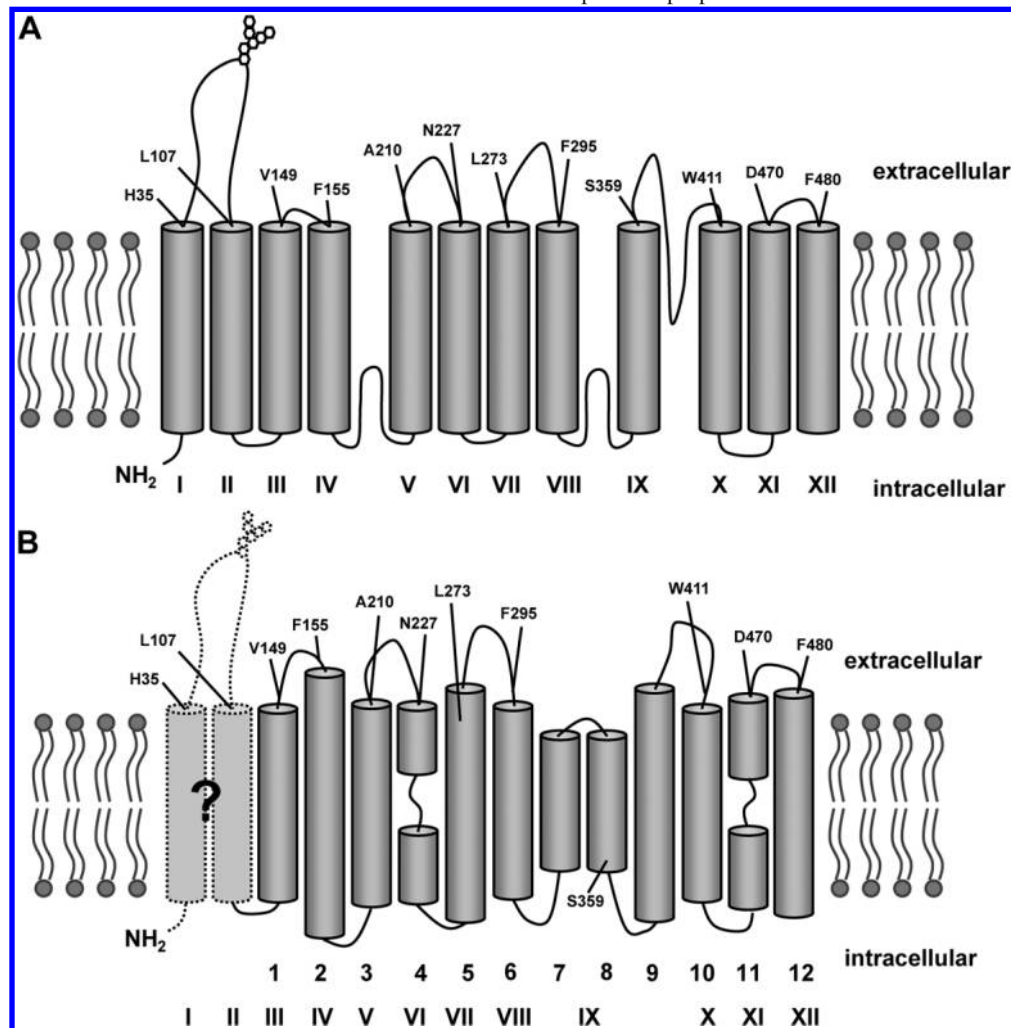
Topological models of NHE1

Detailed knowledge of the structure of NHE1 is necessary to understand its mechanism and to design more effective inhibitors; however, the high-resolution structure of NHE1 has not yet been solved, owing to the difficulty of expressing and crystallizing membrane proteins. Currently most of the information on NHE1 structure comes from two topology models of NHE1, some biochemical studies, a low-resolution electron diffraction envelope, and a crystal structure of a bacterial homologue. NHE1 contains a 500 amino acid N-terminal membrane domain responsible for ion transport and a 300 amino acid C-terminal tail that can modulate the activity of the protein through the binding of various regulatory proteins and modification by phosphorylation. The membrane domain is predicted to contain 12 TM alpha-helices by Kyte–Doolittle hydrophathy analysis (Kyte and Doolittle 1982). Two studies investigated the topology of the membrane domain (Wakabayashi et al. 2000; Landau et al. 2007) and are discussed below and reviewed in detail in Kemp et al. (2008). The C-terminal cytoplasmic tail has been expressed and purified. It was found to contain 35% alpha-helix, 17% beta sheet, and 48% random coil through circular dichroism spectroscopy, with most of the secondary structure predicted to occur in the region of the tail adjacent to the membrane domain (Gebreselassie et al. 1998).

