REVIEW ARTICLE

Functional role of polar amino acid residues in Na^+/H^+ exchangers

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Na^+/H^+ exchangers are a family of ubiquitous membrane proteins. In higher eukaryotes they regulate cytosolic pH by removing an intracellular H^+ in exchange for an extracellular Na^+. In yeast and *Escherichia coli*, Na^+/H^+ exchangers function in the opposite direction to remove intracellular Na^+ in exchange for extracellular H^+. Na^+/H^+ exchangers display an internal pH-sensitivity that varies with the different antiporter types. Only recently have investigations examined the amino acids involved in pH-sensitivity and in cation binding and transport. Histidine residues are good candidates for H^+-sensing amino acids, since they can ionize within the physiological pH range. Histidine residues have been shown to be important in the function of the *E. coli* Na^+/H^+ exchanger NhaA and in the yeast Na^+/H^+ exchanger sod2. In *E. coli*, His^{225} of NhaA may function to interact with, or regulate, the pH-sensory region of NhaA. In sod2, His^{367} is also critical to transport and may be a functional analogue of His^{225} of NhaA. Histidine residues are not critical for the function of the mammalian Na^+/H^+ exchanger, although an unusual histidine-rich sequence of the C-terminal tail has some influence on activity. Other amino acids involved in cation binding and transport by Na^+/H^+ exchangers are only beginning to be studied. Amino acids with polar side chains such as aspartate and glutamate have been implicated in transport activity of NhaA and sod2, but have not been studied in the mammalian Na^+/H^+ exchanger. Further studies are needed to elucidate the mechanisms involved in pH-sensitivity and cation binding and transport by Na^+/H^+ exchangers.

Key words: cation co-ordination, charge relay system, membrane protein, pH regulation, salt tolerance.

1. GENERAL INTRODUCTION

Intracellular pH regulation is essential for growth and a variety of cellular functions. In both prokaryotic and eukaryotic cells, an Na^+/H^+ exchanger plays a key role in regulation of cytosolic pH. The Na^+/H^+ exchanger is a ubiquitous membrane protein localized in cytoplasmic and organellar membranes and is present in virtually all cell types, including bacteria and the cells of plants and mammals. This integral membrane protein transports Na^+ and H^+ in opposite directions across cell membranes. The direction of exchange is dependent solely upon the ions' electrochemical gradient, requiring no additional metabolic energy. In higher eukaryotic cells, Na^+/H^+ exchangers function to remove excess protons from the cytosol by taking up Na^+ from the external environment, a process that is driven by the sodium gradient generated by Na^+/K^+-ATPase (Figure 1A) [1,2]. In bacteria and yeast, Na^+/H^+ exchange is in the opposite direction, and intracellular Na^+ is removed utilizing the H^+ gradient generated by the plasma-membrane H^+-ATPase (Figure 1B) [3]. In mammalian and fungal cells, Na^+/H^+ exchange is electroneutral, with a stoichiometry of 1:1 [1–3]. Conversely, bacterial Na^+/H^+ exchange is electrogenic. In *Escherichia coli*, NhaA exchanges 2 H^+ per 1 Na^+ [4] and NhaB exchanges 3 H^+ per 2 Na^+ [5]. In addition, all Na^+/H^+ exchangers are able to transport Li^+ in exchange for H^+.

Na^+/H^+ exchange serves a variety of physiological functions, depending upon cell type. In mammalian cells its most common and important role is to regulate cytosolic pH [2]. The exchanger is also involved in initiating shifts in intracellular pH which, in turn, stimulate changes in the growth or functional state of the cell [6,7]. Na^+/H^+ exchange serves to protect cells from intracellular acidification. This is demonstrated by the fact that mutant cell lines devoid of the Na^+/H^+ exchanger are extremely sensitive to acidosis [8]. In addition, Na^+/H^+ exchange is involved in regulation of sodium fluxes and cell volume regulation after osmotic shrinkage [9]. The Na^+/H^+ exchanger also plays a pivotal role in the damage to the human myocardium that occurs during ischaemia and reperfusion. It has been demonstrated that inhibition of the exchanger's activity has beneficial effects for the myocardium under these conditions [10].

In bacteria and yeast, Na^+/H^+ exchange serves a central role in the regulation of internal Na^+ concentrations and also intracellular pH under alkaline environmental conditions.

