BJ www.biochemj.org

BJ Structure

Biochem. J. (2007) **401**, 623–633 (Printed in Great Britain) doi:10.1042/BJ20061062

online data

REVIEW ARTICLE

Structural and functional analysis of the Na^+/H^+ exchanger

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The mammalian NHE (Na^+/H^+ exchanger) is a ubiquitously expressed integral membrane protein that regulates intracellular pH by removing a proton in exchange for an extracellular sodium ion. Of the nine known isoforms of the mammalian NHEs, the first isoform discovered (NHE1) is the most thoroughly characterized. NHE1 is involved in numerous physiological processes in mammals, including regulation of intracellular pH, cell-volume control, cytoskeletal organization, heart disease and cancer. NHE comprises two domains: an N-terminal membrane domain that functions to transport ions, and a C-terminal cytoplasmic regulatory domain that regulates the activity and mediates cytoskeletal interactions. Although the exact mechanism of transport by NHE1 remains elusive, recent studies have identified amino acid residues that are important for NHE function. In addition, progress has been made regarding the elucidation of the structure of NHEs. Specifically, the structure of a single TM (transmembrane) segment from NHE1 has been solved, and the high-resolution structure of the bacterial Na^+/H^+ antiporter NhaA has recently been elucidated. In this review we discuss what is known about both functional and structural aspects of NHE1. We relate the known structural data for NHE1 to the NhaA structure, where TM IV of NHE1 shows surprising structural similarity with TM IV of NhaA, despite little primary sequence similarity. Further experiments that will be required to fully understand the mechanism of transport and regulation of the NHE1 protein are discussed.

Key words: cation transport, intracellular pH, membrane protein, Na^+/H^+ exchanger (NHE), NhaA, structure–function analysis.

INTRODUCTION

The mammalian NHE (Na^+/H^+ exchanger) is an integral membrane protein that functions to exchange one intracellular proton for one extracellular sodium ion. By its involvement in ion fluxes, the NHE serves to regulate pH_i (intracellular pH) and cell volume, and to initiate changes in the growth or functional state of cells [1]. Aside from its physiological role, the NHE serves important roles in human pathology. Transport by the NHE plays a pivotal role in the damage caused to the human myocardium during and following a myocardial infarction [2], and it is considered to represent a key step in the oncogenic transformation of cancerous cells [3].

To date, the structure of the mammalian NHE remains elusive. However, a great deal is known about many residues that are required for the activity and regulation of the exchanger. In addition, the structure of a peptide of a single TM (transmembrane) segment from the mammalian NHE has been published recently [4]. This structure has some similarity to the structure of a functionally important TM segment from the *Escherichia coli* Na^+/H^+ antiporter NhaA, suggesting that these two proteins may have a similar structural architecture, although they share little sequence similarity.

MAMMALIAN NHE ISOFORMS

To date, nine isoforms (NHE1–NHE9) have been identified within the mammalian NHE family [5,6]. The isoforms share approx. 25–70 % amino acid identity, with calculated relative molecular masses ranging from approx. 74 000 to 93 000 [5,7]. Hydropathy analysis of the exchangers predicts that they have similar membrane topologies, with an N-terminal membrane domain consisting of 12 predicted TM segments and a more divergent C-terminal cytoplasmic domain [5]. The NHE1 isoform is the 'housekeeping' isoform of the exchanger and is ubiquitously expressed in the plasma membrane of virtually all tissues. It is the primary NHE isoform found in the plasma membrane of the myocardium [2]. The NHE2–NHE5 isoforms are also localized to the plasma membrane, but have more restricted tissue distributions. NHE2 and NHE3 are predominantly located in the apical membrane of epithelia and are highly expressed in kidney and intestine [8,9]. NHE4 is most abundant in stomach, but is also expressed in intestine, kidney, brain, uterus and skeletal muscle [8]. NHE5 is expressed predominantly in brain, but may also be present at low levels in other non-epithelial tissues, including spleen, testis and skeletal muscle [10,11]. The isoforms NHE6–NHE9 are ubiquitously expressed and are present in intracellular compartments [6]. These organellar membrane NHEs are presumed to regulate luminal pH and the cation concentration of the intracellular compartments [6]. NHE6 expression is highest in heart, brain and skeletal muscle and is localized to early recycling endosomes [6,12]. The NHE7 isoform is localized predominantly to the *trans*-Golgi network, and differs from the other NHE isoforms in that it mediates the influx of either Na^+ or K^+ in exchange for H^+ [13]. The highest levels of NHE8 expression are found in skeletal muscle and kidney, and this isoform is mainly localized to the mid- to *trans*-Golgi compartments [6]. The recently identified NHE9 isoform is localized to late recycling endosomes [6].

NHE1 TOPOLOGY AND TRANSPORT PROPERTIES

The NHE1 isoform is the most well-characterized isoform of the NHE family. As shown in Figure 1, NHE1 is 815 amino acids in

Abbreviations used: CAII, carbonic anhydrase II; CaM, calmodulin; CHP, calcineurin homologous protein; EL, extracellular loop; ERK1/2, extracellular-signal-regulated kinase 1/2; ERM, ezrin, radixin and moesin; HSP70, heat-shock protein 70; IL, intracellular loop; MAPK, mitogen-activated protein kinase; NHE, Na^+/H^+ exchanger; pH_i, intracellular pH; PIP_2, phosphatidylinositol 4,5-bisphosphate; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; TM, transmembrane.

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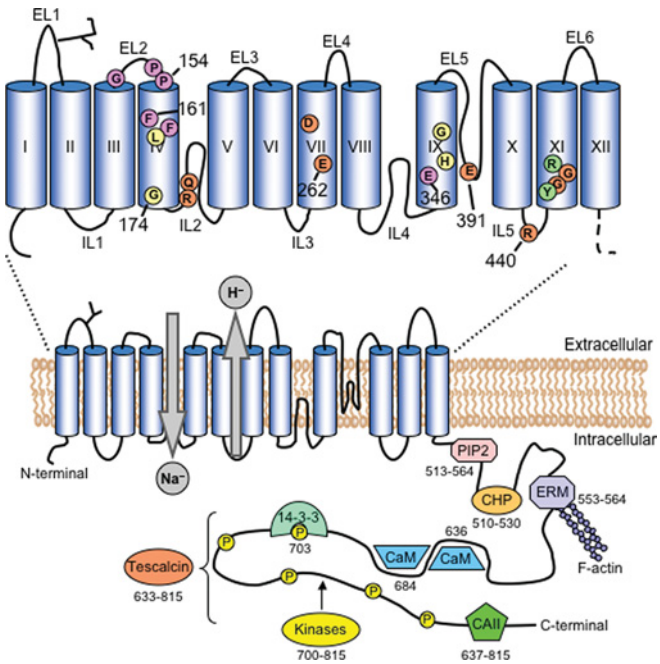