The entire NHE1 protein has also been produced and purified (Moncoq et al. 2008). A low-resolution electron microscopy structure showed that NHE1 exists as a homodimer. Intermolecular cross-linking confirmed this observation and showed that the homodimer interacts through the C-terminal domain of NHE1 (Hisamitsu et al. 2004; Moncoq et al. 2008). This dimerization facilitates some of its transport function (Hisamitsu et al. 2006).

The topology of the membrane domain of NHE1 was first examined in detail by Wakabayashi et al. (2000). They used the substituted cysteine accessibility method (Akabas et al. 1992) to probe the positions of the intracellular and extracellular loops in the membrane domain as predicted by Kyte–Doolittle hydrophathy analysis. Their model (Fig. 1A) suggests that NHE1 contains 12 TM helices, here referred to as TM I – TM XII. It places both the N- and C-termini in the cytoplasm and the N-linked glycosylation site on the extracellular side. Wakabayashi et al. also suggested that two intracellular loops and one extracellular loop may be pore- or membrane-associated, based on the patterns of accessibility in those loops. The second and fourth intracellular loops were found to contain accessible (extracellular) and inaccessible residues adjacent to each other. To resolve this conundrum, Wakabayashi et al. proposed that these loops could

Fig. 1. Topology models of NHE1. (A) Topology model of Wakabayashi et al. (2000). TM helices are numbered underneath with Roman numerals. (B) Topology model of Landau et al. (2007). TM helices are numbered underneath with the corresponding Wakabayashi model numbering (Roman numerals) and Landau model numbering (Arabic numerals). The first two helices in the model by Wakabayashi et al. (2000) are not included in this model. Selected residues are labelled for comparative purposes.



form part of the pore, participating in ion translocation, where they could be accessible from both sides of the membrane. The fifth extracellular loop contains residues inaccessible from outside the membrane, and so it was proposed that this loop could be partially buried in the membrane domain. The topology of the fifth extracellular loop appears to be supported by an *in vivo* topogenesis study (Sato et al. 2004).

A more recent model for the topology of NHE1 was developed by Landau et al. (2007). This model was obtained using computational methods, including evolutionary conservation analyses and fold alignment methods with NhaA, a partially homologous bacterial Na^+/H^+ exchanger whose crystal structure has been determined (Hunte et al. 2005). The model was built under the assumption that since NHE1 and NhaA share a similar function, they may also share similar conserved residues and structure. A comparison of the two models shows some significant differences (Fig. 1B). The Landau model also has 12 TM segments, here referred to as TM 1 – TM 12. However, the model does not include

the first two helices predicted by the Wakabayashi et al. (2000) model, suggesting they are poorly conserved across NHE members (Brett et al. 2005) and may function only as a signal sequence (Sato and Sakaguchi 2005). However, studies have shown the presence of the intracellular N-terminus (Wakabayashi et al. 2000) and an extracellular glycosylation site (Counillon et al. 1994) in this region in the full protein. If the first two TM segments are not cleaved, and the balance of the Landau model is correct, this would in fact make a 14 TM segment protein, which is an intriguing but as yet unproven possibility (Fig. 1B).

The next six TM segments of NHE1 (amino acids 129–315, TM 1–6 in Fig. 1B, corresponding to TM III–VIII in Fig. 1A) have approximately the same sequence assignment in both models. Amino acids 339–398, TM IX, and the TM IX–X loop in the model of Wakabayashi et al. (Fig. 1A) have been reassigned as two short helices (TM 7 and 8) and another TM helix (TM 9), respectively, in the Landau model (Fig. 1B). Interestingly, this rearrangement results in the IX–X loop previously determined to be extracellular to be

placed on the intracellular side as the 8–9 loop; to explain this result, Landau et al. suggested that this loop could be within the ion translocation pore. The last three TM segments, TM 10–12 (amino acids 415–500), are the same in both models (Figs. 1A, 1B).

The crystal structure of NhaA provided the first high-resolution structure of a Na⁺/H⁺ exchanger-family protein, and provides insights into the mechanisms of Na⁺/H⁺ transport (Hunte et al. 2005). The structure of NhaA contains 12 TM helices, similar to what has been predicted for NHE1. The structure includes a novel fold consisting of TM 4 and TM 11, which cross each other at extended segments in the center of the two TM helices. Similar features have been found more recently on other membrane transporters (Screpanti and Hunte 2007). These extended regions are suggested to be important in ion binding and the conformational changes that occur during ion transport (Hunte et al. 2005). Because both TM 4 and TM 11 have interrupted helical structures, they create dipoles with partial positive and negative charges that are compensated for by amino acids D133 and K300, respectively. The assembly is therefore delicately balanced electrostatically and is crucial for NhaA activity (Padan et al. 2009). The crystal structure has been a starting point for defining a detailed mechanism of the protein. Changes in pH are sensed by an intracellular pH sensor that includes charged residues on TM 9 (Padan et al. 2009), the protein N-terminus (Appel et al. 2009), and TM 2 (Herz et al. 2009), which induce structural changes in the protein that result in a more open structure. This makes accessible two key aspartate residues on TM 5 in the core of the protein that are important for binding and transport of Na⁺ and H⁺ through an alternating exchange mechanism (Arkin et al. 2007).