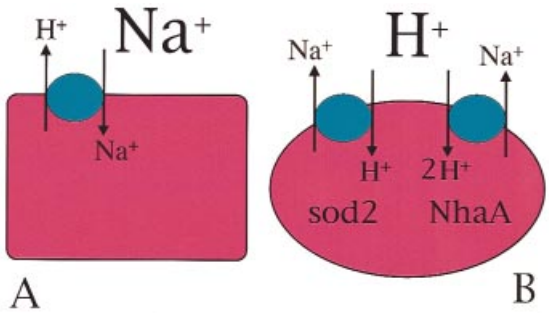

Figure 1 Schematic diagram illustrating typical activity of Na^+/H^+ exchanger isoforms in mammalian cells, yeast and *E. coli*

(**A**) Typical activity of the mammalian Na^+/H^+ exchanger, NHE1 isoform. The large 'Na^+' indicates the higher sodium concentration outside the cell driving activity of the protein. (**B**) Activity of Na^+/H^+ exchangers involved in salt tolerance, the yeast Na^+/H^+ exchanger sod2 of *Schizosaccharomyces pombe* and *NhaA* of *E. coli*. The large 'H^+' indicates the higher H^+ concentration driving the activity of these forms of the protein.

Abbreviations used: UCP-1, uncoupling protein-1; H225R (mutant designation using the one-letter amino acid code), $His^{225} \rightarrow$ Arg etc.; DEPC, diethyl pyrocarbonate; TMS, transmembrane segment.

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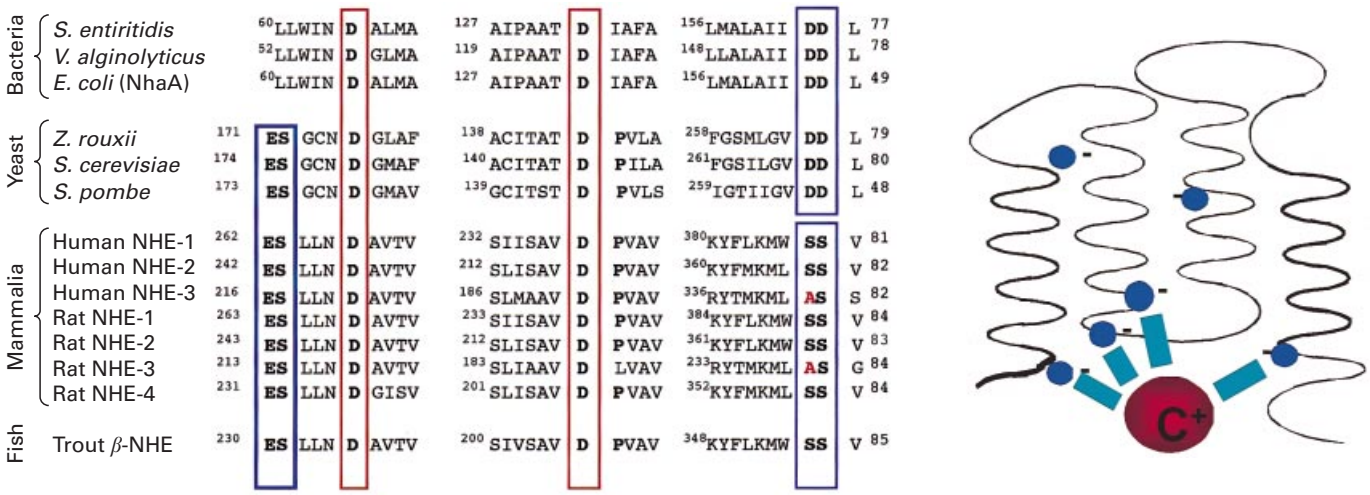

Figure 2 Alignment of prokaryotic and eukaryotic Na^+/H^+ exchangers (left-hand panel) and hypothetical model of cation co-ordination by amino acids of TMSs of Na^+/H^+ exchangers (right-hand panel)

Left-hand panel: Alignment of prokaryotic and eukaryotic Na^+/H^+ exchangers. Conserved polar residues are indicated by a box and are in **bold**. Numbers preceding the sequence indicate the number of the first amino acid, while the number following the sequence indicates the reference number. Red boxes indicate conserved single polar amino acids. Blue boxes indicate conserved pairs of polar amino acids. The red A indicates a non-conserved amino acid within the grouping of amino acids. *S. entiritidis* is *Salmonella entiritidis*. Right-hand panel: the red 'C^{+}' indicates a positively charged cation, whereas blue indicates negatively charged amino acids that may aid in co-ordination. Wavy black lines indicate transmembrane α-helices.

Na^+/H^+ exchange functions both to increase the buffering capacity of the cytosol and to buffer the protonmotive force [11].

Some Na^+/H^+ exchangers have been shown to be sensitive to changes in internal pH. For example, NhaA of *E. coli* is highly pH-dependent, but NhaB activity is pH-independent [12]. Although this phenomenon has not been studied in all Na^+/H^+ exchangers, the mammalian antiporter has been well characterized in this regard. It is activated allosterically by decreases in internal pH that lead to rapid increases in activity over relatively small pH changes. This activation is greater than can be accounted for by a simple Michaelis–Menten process. Kinetic analysis of the most ubiquitous, NHE1, isoform of the Na^+/H^+ exchanger has determined that there is an internal H^+ modifier site that is independent of the H^+-binding site used for transport [13]. Results have also suggested that the N-terminal transmembrane region contains the H^+ sensor site and that the C-terminal domain modulates the value of the set point [14,15].