Figure 1 Model of NHE1 showing the membrane and cytoplasmic domains

Upper panel: topology of the membrane domain which functions to transport cations. This illustration is based on the findings of Wakabayashi et al. [90]. Pink circles, residues implicated in both ion transport and inhibitor binding; orange circles, residues implicated in ion binding and transport; yellow circles, residues implicated in inhibitor binding, green circles, residues implicated in NHE1 folding and targeting to the plasma membrane. Lower panel: representation of the cytoplasmic domain which functions to regulate the membrane domain through interactions with signalling molecules.

length, with residues 1–500 comprising the membrane domain and residues 501–815 comprising the cytoplasmic tail. The membrane domain of NHE1 is both necessary and sufficient for ion transport, whereas the cytosolic domain is involved in regulation of the activity of the exchanger [14]. Ion flux via the exchanger is driven by the TM Na^+ gradient and requires no direct metabolic energy input. NHE1 exhibits a simple Michaelis–Menten dependence on extracellular Na^+, with a reported apparent K_m of 5–50 mM [15]. Extracellular Li^+ and H^+ compete with Na^+ for binding at the Na^+-binding site, and high extracellular concentrations of K^+ inhibit NHE1 [16]. In contrast with the simple Michaelis–Menten dependence on extracellular Na^+, intracellular acidification allosterically increases the activity of NHE, resulting in a rapid increase in pH_i.

NHEs are targets for inhibition by the diuretic compound amiloride and its analogues, and by novel benzoylguanidine derivatives [17]. Comparisons of the different NHE isoforms show that they have varying affinities for these inhibitors, with the following order of sensitivity under similar experimental conditions: NHE1 $\geqslant$ NHE2 > NHE5 > NHE3 > NHE4 [5,16]. Because NHE1 is the isoform that is most sensitive to inhibition, and is the only isoform that is present in the plasma membrane of the myocardium, the selective properties of these inhibitors can be exploited therapeutically.

PHYSIOLOGICAL FUNCTIONS OF NHE1

Na^+/H^+ exchange is critical for a variety of physiological functions. The mammalian NHE protects cells from intracellular acidification, as shown by the fact that mutant cell lines devoid of Na^+/H^+ exchange activity are extremely sensitive to acidosis [18,19]. Because NHE is activated by decreased pH_i, when acidosis occurs it increases NHE1 activity, resulting in the return of pH_i to resting values. NHE also serves as a major Na^+ entry pathway in many cell types, and as such it regulates both sodium fluxes and cell volume after osmotic shrinkage [20–22]. Although the mechanism by which NHE1 regulates cell volume remains elusive, it is known that the volume- or osmolarity-sensitive site within NHE1 is located in the N-terminal 566 amino acids of this protein [23].

In addition to its role in regulating cellular pH and volume, NHE also initiates shifts in pH_i that stimulate changes in the growth or functional state of cells [18,24]. NHE1 is required for normal cell growth and proliferation, and cell proliferation is significantly reduced in cells expressing an inactive NHE1 mutant [25,26]. NHE1 activity is also important for cell differentiation. Transcription of NHE1 increases during differentiation in both human leukaemic cells and P19 embryonal carcinoma cells, and the P19 cells that have NHE1 inhibited or deleted are markedly deficient in their ability to differentiate [26–28]. NHE1 also plays a role in either promoting or inhibiting apoptosis, with its role varying depending on cell type [29–32].

NHE1 AS A STRUCTURAL ANCHOR

NHE1 acts as a structural anchor that is involved in regulating cytoskeletal organization. NHE1 is restricted to specialized membrane domains in some cells, such as in the lamellipodia of fibroblasts. There, NHE1 associates with the cytoskeleton via a direct interaction with the actin-binding ERM (ezrin, radixin and moesin) proteins at residues 553–564 in the C-terminal tail of NHE1 [33,34]. Fibroblasts expressing NHE1 with mutations that disrupt the interaction show impaired formation of focal adhesions and actin stress fibres. The cells also have an irregular shape, and, in wounding assays, migration of the cells is greatly impaired [33,35,36]. NHE1 therefore has a key role in cell migration through its interaction with the cytoskeleton and its restricted location. These observations, plus the fact that NHE1 binds several other proteins in the cytoplasmic regulatory domain, have led to the hypothesis that NHE1 can act as a plasma membrane scaffold that brings together many proteins so they can interact functionally [37]. For example, in addition to its association with ERM proteins and the cytoskeleton, NHE1 associates with signalling molecules such as PIP_2 (phosphatidylinositol 4,5-bisphosphate). PIP_2 binds in the same region as ERM proteins and ERM proteins require PIP_2 for F-actin binding. It is hypothesized that the proximity of PIP_2 on NHE1 facilitates ERM association with F-actin [37]. However, further investigation is necessary to confirm if the binding of NHE1 with a variety of proteins facilitates their interaction in complicated processes.

NHE1 IN DISEASE

NHE1 has been implicated in the physiology of several diseases, with the majority of research focusing on the role of NHE1 in heart disease and cancer. In the myocardium, under normal conditions, NHE1 removes excess intracellular acid in exchange for extracellular sodium. The increased intracellular sodium is removed by regulatory membrane proteins, including the Na^+/K^+ ATPase and the Na^+/Ca^{2+} exchanger. Problems arise in the myocardium with the increased production of protons that occurs in the human myocardium during and following a myocardial infarction [38]. The mechanism by which this occurs has been the subject of much investigation. In the first part of this mechanism, ischaemia results in increased anaerobic glycolysis, which leads to a large increase in the production of protons. This serves to

activate NHE1, which then exchanges intracellular protons for extracellular sodium, leading to a rapid accumulation of sodium in the cell [39–41]. Na^+/K^+ ATPase function may be inhibited by the decrease in high-energy phosphate stores that occurs during ischaemia. The increased sodium concentration within the cell drives an increase in calcium within the cell via reversal of the Na^+/Ca^{2+} exchanger. This results in a buildup of calcium in the cell that triggers various pathways, leading to cell death. It is known that inhibiting NHE1 can have beneficial effects on the myocardium during ischaemia and reperfusion, and the use of NHE1 inhibitors to protect the heart against ischaemic damage has been well established in animal studies [42–44]. The importance of NHE1 in ischaemia–reperfusion injury is further supported by the fact that genetic ablation of NHE1 in mice protects the heart from ischaemia–reperfusion injury [45].