The Landau et al. (2007) three-dimensional model of NHE1 contains features modelled on the NhaA crystal structure. The model predicts that two of the helices, TM 4 (TM VI) and TM 11 (TM XI), contain the same structure as TM 4 and TM 11 of NhaA. The model also shows an aspartate and asparagine on TM 5 (TM VII) that Landau et al. suggest are equivalent to the functionally important pair of aspartates in NhaA. The model is supported by the presence of functionally important, conserved residues in the core of the TM domain that are suggested to play roles similar to those of the important residues in NhaA. The model is also supported by the clustering of residues involved in inhibitor binding in 3D space. It should be noted, however, that NhaA has a different stoichiometry than NHE1, as it transports two H⁺ for one Na⁺, is activated at high pH, and functions to export Na⁺ using the proton electrochemical gradient (Padan et al. 2009).

A recent model of a related protein, human NHA2, has also been published (Schushan et al. 2010), with results suggesting a structure similar to that of NhaA but with unique features. The study included model-based mutagenesis experiments. Mutagenesis of conserved charged residues and residues in the core and comparison with the model suggested that NHA2 may have a TM segment assembly similar to that of NhaA. The dipoles of this assembly can be compensated for by polar but uncharged residues.

Use of NMR in membrane protein structure studies

NMR is an attractive method for studying the structure and function of membrane proteins and proteins in general, in that proteins can be studied in physiologically relevant conditions and thus the need for crystallization is avoided. Two approaches to studying the structure of membrane proteins with NMR are commonly used. One approach is the study of the entire protein. Expression of large quantities of eukaryotic membrane proteins can be difficult. Other difficulties include poor resolution in the NMR spectra, which is caused by both the primarily helical structure combined with the large number of residues, resulting in crowded spectra. In addition, the large size of protein–lipid complexes in solution results in slow tumbling times and broadening of spectral peaks. Methods such as transverse relaxation optimized correlation spectroscopy, perdeuteration, and selective labelling schemes (Sanders and Sönnichsen 2006) as well as solid-state magic angle spinning NMR (McDermott 2009) have been used to overcome this problem.

Because of the problems in producing and analyzing full-length multi-spanning membrane proteins, we have used a “divide and conquer” approach to study the NHE1 protein (White et al. 2001; Bordag and Keller 2010). That is, we have studied individual TM segments that are components of the larger multi-TM domain. Such smaller peptides tend to be easier to produce, both chemically and biosynthetically, than full membrane proteins and provide better resolved NMR spectra than the larger full-length proteins. This approach is based on the two-stage TM folding model (Popot and Engelman 1990), which reasons that TM helices form energetically stable, independently folding domains in membrane environments that subsequently associate to form the final three-dimensional structure. Therefore, TM helices might be expected to have similar structures both isolated and in the complete protein. This model has been further expanded to include the binding of cofactors and the folding of interhelical loops (Engelman et al. 2003) and the role of the solvent–membrane interface in folding (White and Wimley 1999). NMR studies of individual TM helices and loops have shown that the structures are often very similar to the structures of helices found in crystal structures, and kinks or unstructured regions in these helices have functional relevance, supporting this approach (Katragadda et al. 2001; Bordag and Keller 2010).

Methods other than NMR have also been used for the investigation of TM segment structure and folding. Biophysical methods such as fluorescence or circular dichroism spectroscopy are commonly used for both whole proteins and isolated fragments. Biochemical methods such as mutagenesis, cross-linking, and fusion protein assays commonly look at the entire protein. However, NMR can provide atomic resolution of details of structures or conformational changes in proteins, which is not possible with these other methods.