Although the various kinds of Na^+/H^+ exchanger proteins all exchange Na^+ for H^+, the Na^+/H^+ antiporter serves different functions in different cell types. An analysis of the evolutionary relationships between the antiporters [16] has shown that the various mammalian isoforms are closely related. Comparison of more distantly related Na^+/H^+ exchangers, such as yeast and *E. coli* exchangers, with the mammalian isoforms suggests that these antiporters do not have long overall sequences of amino acids that are homologous. Rather, there are some short amino acid sequences of identity that might indicate a common evolutionary origin or function [16]. An alignment of selected regions of Na^+/H^+ exchangers is shown in Figure 2 (left-hand panel). It is clear from this and other alignments [16] that the mammalian isoforms are closely related, but that there is less identity when comparing them with bacteria or yeast exchangers. This low similarity makes it difficult to identify amino acid residues that are functionally important between distantly related isoforms, though it appears as though some polar amino acids may be conserved (Figure 2, left-hand panel). It has been suggested that a relatively small number of conserved amino acids may be sufficient to carry out and regulate Na^+/H^+ exchange, even when they are dispersed throughout the protein [11,17]. Therefore it is possible that these and other key conserved amino acids [16] may be important in transport. A hypothetical model for such cation binding and co-ordination is shown in Figure 2 (right-hand panel). We propose that this conservation of polar residues, together with their spatial arrangement within the protein, represents a general mechanism for cation binding and translocation by exchangers. This binding could be based on a crown-ether-like arrangement of electronegative atomic groups that co-ordinate the substrate cation [18]. Side chains of different amino acids from different transmembrane segments could aid in co-ordination of Na^+ or H^+. Thus, rather than the conservation of primary amino acid sequence, three-dimensional motifs of polar amino acids may be conserved among the various Na^+/H^+ exchangers throughout evolution. The amino acids may be on adjacent or more separate transmembrane segments. Whether such co-ordination occurs at the membrane/aqueous interface or within the membrane is not known.

Studies have only recently begun to systematically examine amino acids that are important in cation binding and transport and in pH-sensitivity. The purpose of the present review is to examine recent advances in understanding Na^+/H^+ exchanger function. Specifically, we examine recent results that have led to a greater understanding of specific amino acids involved in cation binding, transport and pH-sensitivity of Na^+/H^+ exchangers. Histidine residues are of particular interest. Several research groups have recently investigated the role that histidine residues play in Na^+/H^+ exchanger function. We examine the exchangers in which histidine residues have been studied, including NhaA of *Escherichia coli*, sod2 of the fission yeast *Schizosaccharomyces pombe*, and the mammalian NHE1 isoform of the Na^+/H^+ exchanger. In addition, histidine residues have been shown to be important in a number of other membrane-transport proteins. We also examine other polar amino acids that are important in cation binding and transport. Our review suggests that histidine residues play a vital role in activity of

these proteins and that other specific polar amino acids are also critical in Na^+/H^+ exchanger function. Further studies are necessary for a greater understanding of amino acids involved in cation binding and transport of this family of proteins.

2. HISTIDINE RESIDUES

2.1 Histidine residues in other membrane proteins

Histidine residues are good candidates for amino acids that are involved in pH-sensing or H^+ transport. The imidazole side chain has a p*K* of 6.0 and is the only side chain that ionizes within the physiological pH range. Depending on its local environment, histidine can therefore exist uncharged or positively charged when under acidic pH conditions. The imidazole ring can readily switch between the two states to catalyse the formation and breaking of bonds. Therefore histidine is often found in the active site of enzymes and is often an integral component of 'charge-relay' systems. As a result of ionization, histidine is capable of forming intra- and inter-molecular hydrogen bonds. These have a variety of effects, from inducing protein conformational changes and changes in pore size, to influencing activity and transport of H^+ and other ions [19–22].

A functional role for histidine residues has previously been implicated in a variety of membrane transport proteins. In several instances this involves membrane proteins that are involved in H^+ translocation. Histidine residues are involved in H^+ translocation of uncoupling protein (UCP-1) of brown-adipose-tissue mitochondria. UCP-1 acts as a H^+ transporter that 'short circuits' H^+ pumped by the respiratory chain, thereby generating heat. H^+ transport is dependent on a histidine pair (His^{145}/His^{147}), which act as H^+ donor/acceptor groups. Furthermore, H^+ transport activity of UCP-1 is inhibited by purine-nucleotide binding in a pH-dependent manner [23]. An additional histidine residue, His^{214}, was identified as functioning as a pH-sensor specific for nucleoside triphosphate binding, thus providing additional pH regulation of H^+ transport by UCP-1 [19].