Despite the promising results generated in animal models, results from clinical studies of NHE1 inhibition have not been very positive. Two large studies have failed to find any significant benefit of an NHE1 inhibitor. Any benefits were restricted to a subset of patients who underwent coronary artery bypass graft surgery. Additionally, a small study of 100 patients did reveal that the NHE1 inhibitor cariporide reduced infarct size and improved left ventricular function in post-infarction patients undergoing angioplasty [46–48]. A more recent study [49] showed that cariporide administration caused a modest, but significant, reduction in myocardial infarction after coronary artery bypass graft surgery; however, in this study a concurrent increase in cerebrovascular events occurred in high-risk patients, and this risk outweighed the positive results.

It has long been known that NHE is important for tumour growth, because tumour cells deficient in Na^+/H^+ exchange activity either fail to grow tumours or show severely retarded growth when implanted into immune-deprived mice [50]. It is now evident that NHE1 causes a reversal of the pH gradient in many types of transformed and/or malignant cells so that the intracellular environment is alkaline and the extracellular environment is acidic [3]. This 'malignant acidosis' is considered to represent a key step in oncogenic transformation and is necessary for the development and maintenance of a transformed phenotype [31,51,52]. Whereas NHE1 functions normally to regulate pH in untransformed cell types, in at least some transformed cells the tumour microenvironment aberrantly activates the protein. For example, in breast cancer cells, serum deprivation is a common tumour microenvironmental condition and this results in abnormal activation of NHE1 in this cell type. The activation of NHE1 was shown to be mediated by effects of RhoA and Rac1, which were specific to tumour cells. In cancer cells, serum deprivation provoked inhibition of RhoA activity in the leading edge of pseudopodia, whereas in non-tumour cells this did not occur [53,54]. This resulted in increased motility and invasion, characteristics that are required for metastasis to occur [3,53]. A recent study [55] has also demonstrated that the microenvironment of breast tumours can activate NHE1 through CD44. This results in acidification of the extracellular environment and promotes breast cancer progression. Thus NHE1 inhibitors have a potential use in treating various types of cancers. To date, NHE1 inhibitors have been shown to induce apoptosis in leukaemic and breast cancer cell lines [31,56]. The efficacy of NHE1 inhibitors for treating cancer currently awaits preclinical and clinical trials.

REGULATION OF NHE1

The NHE1 isoform is highly regulated. Intracellular acidosis is the major stimulus that regulates NHE1 activity, which is negligible under normal physiological conditions, but is rapidly activated as the pH_i decreases [57]. This activation exhibits a Hill coefficient of approx. 3, indicating that more than one proton binds to NHE1 during the transport cycle [58]. Thus it has been suggested that NHE1 contains a non-transporting H^+-binding site, sometimes referred to as a 'proton-modifier site', which allows an allosteric regulatory mechanism to lead to a greater increase in NHE activity than would be expected based on pH_i [58]. In addition to responding to intracellular protons, NHE1 is regulated by phosphorylation by various kinases and by interactions with other cellular proteins. NHE1 is also regulated at the transcriptional level, allowing both mRNA levels and the amount of NHE1 protein produced to be controlled [59–62].

REGULATION BY PHOSPHORYLATION

The distal region of the C-terminal tail of NHE1, which comprises amino acids ~ 700–815, contains a number of serine and threonine residues that are phosphorylated by protein kinases in response to sustained acidosis or to hormone and growth-factor stimulation [63–65]. Phosphorylation of residues in this region moves the set point of the exchanger, shifting the exchange activity to be more active at more alkaline pH values. Kinases that phosphorylate NHE1 and stimulate its activity include: ERK1/2 (extracellular-signal-regulated kinase 1/2), via the MAPK (mitogen-activated protein kinase) cascade [66–68]; $p90^{rsk}$ (p90 ribosomal S6 kinase), a downstream substrate of ERK1/2 [66,69,70]; the Rho-associated kinase, $p160^{ROCK}$ [71]; NIK (Nck-interacting kinase) [72]; and CaMKII (Ca^{2+}/calmodulin-dependent kinase II) [73]. NHE1 is also directly phosphorylated by p38 MAPK [32]. This kinase inhibits NHE1 activity in response to angiotensin II via inhibition of ERK1/2 [74], but it may also stimulate NHE1 and induce alkalinization in an apoptotic pathway [32]. Protein kinases C and D are also able to regulate the exchanger, but do not appear to phosphorylate it directly [68,73,75,76]. In addition, NHE1 is also regulated by dephosphorylation via protein phosphatase 1 [64,77].

INTERACTION WITH SIGNALLING MOLECULES

NHE activity is regulated by interaction with a variety of signalling molecules, and these interactions are shown in Figure 1 (lower panel). To date, three calcium-binding proteins have been shown to interact with the exchanger: CaM (calmodulin) and CHP (calcineurin homologous protein) act to stimulate NHE1, whereas tescalcin inhibits NHE1. CaM binds to the cytoplasmic C-terminal tail of NHE1 at two sites: a high-affinity site that is located at amino acids 636–656 (CaM-A; $K_d \sim 20$ nM) and a low-affinity site that is located at amino acids 657–700 (CaM-B; $K_d \sim 350$ nM) [78]. The high-affinity site regulates NHE1 activity by functioning as an auto-inhibitory domain, and either deletion of this site or binding of Ca^{2+}/CaM to this site abolishes the inhibitory effect [79]. A second Ca^{2+}-binding protein that interacts with NHE1 is CHP [80,81]. Endogenous CHP always contains two tightly bound Ca^{2+} ions when it is associated with NHE1, and it binds to amino acids 515–530 in the C-terminal cytoplasmic domain of NHE1 [80,82]. Finally, tescalcin binds to the final 180 amino acids in the C-terminal tail of NHE1 and functions to inhibit the activity of the exchanger [83,84].

Other signalling molecules that bind to NHE1 are: CAII (carbonic anhydrase II), the adaptor protein 14-3-3, PIP_2, and the mammalian HSP70 (heat-shock protein 70). CAII binds to amino acids 790–802 at the distal end of the C-terminal tail of NHE1 and increases the activity of the exchanger [85,86]. Phosphorylation of NHE1 at a site within amino acids 634–789 causes an increased interaction between NHE1 and CAII [85,86]. The binding of

14-3-3 protein to NHE1 is also dependent on phosphorylation of the exchanger, with binding occurring only when Ser^{703} is phosphorylated [87]. In Chinese-hamster fibroblasts, 14-3-3 protein binding is thought to participate in serum-stimulated exchanger activation by preventing dephosphorylation of Ser^{703} and by stabilizing an active conformation [87]. The signalling molecule PIP_2 binds to NHE1 at two putative PIP_2-binding motifs within the C-terminal domain at residues 513–520 and 556–564, and deletion of these binding motifs results in reduced transport activity *in vivo* [88]. Binding of PIP_2 to NHE1 is required for optimal activity of the exchanger, and this interaction, at least partially, accounts for the ATP dependence of NHE1. Finally, HSP70 binds directly to the C-terminal regulatory domain of NHE1, an interaction that is probably involved in the folding and processing of the antiporter [89].