NMR structures and functions of NHE1 helices

We have determined the structures of several isolated TM helices of NHE1 using NMR. These were TM IV (residues

155–180) (Slepkov et al. 2005), TM VII (250–275) (Ding et al. 2006), TM IX (338–365) (Reddy et al. 2008), and TM XI (447–472) (Lee et al. 2009), based on the Wakabayashi et al. (2000) model. These helices were chosen on the basis of their functional importance implied by the presence of charged or helix-breaking residues and shown in previous mutagenesis studies. Each putative TM helix was found to display irregular structural properties, adopting conformations with varying degrees of non-helical structure present, mainly in the center of what were putatively helical peptides. Furthermore, these unusual features tended to correspond to residues found to be functionally significant in the full protein. Non-helical regions in TM helices have been found in the structures of many transporter proteins and are suggested to play key roles in their function (Screpanti and Hunte 2007).

Milligram amounts of peptide are needed for NMR structural analysis. A peptide representing TM IV was expressed in *E. coli* as a fusion protein with an N-terminal streptococcal immunoglobulin binding domain (GB1 domain) for solubility and a C-terminal poly-histidine tag for purification (Lindhout et al. 2003). Methionine residues separated the three regions of the fusion protein, allowing for purification of the TM IV peptide by cyanogen bromide cleavage and subsequent high performance liquid chromatography (HPLC). This expression system allowed for stable isotopic labelling of TM IV for detailed NMR study. Attempts at expression or purification of TM VII and TM XI using the GB1 system resulted in poor yield of purified peptide, so these peptides were made synthetically (Ding et al. 2007; Lee et al. 2009). Multiple naturally occurring charged residues and (or) additional non-natural lysine residues were placed at the termini. Lysine tags have been shown to increase peptide solubility during purification (Melnik et al. 2003).

The NMR structural studies were performed simultaneously with complementary functional assays involving detailed site-directed mutagenesis along the TM helices being investigated. The effects of these mutations on transport activity and inhibitor binding were measured in cultured cells (AP1 cells) through measured changes in pH. Cysteine-substituted mutants could be further reacted with sulfhydryl reactive reagents, such as negatively charged MTSES and positively charged MTSET. Treatment of specific mutants with MTSES or MTSET resulting in inhibition of transport activity suggests that residues are extracellularly accessible and line the pore of the protein.

TM IV (residues 155–180)

The NMR structure of TM IV (residues 155–180) was determined in an organic solvent mixture of CD₂OH:CDCl₃:H₂O (4:4:1 v/v) as a membrane mimetic (Slepkov et al. 2005). The low dielectric constants of the organic solvent solutions mimic the conditions found within a membrane bilayer or protein interior, and so may be suitable for structure determination of TM segments of polytypic membrane proteins. TM IV was found to be irregularly structured, not resembling a canonical alpha-helix as might be suggested in the topology model of Wakabayashi et al. (2000). The structure contains 3 structurally convergent re-

gions: residues 159–163, consisting of overlapping beta turns; residues 165–168, an extended region containing two proline residues, P167 and P168; and residues 169–176, including a short alpha-helical turn at residues 170–174. These three structured regions are connected by two pivot points at F164 and P168–I169, around which the structured regions rotate freely with respect to one another in the ensemble of NMR structures.

TM IV was found to be very sensitive to mutations, with mutation of 11 out of 23 residues to cysteine resulting in a decrease in activity to 20% or less compared with the wild-type NHE1. Mutation of P167 and P168 in the central, extended region to cysteine, glycine, or alanine (Slepkov et al. 2004) abrogated the activity of NHE1, suggesting the importance of these two prolines in the function of the exchanger, possibly through maintaining the extended structure of the region. An F161C mutant was found to react with both positively charged MTSET and negatively charged MTSES. This suggests this residue is accessible from the extracellular pore and would also be consistent with this residue having a role in inhibitor binding. Since residue D159 is consistently located on the same face as F161 in the NMR structures, it is likely that this residue would also be pore lining, with the negatively charged aspartate potentially aiding in the attraction of positively charged ions or participating in ion binding. Mutation of this residue to cysteine significantly decreased the activity and expression of NHE1, precluding the determination of its pore-lining orientation. It is not clear what role D159 or the second aspartate in TM IV, D172, might play, as mutations to asparagine, glutamate, or glutamine, changing the charge and size of the side chains, had varying results on each of the two residues (Murtazina et al. 2001).

TM VII (residues 250–275)

The NMR structure of TM VII, as well as subsequent studies of TM helices of NHE1, was determined in the presence of dodecylphosphocholine micelles as a membrane mimetic. The choline head groups mimic a phosphatidylcholine-like bilayer, and the small size of the detergent micelles compared with bilayer membrane preparations makes them favourable as a membrane mimetic for solution-state NMR studies (Kallick et al. 1995). The structure of TM VII (residues 250–275) was found to be helical over residues 255–260 and 264–272, with the central residues at 261–263 allowing for the two helical regions to adopt different conformations relative to each other (Ding et al. 2006). A structure calculation strategy taking into account possible heterogeneity or oligomerization (Rainey et al. 2006) suggested TM VII adopts two major conformations in dodecylphosphocholine micelles: one where the TM segment adopts only a mildly kinked structure, and one where the TM segment adopts a sharply kinked structure, with the two helical regions coming into close contact. Both NMR structure determination and ¹⁵N NMR relaxation studies suggest that the segment is undergoing microsecond to millisecond interconversion between the different conformations (Reddy et al. 2010).