STRUCTURE OF THE MEMBRANE DOMAIN

Relatively little is known about the structure of NHE1, because of the inherent difficulties associated with crystallizing membrane proteins. Thus molecular biology techniques have been used to gain some understanding of the general structure of NHE1. For example, the membrane topology of NHE1 shown in Figure 1 (upper panel) was determined experimentally by means of substituted-cysteine-accessibility analysis [90]. This analysis confirmed predictions that NHE1 has 12 TM segments, with both the N- and C-termini located in the cytosol. In addition, it identified three membrane-associated loop regions, IL2 (intracellular loop 2), IL4 and EL5 (extracellular loop 5) which may be involved in NHE1 function.

The mature form of NHE1 is localized to the plasma membrane and is glycosylated at both N- and O-linked sites. The N-linked glycosylation is not necessary for Na^+/H^+ exchange function and biosynthesis [91,92]. In addition, it is known that NHE1 forms homodimers in intact cells, and that dimer formation is not required for Na^+/H^+ exchange activity [93–95]. Although it was originally thought that the membrane domain of NHE1 alone is sufficient to allow for dimerization, a recent study showed that the proximal C-termini (amino acids 503–580) have a strong propensity to interact directly with each other, suggesting that the two C-termini of the NHE1 dimer may also interact with each other [94,95]. Finally, structural information has been deduced about the C-terminal cytoplasmic domain of NHE1 using CD spectroscopy. This method revealed that the cytoplasmic tail of NHE1 is 35 % α-helix, 17 % β-turn and 48 % random coil, and that the structure of the cytoplasmic tail is more compact at regions proximal to the membrane domain, whereas regions distal to the membrane domain are more flexible and display calcium-dependent conformational changes [83,96].

We recently published the first high-resolution structure of a TM segment of the human Na^+/H^+ exchanger [4]. A TM IV peptide was expressed and purified, and its structure was determined in a membrane-mimetic environment (Figure 2). From this NMR structure it is clear that TM IV does not, as a whole, assume a single conformation. Rather, sections within TM IV have distinct structural characteristics. Specifically, TM IV is composed of three sections of four to nine residues that converge structurally and only one region is α-helical: Asp^{159}–Leu^{163} form a series of β-turns; Leu^{165}–Pro^{168} has an extended structure; and Ile^{169}–Pro^{176} form an α-helix. As the structure of the peptide was determined in a membrane-mimetic solvent in the absence of the balance of the protein, these three structured regions rotated quite freely with respect to each other at swivel points that are located at Phe^{164} and Pro^{168}/Ile^{169} (Figure 2). It must be presumed that in the entire NHE1 protein the interaction of TM IV with other TM

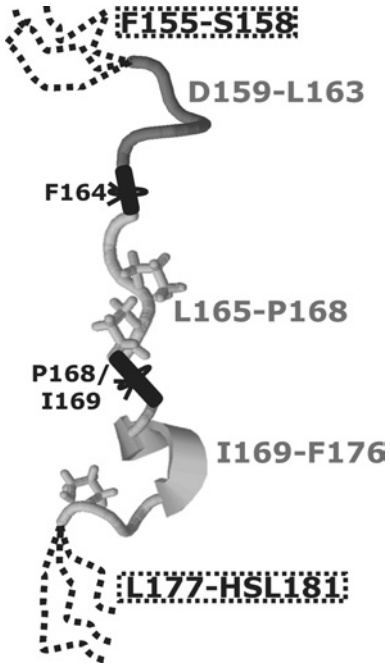

Figure 2 Structure of a TM IV peptide in a membrane -mimetic environment

Convergent stretches (grey) of residues Asp^{159}–Leu^{163}, Leu^{165}–Pro^{168}, and Ile^{169}–Phe^{176} in relation to pivot residues Phe^{176} and Pro^{168}/Ile^{169} are shown [137]. The flexible N- and C-termini are represented by dashed lines. Note that only the proline side chains are indicated. Reproduced from [4] with permission.

segments would restrict the rotation about these swivel points. Aside from this TM segment, no other information has been published regarding the structure of NHE1. However, in contrast with results from TM IV, we have recently determined TM VII is a kinked α-helix [96b].

RESIDUES INVOLVED IN NHE1 FUNCTION

Although the exact mechanisms of transport and inhibitor binding by NHE1 are not known, specific residues within the membrane domain of NHE1 have been implicated as being important for ion binding and transport. The location of these residues is highlighted in Figure 1 (upper panel), and Table 1 summarizes the effects of these mutations.

TM IV

Numerous residues in TM IV have been implicated in NHE1 function. For example, a $Phe^{165} \rightarrow Tyr$ mutation in TM IV of the hamster NHE1 sequence (corresponding to human Phe^{161}) causes both an increase in resistance to inhibitors and a decrease in V_{max} for Na^+ [97]. In addition, a $Leu^{167} \rightarrow Phe$ mutation (corresponding to human Leu^{163}) causes increased inhibitor resistance with no effect on Na^+ transport. In 1997, Counillon et al. [98] used random mutagenesis and found that a $Gly^{174} \rightarrow Ser$ mutation in TM IV causes a modest increase in resistance to amiloride with no effect on Na^+ transport. They also made an NHE1 mutant with a $Leu^{163} \rightarrow Phe$/$Gly^{174} \rightarrow Ser$ double mutation, and this mutant possessed a strongly reduced affinity for HOE 694 [(3-methylsulfonyl-4-piperidinobenzoyl)guanidine methanesulfonate] and a 2-fold decrease in sodium affinity. A $Phe^{162} \rightarrow Ser$ substitution in TM IV has been found to cause a dramatic decrease in affinity for cariporide and a 10-fold decrease in Na^+ affinity [99].

Table 1 Amino acids known to be involved in NHE1 structure and function

Summary of residues in the membrane domain of NHE1 required for ion transport, inhibitor binding and/or expression and targeting of the exchanger. Amino acid numbering corresponds to the human NHE1. For mutants that were studied in other mammalian models, the species used and the corresponding amino acid number are indicated in parentheses. The location of the residue within the topology of the exchanger, the specific mutations studied and the effects of the mutations are indicated. EIPA, 5-(*N*-ethyl)-*N*-isopropylamiloride; HOE 694, (3-methylsulfonyl-4-piperidinobenzoyl)guanidine methanesulfonate; MPA, 5-(*N*-methyl)-*N*-propylamiloride; MTSET, 2-(trimethylammonium)ethyl methanethiosulfonate bromide.

Amino acid	Location	Mutation	Effect of mutation	Reference
Gly^{148} (rat: Gly^{152})	EL2	Gly → Ala	Increase in K_i for EIPA	[7]
Pro^{153}/Pro^{154} (rat: Pro^{157}/Pro^{158})	EL2	Pro → Ser/Pro → Phe	Increase in K_i for EIPA Decrease in transport	[7]
Phe^{161} (hamster: Phe^{165})	TM IV	Phe → Tyr	Increase in K_i for amiloride and MPA Decrease in transport	[97]
Phe^{161}	TM IV	Phe → Cys	Lines ion-transport pore	[4]
Phe^{162}	TM IV	Phe → Ser	1500-fold increase in K_i for cariporide Increase in K_m for Na^+	[99]
Leu^{163} (hamster: Leu^{167})	TM IV	Leu → Phe, Ala, Arg, Trp Leu → Tyr	Increase in K_i for amiloride, MPA and HOE 694 Eliminates Na^+/H^+ transport	[97]
Pro^{167}	TM IV	Pro → Gly, Ala, Cys	Decreased Na^+/H^+ transport, expression and plasma membrane targeting	[100]
Pro^{168}	TM IV	Pro → Gly, Ala, Cys	Decreased Na^+/H^+ transport	[100]
Gly^{174}	TM IV	Gly → Ser,Asp	Increase in K_i for amiloride and HOE 694	[98]
Leu^{163}/Gly^{174}	TM IV	Leu → Phe/Gly → Ser	Increase in K_i for HOE 694 Increase in K_m for Na^+	[98]
Arg^{180}	IL2	Arg → Cys	MTSET treatment decreases activity	[90]
Gln^{181}	IL2	Gln → Cys	MTSET treatment decreases activity	[90]
Glu^{262}	TM VII	Glu → Gln Glu → Asp	Eliminates Na^+/H^+ transport Restores partial Na^+/H^+ transport Increases K_m for Li^+	[102]
Asp^{267}	TM VII	Asp → Asn Asp → Glu	Eliminates Na^+/H^+ transport Restores Na^+/H^+ transport	[102]
His^{349}	TM IX	His → Gly, Leu His → Tyr, Phe	Increase in K_i for amiloride Decrease in K_i for amiloride	[103]
Glu^{346} (rat: Glu^{350})	TM IX	Glu → Asp	Increase in K_i for EIPA and HOE 694 Decrease in transport Increase in K_m for Na^+	[7,105]
		Glu → Asn, Gln	Increase in K_i for EIPA Decrease in transport	
Gly^{352} (rat: Gly^{356})	TM IX	Gly → Ala, Ser, Asp	Increase in K_i for EIPA Decrease in transport	[7]
Glu^{391}	EL5	Glu → Gln Glu → Asp	Decreased Na^+/H^+ transport Restores Na^+/H^+ transport	[102]
Arg^{440}	IL5	Arg → Cys, Lys, His, Asp, Glu, Leu	Shifts pH_i dependence to acidic side	[107]
Tyr^{454}	TM XI	Tyr → Cys	Retained in endoplasmic reticulum	[106]
Gly^{455}	TM XI	Gly → Cys, Gln, Thr, Val Gly → Ala, Asp, Asn, Ser	Shift pH_i dependence to alkaline side No effect on pH_i dependence	[107]
Gly^{456}	TM XI	Gly → Cys	Shifts pH_i dependence to alkaline side	[107]
Arg^{458}	TM XI	Tyr → Cys	Retained in endoplasmic reticulum	[106]

We have been investigating the importance of residues in TM IV of NHE1 for several years, and we have found numerous residues in this TM segment that are required for normal Na^+/H^+ exchange activity. For example, we found that both Pro^{167} and Pro^{168} are required for normal NHE1 activity, whereas Pro^{178} is not [100]. In addition, mutation of Pro^{167} affects the expression and membrane targeting of NHE1 [100]. Thus both Pro^{167} and Pro^{168} are critical for normal NHE1 function, and may be required to directly interact with transported cations, or to allow TM IV to assume a unique structure or undergo a conformational change that is required for NHE1 activity. Mutation of these prolines may also affect the structure or folding of NHE1 that may cause aberrant targeting of the protein.

We also used the substituted-cysteine-accessibility method [101] to examine both functional and structural aspects of TM IV in NHE1. We found that TM IV is exceptionally sensitive to mutation, with each of the 23 single-cysteine mutations resulting in a significant decrease in exchanger activity [4]. In addition, we examined the sensitivity of the active single-cysteine mutants to thiol-reactive reagents and found that the mutant $Phe^{161} \rightarrow Cys$ was significantly inhibited by this treatment [4]. Therefore, in addition to being extremely sensitive to mutation, TM IV, and specifically Phe^{161}, line the ion-transport pore of NHE1.

The loop regions at either end of TM IV also contain residues that are important for NHE1 function. Mutation of three residues in the second exomembrane loop at the N-terminal end of TM IV (EL2) affects both the drug sensitivity and the activity of the exchanger [7]. IL2, at the C-terminal end of TM IV, also contains residues that may line the ion-transport pore, because treating the mutants $Arg^{180} \rightarrow Cys$ or $Gln^{181} \rightarrow Cys$ at IL2 with membrane-impermeant thiol reagents severely inhibits transport [90].

TM VII

TM VII is clearly involved in the ion-binding and transport capabilities of NHE1, because both Glu^{262} and Asp^{267} are essential for NHE1 activity [102]. Both of the mutations $Glu^{262} \rightarrow Gln$ and $Asp^{267} \rightarrow Asn$ abolished Na^+/H^+ exchange activity, whereas the

conservative mutations $Glu^{262} \rightarrow Asp$ and $Asp^{267} \rightarrow Glu$, which retain the acidic side chain, restored Na^+/H^+ exchange activity. In addition, the mutant $Glu^{262} \rightarrow Asp$ had a lower affinity for Li^+ than the wild-type exchanger. Because Li^+ has a smaller ionic radius than Na^+, this decreased affinity may be due to the shorter side-chain length of the aspartate residue reducing the ability of the exchanger to co-ordinate the smaller Li^+ ion. Thus TM VII is involved in the cation-exchange mechanism of NHE1, and Glu^{262} is a likely target for directly interacting with transported cations.

TM IX

The importance of TM IX for NHE1 activity was first demonstrated when it was determined that mutating His^{349} affects the amiloride sensitivity of NHE1 [103]. Moreover, chimaeric NHE proteins made by interchanging a 66-amino-acid segment containing TM IX and its adjacent loops from NHE1 and NHE3 displayed reciprocal alterations in their sensitivities to inhibitors, but retained normal Na^+ transport properties [104]. Recently, two residues within TM IX of rat NHE1, Glu^{350} and Gly^{356} (corresponding to Glu^{346} and Gly^{352} in human NHE1), were identified as major determinants of drug sensitivity [7]. In addition, mutation of Glu^{346} also affects the Na^+ affinity of NHE1 [105].