Alanine scanning mutagenesis experiments suggest that TM VII is less sensitive to mutation than TM IV; however,

there are several residues on this TM segment that are particularly important in the function of NHE1. Mutagenesis experiments suggest that E262 and D267 are critical in the activity of the transporter, tolerating only mutations that conserve the charges of the residues. Furthermore, a double mutation consisting of a glutamate inserted between residues 261 and 262 plus an N266D mutation resulted in a functional protein, suggesting the positioning of D267 on the helix is crucial in function. Owing to the helical nature of the structure, these two critical residues are on the same face of the C-terminal helix. T270, also on the same face, appears to be important for function as well. These three residues likely line the pore, where they would play an important role in ion binding and (or) transport. Replacement of residue F260, G261, or S263 with either alanine or isoleucine resulted in a decrease in activity, indicating the importance of the conformational flexibility (suggested by NMR) imparted to this region by the small glycine and serine residues. Further experiments have also identified mutants L255C and L258C as reactive to MTSET, indicating that these residues line the pore and are accessible from the extracellular side of the membrane (Ding et al. 2007). Some residues were also suggested to be important for inhibitor sensitivity. L255, L258, and L265 are suggested to be accessible or near to the pore based on the NMR structure and helical wheel analysis, suggesting they may have roles in inhibitor binding. V272 is on the opposite face, however, so it is more likely to play a role in helix packing and inhibitor-induced conformational changes.

TM IX (residues 338–365)

TM IX (residues 338–365) contains two structurally conserved regions containing alpha helix structure at residues 340–344 and 353–359, with a 90° kink at S351 between the two regions, which could potentially provide flexibility (Reddy et al. 2008). In contrast to TM IV, but similar to TM VII, TM IX is not particularly sensitive to mutation, with only 5 out of 25 residues tested having a significant effect on activity when mutated to cysteine. There does not appear to be a clear correlation between the functionally important residues determined through mutagenesis and the location of the residues in the NMR structure, with the exception of S351, which has a strong effect on activity when mutated to cysteine and is located at the sharp kink in the structure. Reaction with MTSET suggests that both S351 and E346, between the helical regions, are pore lining. Two residues between the two alpha-helical regions, H349 and E346, appear to be important for inhibitor sensitivity. E346 was also found to be important for activity.

TM XI (residues 447–472)

TM XI contains two helical regions, residues 447–454 and 460–471 (Lee et al. 2009). These are connected by an extended region containing residues 455–459, which allows for variation of the orientation of the two helical regions with respect to each other. Similar to TM IV, this helix is particularly sensitive to mutation, with mutation of 13 of 22 residues resulting in less than 20% of the activity of the wild type. Of note, residues within and around the extended region, which contains three glycines and an arginine, were

particularly sensitive to mutation to cysteine. L465 was unambiguously identified as pore accessible through cysteine scanning mutagenesis and reaction with MTSET. L457C and I461C mutant proteins were partly inhibited upon reaction with MTSET, suggesting they could be deeper in the pore or partially buried, both possibilities resulting in decreased accessibility of these residues to MTSET.