TM XI

A number of residues in TM XI may be involved in either ion transport or proper targeting to the plasma membrane, as shown by the fact that mutation of these residues alters NHE1 function [106]. Specifically, two mutants in TM XI, $Tyr^{454} \rightarrow Cys$ and $Arg^{458} \rightarrow Cys$, are retained in the endoplasmic reticulum [106]. In addition, the mutations $Gly^{455} \rightarrow Cys$ and $Gly^{456} \rightarrow Cys$ in TM XI shift the pH_i dependence of the exchanger to a more alkaline value ($pK \sim 7$), whereas the mutation $Arg^{440} \rightarrow Cys$ in IL5 at the N-terminal end of TM XI shifts the pH_i dependence to a more acidic value ($pK < 6.2$) [107]. The shift in pK observed with mutation of Gly^{455} was dependent on the size of the substituted side-chain residue, implying that these mutations may perturb the structure of TM XI, thereby indirectly affecting the H^+-modifier site. In the case of Arg^{440}, mutation to a lysine residue had a more modest effect on the pK than other mutations, indicating that a positive charge at this site may be important for normal pH_i sensitivity. Further experiments measuring $^{22}Na^+$ efflux from cells in which the Na^+/H^+ exchanger was functioning in the reverse mode also support the conclusion that both IL5 and TM XI play a crucial role in the proper functioning of the H^+-modifier site [107].

Other proximal membrane regions involved in NHE1 function

The first extracellular loop (EL1), the membrane-associated segment (EL5) and the proximal region of the cytoplasmic domain all contain residues that are involved in NHE1 function. EL1 is involved in the differences in volume sensitivity between isoforms of the Na^+/H^+ exchanger. Specifically, amino acids 41–53 are responsible for inhibiting hyperosmolarity-induced activation of NHE2, resulting in the lack of RVI (regulatory volume increase) that is evident in this isoform [108]. However, the N-terminal region (amino acids 1–95) of NHE1 do not appear to be involved in the volume-sensing mechanism of this isoform [108].

The possible functional involvement of the membrane-associated segment within NHE1 is of interest, because this segment contains several polar amino acids and is reminiscent of the selectivity filter of potassium channels [102]. It has been demonstrated that Glu^{391}, located in the membrane-associated segment, is important for activity [102]. A $Glu^{391} \rightarrow Gln$ mutation resulted in a partial reduction in activity, and a $Glu^{391} \rightarrow Asp$ mutation, an alternative acidic residue, restored Na^+/H^+ exchanger activity. Thus the membrane-associated segment plays a role in the ion-binding and transport properties of NHE1.

A highly conserved histidine-rich sequence of amino acids in the proximal region of the cytoplasmic domain, $H^{540}YGHHH^{545}$, is also involved in NHE1 function. Mutation of this sequence to $H^{540}HHHHH^{545}$ has no effect on the activation of NHE1 by protons, but did cause a decrease in the maximal velocity of the exchanger [109]. Thus this conserved sequence is involved in NHE1 function, but is not involved in proton sensing.

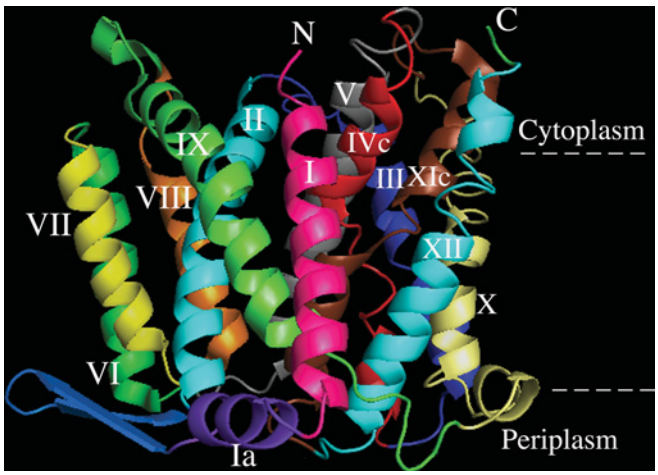

Figure 3 Structure of the *E. coli* Na^+/H^+ antiporter NhaA

Ribbon representation of NhaA viewed in parallel with the membrane. The 12 TM segments are labelled with Roman numerals. TM segments IV and XI have a helix-extended chain–helix conformation. The cytoplasmic (upper) and periplasmic (lower) faces of the membrane are indicated by broken lines. TM segments are colour coded as follows: I, pink; II, cyan; III, blue; IV, red; V, grey; VI, green; VII, yellow; VIII, orange; IX, green; X, pale yellow; XI, brown; XII cyan. This Figure is adapted from [112] with permission from *Nature* © 2005 Macmillan Magazines Ltd. (http://www.nature.com/).

STRUCTURE OF PROKARYOTIC Na^+/H^+ ANTIPORTERS

NHEs are ubiquitous throughout the Animal Kingdom. In bacteria, Na^+/H^+ exchange serves a role in osmotic regulation and removes an internal sodium ion in exchange for external H^+. *E. coli* has two antiporters, NhaA and NhaB. They exchange Na^+ or Li^+ for H^+. NhaA is indispensable for transport and is electrogenic with a stoichiometry of $2H^+/Na^+$ [110]. It is the most well studied of the prokaryotic Na^+/H^+ exchangers and has been well characterized by site-specific mutagenesis, and has also been overexpressed, purified and crystallized. Despite their similarity in function with the mammalian NHEs, NhaA, NhaB and other Na^+/H^+ exchangers, such as those found in yeast, do not share a large amount of similarity in their primary sequence. It may be that some critical amino acids involved in transport are conserved in their position and in their function in cation co-ordination [111]; however, this has yet to be determined.

Crystal structure of the Na^+/H^+ antiporter from *E. coli*

Recently, the crystal structure of the Na^+/H^+ antiporter, NhaA, from *E. coli* has been solved. Although NhaA shares little sequence homology with NHE1, these proteins share a similar basic topology with 12 membrane-spanning segments and both the C- and N-termini in the cytoplasm. Figure 3 shows the 3.45 Å