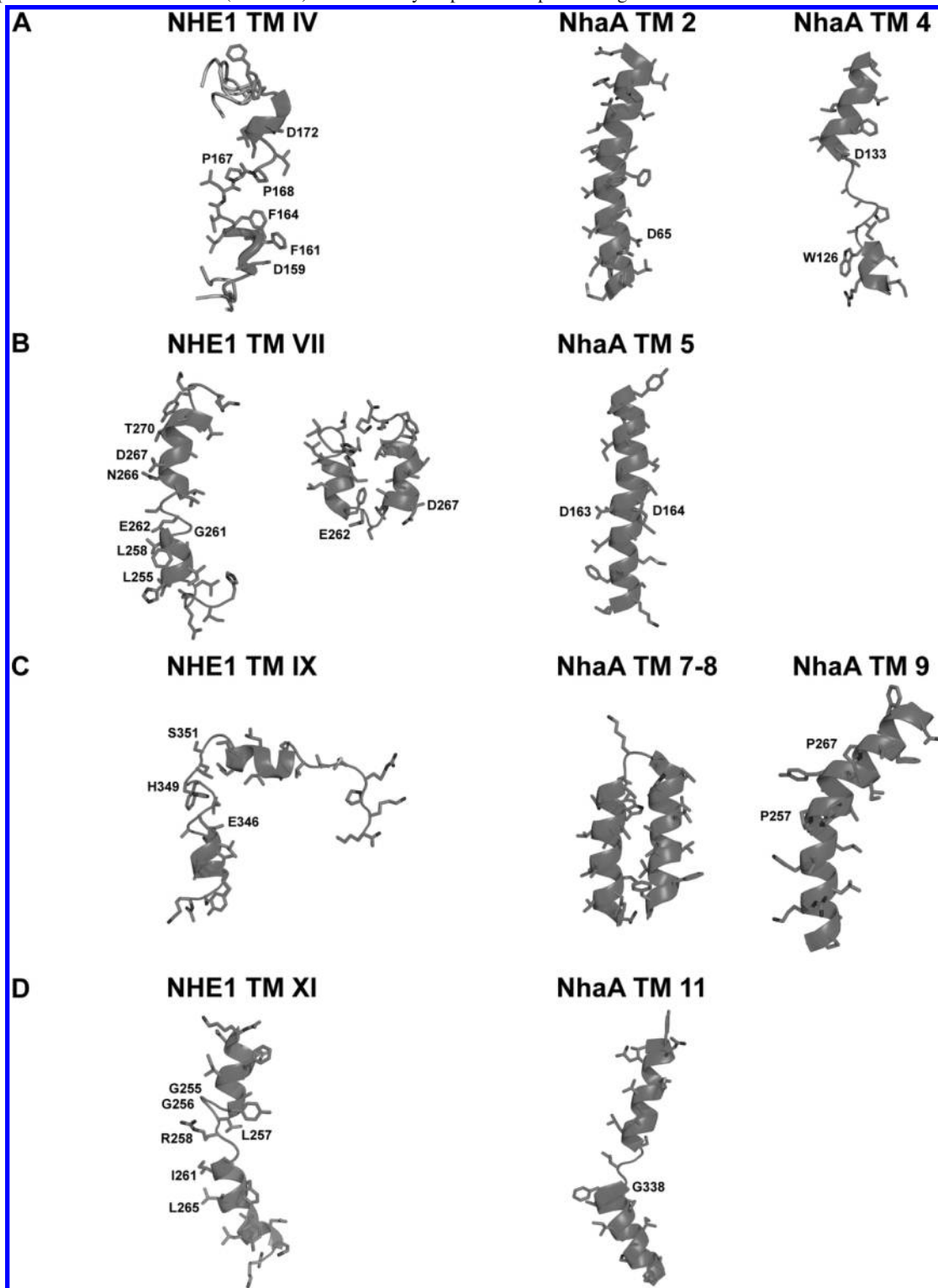
Insights obtained from comparison of NhaA with NHE1

Functional and evolutionary data suggest that bacterial NhaA could have a similar structure to other related sodium/proton exchangers (Brett et al. 2005). Furthermore, insights into NhaA function may apply to NHE1 and related mammalian transporters as well. Based on these premises, Landau et al. (2007) built a model of NHE1 based on the NhaA structure. Our approach to investigating the structure of NHE1 has been to solve the structures of isolated TM helices using NMR, based on the presumption that these peptides adopt structures that are similar to their structures in the full protein. Complementary functional studies, in combination with the structures of these individual helices, have provided insight into the structure–function relationships in NHE1. In a similar manner, a comparison of the NHE1 data with the structural and functional data available for bacterial NhaA may provide further insights into NHE1.

Comparison of TM IV of NHE1 with TM 4 and TM 2 of NhaA

The NMR structure of TM IV was found to roughly resemble the crystal structure of TM 4 of NhaA (Slepkov et al. 2007), initially suggesting that there could be a one-to-one direct correlation between the 12 helices predicted by Wakabayashi et al. (2000) and the 12 helices in NhaA. Despite the primarily non-helical nature of the TM IV NMR structure, the comparison showed there was a significant degree of similarity between the two structures. The N-terminal beta turns in the NMR structure could be superimposed onto the N-terminal helix of the crystal structure, while the central extended and C-terminal helical regions of TM IV could be superimposed on the same regions of TM 4 (Fig. 2A). However, the N-terminus of TM IV is clearly not alpha helical, unlike TM 4 of NhaA. Owing to the presence of pivot points in the NMR structure, only the structured regions and not the entire peptide could be superimposed. A lesser resemblance to TM 11 was also observed; however, the NMR structure of TM XI discussed below suggests that NHE1 TM XI and NhaA TM 11 may be equivalent. In this alignment, the pore-lining residue F161 would correspond to a tryptophan pointing towards the membrane bilayer; however, it is near enough to the surface of the membrane that it could nevertheless be accessible to chemical labelling. The NhaA structure also contains a critical residue, D133, which corresponds to P168. Both proline and aspartate residues can be found to cap the N-terminus of helices (Aurora and Rose 1998), lending support for this alignment. However, D133 is also thought to play a role in electrostatic balance and conformational changes resulting from ion binding (Padan et al. 2009). In TM IV, no such residue is present in the extended region, although it is also possible other nearby

Fig. 2. Structural comparisons of isolated TM segments of NHE1. The left column displays the deduced NMR structures of NHE1 and the right column illustrates the TM helices from the NhaA crystal structure. (A) NHE1 TM IV (residues 155–180) in comparison with NhaA TM 2 (59–85) and 4 (121–143). (B) Two conformations of NHE1 TM VII (residues 250–275) in comparison with NhaA TM 5 (152–175). (C) NHE1 TM IX (residues 338–365) in comparison with NhaA TM 7–8 (205–237) and TM 9 (246–272). (D) NHE1 TM XI (residues 447–472) in comparison with NhaA TM 11 (324–350). Functionally important or pore-lining residues in the TM helices are labelled.



helices could contribute a charged residue with a function similar to that of D133.

The Landau model (Landau et al. 2007) suggests instead

that TM IV is equivalent to TM 2 of NhaA. TM 2 has a bent shape different than the much more irregular structure of TM IV determined by NMR. While prolines are usually

considered helix-breaking, prolines are actually common in TM helices, and structures have been determined where prolines result in a kink rather than a break in the helix (Cordes et al. 2002). Furthermore, TM 2 lines both sides of the pore, consistent with the pore accessibility of NHE1 TM IV. F161 would be equivalent to I63, which would place F161 near the external face of the membrane, where it might be accessible to labelling. TM 2 of NhaA also contains residues involved in pH sensing, particularly D659 (Herz et al. 2009). In NHE1, D159, on the extracellular half of TM IV, could play a similar role. Overall, it is unclear whether TM IV of NHE1 is functionally equivalent to TM 2 or TM 4 of NhaA.

Recently (unpublished observations), we have examined TM VI of NHE1, which is the equivalent of TM 4 of the Landau model (Fig. 1B). We have found that TM VI is even more similar in structure to TM 4 of NhaA than TM IV. TM VI has two helical regions separated by an extended region. A critical feature of TM 4 of NhaA is the dipoles resulting from a break in the middle of the two helices. The dipoles contribute charges that are neutralized by specific residues. TM VI of NHE1 has an equivalent structure with two helices surrounding an extended segment, while TM IV does not. These results suggest that TM VI of NHE1 is a better candidate than TM IV for having a role equivalent to TM 4 of NhaA. They support the alignment of the Landau model with TM IV and TM VI of NHE1 aligning with TM 2 and TM 4 of NhaA, respectively. Future experiments will explore this possibility.

Comparison of TM VII of NHE1 with TM 5 of NhaA

It seems unlikely that TM VII (residues 250–275) of NHE1 could be equivalent to TM 7 of NhaA. TM 7 is a short helix containing only about 15 residues instead of the approximately 20 residues predicted for TM VII. Alignment of the sequences of TM VII and TM 7 suggested that the N-terminal half of TM VII would align with TM 7, while the C-terminal half would form part of TM 8. This would suggest that the break at residues 261–263 in the NMR structure could consist of an extracellular loop, with the critical E262 and D267 at either end of the loop. However, the peripheral location of TM 7/8 in NhaA contradicts the critical role and positioning of the residues in TM VII in NHE1 activity.

Landau et al. (2007) suggested that TM 5 is likely a better candidate for equivalence to TM VII. The charged residues on TM VII are then located to the center and extracellular portion of the helix, where they would line the pore and play direct roles in cation binding, similar to TM 5. The positioning of the negatively charged residues was found to be critical in TM VII, as would be suggested if their positioning were important in binding cations. TM 5 is relatively straight, however, and not kinked like the NMR structure of TM VII. It is possible that TM VII is not kinked in the full structure, as this might be determined by additional helix–helix interactions within the protein. TM 5 in NhaA is suggested to undergo movements in translocation (Arkin et al. 2007), and in TM VII the flexibility could aid in this movement. Furthermore, some of the structures in the ensemble determined by NMR show that the peptide is able to adopt

a more canonical helical conformation similar to TM 5 (Fig. 2B).