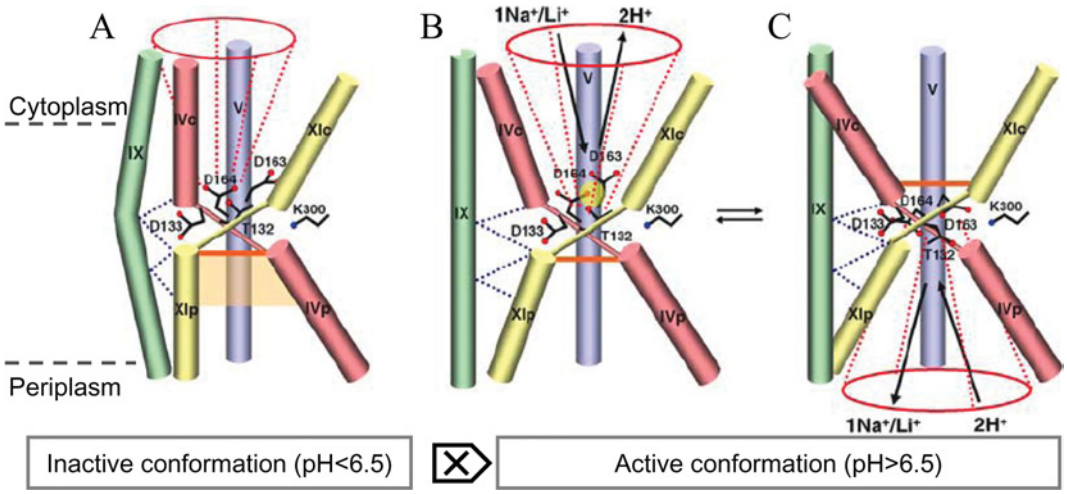

Figure 4 Proposed mechanism of transport by the *E. coli* Na^+/H^+ antiporter NhaA

The TM IV–TM XI assembly and its interaction with TM IX is shown. (**A**) Acidic pH-locked conformation. TM IX is bent, and the conformation of the TM IV–TM XI assembly only partly exposes the Na^+-binding site. (**B**) Alkaline pH causes a conformational change in helix IX that results in a reorientation of the TM IV–TM XI assembly. This exposes the Na^+-binding site (yellow circle) to the cytoplasmic funnel (red broken lines and red circle) and blocks it from the periplasm (orange line). (**C**) Na^+ binding causes the cation-loaded binding site to be exposed to the periplasm. Upon release of the cation, key aspartic acid residues are protonated, shifting NhaA back into the cytoplasm-exposed conformation in (**B**). This Figure is adapted from [112] with permission from *Nature* © 2005 Macmillan Magazines Ltd. (http://www.nature.com/).

resolution structure of NhaA in an acid-locked conformation. This structure reveals a negatively charged funnel that opens to the cytoplasm and ends in the middle of the membrane at the putative ion-binding site [112]. Of the 12 TM segments, ten span the bilayer as α-helices, whereas two (TMs IV and XI) are composed of a short helix, an extended polypeptide chain and a short helix (Figure 3). It should also be noted that, despite weak sequence similarity, TM segments III, IV and V form a bundle with strong structural similarity to the bundle formed by TM segments X, XI and XII. These bundles are in the opposite orientation relative to the membrane and bring TM segments IV and XI into close proximity at the ion-binding site while providing a balanced electrostatic environment. Extended (also referred to as 'unwound') segments in pairs of TM segments at active sites have also been observed in the SERCA1a (sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 1a); [113] and in a bacterial homologue of Na^+/Cl^--dependent neurotransmitter transporters ($LeuT_{AA}$) [114]. In each case, the extended portions of otherwise helical TM segments are in close proximity to each other and form ion- and substrate-binding sites.

The mechanism of NhaA regulation is thought to be dependent on TM IX, which is known to contribute to the 'pH sensor' and undergo a pH-induced conformational change. In the structure, TM IX is a distorted helix that is in contact with the TM IV–TM XI assembly at the centre of the membrane. Thus it is thought that at alkaline pH the conformation of TM IX changes, resulting in a reorientation of the TM IV–TM XI assembly that would fully expose the Na^+-binding site to the cytoplasm. Subsequent binding of Na^+ would then trigger another small movement of the TM IV–TM XI assembly, thereby exposing the cation-loaded binding site to the periplasm. Figure 4 shows this proposed mechanism of pH regulation and ion translocation. This mechanism, which requires only a small conformational change, is consistent with the extremely high catalytic activity of NhaA.

STRUCTURAL SIMILARITIES BETWEEN NHE1 AND NHAA

It has been proposed that the three-dimensional architecture of NhaA may be similar to that of the mammalian NHEs, despite the fact that these proteins share little sequence similarity in alignments of primary structure and have different transport stoichiometry [111,115,116]. In a number of membrane proteins, intramolecular structural homology has been observed within three-dimensional structures for regions having limited primary sequence similarity [112,114,117–122], including the case of NhaA, as described above [112,119,120]. Despite differences in transport mechanism, such as differing stoichiometery, the distantly related proteins NHE1 and NhaA do share a number of similar characteristics. In each of these proteins the pH regulatory site is different from the active site, a loop participates in the pH response and each protein exists as an oligomer within the membrane [95,115,123,124].

In the NMR structure of TM IV from NHE1 (Figure 2), an extended structure in the middle of the segment can be observed which is reminiscent of those seen in the ion-/substrate-binding sites of NhaA, SERCA1a [113] and $LeuT_{AA}$ [114]. Although it is certainly a possibility that an isolated TM peptide in membrane-mimetic conditions will assume a non-physiologically relevant structure, a number of isolated TM segments under such conditions have been shown to be both functional and properly structured where a structure of the full-length protein exists [125–132]. Beyond the general motif of paired TM segments in close proximity with extended regions interrupting structure, direct comparison of the crystal structures of NhaA, SERCA1a and $LeuT_{AA}$ shows that neither TM IV or TM XI of NhaA has strong structural similarity with M4 or M6 of SERCA1a or to TM I or TM VI of $LeuT_{AA}$ (results not shown). At best, a five-residue stretch of M4 of the extended portion of SERCA1a has 0.98 Å Cα RMSD (root mean square deviation) overlap with TM IV of NhaA. Comparison of TM IV of NHE1 with TM IV and TM XI of NhaA demonstrates strong structural similarity, particularly between the pair of TM IV segments.

Specifically, when aligned as shown in Figure 5, 14 residues of the TM IV segments of each exchanger are structurally homologous (Figure 5; see also detailed superimpositions in the Supplementary Figures, http://www.BiochemJ.org/bj/401/bj4010623add.htm). Most notably, the extended segment in NHE1 at residues 165–168 superimposes extremely well on to the