L255 and L258 on the N-terminal side of TM VII were shown to be accessible to externally applied MTSET (Ding et al. 2007). In both models of NHE1, for the equivalent helices for TM VII, the N-terminus of the TM helix would be on the intracellular side, where residues would not normally be predicted to be accessible to MTSET. However, the topology model of Wakabayashi et al. (2000) contains intracellular loops containing extracellularly accessible residues. These loops were suggested to be in the pore of the protein, possibly involved in ion translocation, and a similar interpretation could be made for the residues in TM VII. This would also support the Landau et al. (2007) assignment.

TM IX, unusual structure or extracellular segment

The NMR structure of TM IX (residues 338–365) of NHE1 contains a sharp kink at residue S351 that may reflect a flexible kink or loop in the full structure. There is the possibility that TM IX represents TM 9 in NhaA. TM 9 is kinked, with the bend occurring near an outward distortion of the helix N terminal to a proline residue in the center of TM 9. This kink, and residues on the extracellular side of the helix, are thought to be important in pH sensing and the resulting conformational changes. TM IX does not contain a proline; however, the small amino acids S351 and G352, which result in a kink in the center of TM IX, may contribute to the flexibility of the helix. The reactivity of amino acids S351 and E346 near the center of TM IX with MTSET would also be consistent with a pore-lining location.

The Landau et al. (2007) model suggests that TM IX comprises the extracellular half of TM 7 and all of TM 8. The sharp kink at S351 and the non-helical central region could suggest an extracellular loop (Fig. 2C). There is a lack of interaction between the helices that would be suggested by the crystal structure. If TM IX and TM 7–8 are equivalent, the lack of interaction may be due to the removal of interactions with surrounding helices or the choice of sequence that spans only half of the model TM 7. TM 8 of NhaA is near the pore in the crystal structure, and thus it is likely that the extracellular loop containing S351 and E346 is near the pore, such that reactivity to MTSET could block the pore. The residues E346 and H349 have been implicated in sensitivity to inhibitors (Wang et al. 1995; Noël et al. 2003). Both a pore-lining and an extracellular location would be able to explain these results. Furthermore, in the Wakabayashi et al. (2000) model, the termini of TM IX are on opposite ends of the membrane, while in the Landau et al. (2007) model, both ends are on the cytoplasmic side. However, the exact topology of TM IX in NHE1 could not be conclusively determined (Reddy et al. 2008).

TM XI, comparison with TM 11 of NhaA

TM XI contains a helix–extended segment–helix topology, similar to the NMR structure of TM IV of NHE1 and similar to TM 4 and TM 11 in the NhaA crystal structure. Both topology models agree this helix is the 11th TM helix, and thus it is likely that this assignment is unambiguous. The

NMR structure of TM XI also suggests this helix is structurally equivalent to TM 11 from NhaA (Lee et al. 2009). The N-terminal helix in the NMR structure is more unwound than the N-terminal helix in the crystal structure, possibly because of the presence of two glycines just before the extended region, G455 and G456 (Fig. 2D). These two glycines have been implicated in the pH response of NHE1, possibly allowing for packing interactions with other TM helices or facilitating the unwinding of TM XI. G338C, in a similar location of NhaA, is also considered important in the pH response (Galili et al. 2004). TM XI contains an important arginine residue, and the Landau et al. (2007) model suggests a second arginine residue that is contributed by TM 10. NhaA has only a single lysine in this region from TM 10, which has been suggested to be involved in the electrostatic balance in the TM 4/11 assembly. The NHE1 arginine residues could play a similar role. The identified pore-lining residues of TM XI would be placed in the extracellular half of TM XI, which agrees with their location in the Landau model. This segment was also found to be important in drug binding (Pedersen et al. 2007). Given the potential central role of this helix in the transport activity of the protein, it may be that this is due to the need for this segment to undergo conformation changes, rather than the direct binding of inhibitors.

Conclusions

The bulk of the data suggest that much of the topology proposed by Landau et al. (2007) based on NhaA may be reflective of NHE1 topology. While it seems unlikely that TM I and TM II are absent in NHE1, it seems likely that TM VII and TM XI are equivalent to TM 5 and TM 11 in NhaA, both structurally and functionally. It is not as clear which helices TM IX and TM IV are related to in NhaA. However, the structural and functional data can be explained when these helices are assigned as in the Landau et al. (2007) model. The question remains, particularly for the first half of the helix, whether TM IV corresponds to TM 2 or TM 4 of NhaA. The functional data suggest specific roles for the critical residues identified in NHE1, through comparison with NhaA. The structure of NhaA provides much promise in terms of structure–function studies of the protein and understanding the mechanism of transport. In combination with structures of individual helices, some of this data may be useful in interpreting the functional results obtained in NHE1. The NMR structure determination of further TM helices of NHE1 could provide additional insights into the topology and function of NHE1 and how it compares with NhaA.

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