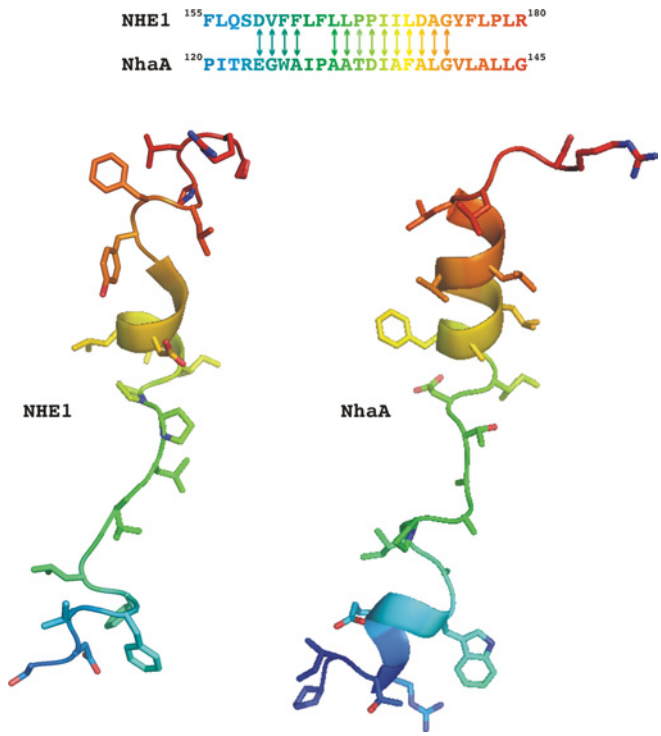

Figure 5 Structural similarity between TM IV segments of NHE1 and NhaA

Upper panel: sequence alignment of TM IVs of NHE1 and NhaA with arbitrary coloration. Lower panel: representative NHE1 TM IV structure [4] (arbitrary orientation at swivel point Phe^{164}) shown alongside the TM IV segment of NhaA [112], with colouring as indicated in the sequence alignment. Despite little sequence similarity, alignment of the TM IV segments of NHE1 and NhaA over the residues illustrated allows structural superimposition at the 14 pairs of residues indicated by arrows. Differences in structure between the Leu^{163}–Phe^{164} swivel point of NHE1 and the crystal structure of inactive NhaA at Ile^{128}–Pro^{129} mean that the entire segment does not superimpose well. However, Asp^{159}–Phe^{162} of NHE1 shows extremely similar structure to Ile^{121}–Trp^{126} of NhaA, and a subset of the NMR structures with the appropriate Pro^{168}/Ile^{169} swivel point orientation gives excellent superimposition of Leu^{165}–Gly^{174} of NHE1 on Ale^{130}–Gly^{139} of NhaA.

extended region of NhaA at residues 130–133. There would be little chance of predicting this structural similarity based on primary sequence, since in NHE1 this region has the sequence Leu-Leu-Pro-Pro versus Ala-Ala-Thr-Asp in NhaA. As would be expected, the helical segment 169–174 of NHE1 shows a good superimposition on to the helical segment TM IVc of NhaA, but the flexibility we observed in the NMR structures at the swivel point between Pro^{168} and Ile^{169} (Figure 2) means that only a subset of the TM IV structures of NHE1 superimposes well over both the extended segment and the C-terminal helical region IVc of NhaA. An example of another structure showing good superimposition over this entire region is presented in Figure 5. Finally, the turn structure observed over Asp^{159}–Phe^{162} of NHE1 superimposes exceptionally well on to Glu^{124}–Ala^{127} of NhaA. This covers the C-terminus of the TM IVp helix, which extends from Ile^{121}–Trp^{126} in the X-ray structure of the pH-inactivated form of NhaA, and the initiation of the β-bend observed at Ala^{127}–Ala^{130}. TM IV of NHE1 also shows a lesser structural similarity to TM XI of NhaA (results not shown), which would be expected given the quasi-symmetrical symmetry between TM IV and XI in the NhaA crystal structure [112,119,120]. Finally, TM IV of NHE1 does not superimpose well on to the M4 segment of SERCA1a, even though the M4 segment shows some structural similarity to TM IV of NhaA. Therefore, despite extremely low amino acid sequence similarity, the NMR structure of TM IV of NHE1 over residues Asp^{159}–Gly^{174} shows strong structural similarity to the TM IV segment of NhaA over residues Glu^{124}–Gly^{139}, with the exception of the swivel point region Leu^{163}–Phe^{164}.

Because biochemical studies have shown that TM IV in NHE1 lines the ion-transport pore and contains numerous residues that are important for NHE1 function [4], it is reasonable to assume that this TM segment is likely to play a central role in the mechanism of NHE1. As shown in Figure 4, conformational change of TM IV in NhaA about the extended non-helical region is proposed to play a major role in exposing the Na^+-binding site in the active versus inactive protein. Given the structural similarity of the extended segment of TM IV in NHE1, alongside its inherent flexibility, as exhibited by the pair of swivel points immediately N- and C-terminal to the extended segment, it is very possible that TM IV of NHE1 is involved in a similar mechanistically important conformational change. Furthermore, despite an apparent lack of sequence similarity, it is possible that further structural similarity exists and that these proteins share a similar tertiary fold. However, a definitive analysis of the comparative structure of NhaA and NHE1 will await the resolution of the structure of the entire membrane domain of NHE1.

CONCLUSIONS AND FUTURE DIRECTIONS

In recent years, there have been many advances made in our understanding of the function and regulation of the mammalian NHE. Several regions of the exchanger have been shown to be involved in ion-exchange activity, inhibitor binding, interaction with signalling molecules and regulation by phosphorylation. However, many unanswered questions still remain. For example, although it is evident that TM IV lines the ion-transport pore of NHE1 and contains many residues that are important for NHE1 function and inhibitor binding, less is known about other TM segments. Thus further mutagenesis studies are required to identify pore-lining and functionally important residues in other TM segments of NHE1. In addition, once further pore-lining residues are identified in TM segments other than TM IV, site-directed chemical cross-linking experiments may be of use to elucidate structural information about NHE1. This technique has been used to probe the three-dimensional structures of many polytopic membrane proteins and can be used to develop a model for the arrangement of TM segments [133–136]. Finally, although some structural information can be deduced about NHE1 based on the NMR structure of TM IV and similarities between NHE1 and NhaA, a major goal in the field of NHE research is the elucidation of high-resolution structural information about NHE1. One way to accomplish this is to determine the structure of TM segment peptides. It may also be possible to express and purify larger sections of the NHE1 protein that encompass several TM segments. The structures of larger sections of the NHE1 protein would be especially useful for understanding results from biochemical studies, because interactions between the TM segments may limit rotation within the TM segments, allowing the segments to adopt the conformation that they have in full-length NHE1. Finally, we await the structure of full-length NHE1, which will allow us to understand at a molecular level how this protein binds and transports cations and interacts with inhibitors.

E. R. S. was supported by an AHFMR (Alberta Heritage Foundation for Medical Research) Studentship. J. K. R. is supported by a Fellowship from the CIHR (Canadian Institutes of Health Research) Strategic Training Program in Membrane Proteins and Cardiovascular Disease. B. D. S. receives support as a Canada Research Chair in Structural Biology. L. F. is supported by an AHFMR Senior Scientist Award. Research by L. F. in this area is supported by the CIHR.

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Received 13 July 2006/28 September 2006; accepted 30 October 2006
Published on the Internet 12 January 2007, doi:10.1042/BJ20061062