Similarly, His^{322} of the *E. coli* lactose permease is one of only four residues that are irreplaceable for coupling substrate and H^+ translocation [21,24]. However, in this instance the histidine residue may function in substrate recognition, interaction of helices and for coupling conformational changes between helices rather than directly in H^+ translocation.

A single transmembrane-region histidine residue has been shown to be important in transport activity of a metal-tetracycline/H^+ antiporter and was hypothesized to play a role in tetracycline/H^+ coupling activity [25,26]. A single histidine residue has also been shown to be associated with H^+ translocation and energy coupling in vesicular monoamine transporters [27]. In addition, histidine is important in electron-transfer reactions in Photosystem II and cytochrome *b* subunits and in nickel transporters [28–30].

There is also accumulating evidence that histidine is a pivotal amino acid in the pH-dependent modulation of a number of membrane proteins. For example, protonation of a single histidine residue is responsible for the pH-dependent oligomerization of aerolysin, a bacterial toxin that inserts into plasma membranes and forms channels [31]. A single extracellular histidine residue has been identified to be responsible for a pH-dependent metal-cation-sensitivity of γ-aminobutyric acid ('GABA') rho 1 receptors [32]. Similarly, an extracellular histidine residue was found to be responsible for the pH modulation of aquaporin 0 [33].

Histidine residues are also important in the pH-dependent regulation of a number of ion channels, including several types of K^+ channels [34–39]. A single histidine residue also determines the pH-sensitivity of the cardiac and neuronal pacemaker channel, HCN2 [40], and a single histidine residue has been shown to be important in pH regulation of the cardiac gap-junction protein connexin 43 [41].

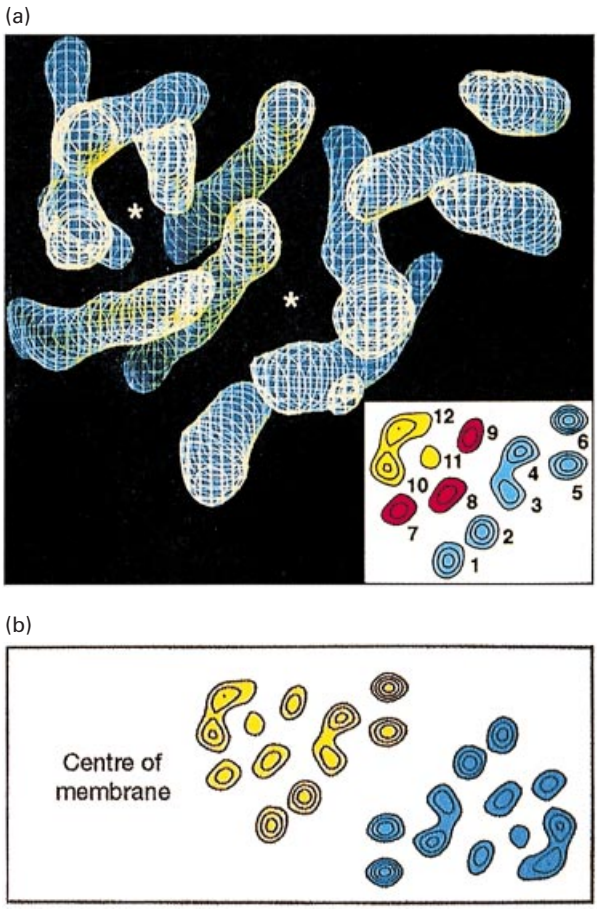

Figure 3 Three-dimensional map of *E. coli* Na^+/H^+ exchanger NhaA at 7 Å (0.7 nm) resolution and illustrated normal to the membrane plane (a), and horizontal slice through the NhaA dimer at the centre of the membrane illustrating the association of the two monomers (b)

(**a**) A total of 12 rod-shaped density features are illustrated that correspond to the transmembrane helices. It is uncertain whether the view is the periplasmic or cytoplasmic surface. The inset corresponds to a horizontal section from the centre of the three-dimensional map and with helices arbitrarily numbered 1–12. * Indicates putative ion translocation pathways. (**b**) A total of 12 peaks are seen for a monomer of NhaA. One monomer is yellow and one is blue. This Figure is taken from [42] and is reprinted with permission from the author and from *Nature* © 2000 Macmillan Magazines Ltd. (http://www.nature.com/).

Overall it is quite evident that histidine residues play a critical role in the activity of many membrane-transport proteins. This is particularly the case for membrane proteins involved in H^+ translocation or those that exhibit H^+ sensing.

2.2 *Escherichia coli* NhaA

The first Na^+/H^+ exchanger in which the role of histidine residues was thoroughly investigated was NhaA, the Na^+/H^+ exchanger of *E. coli*. This antiporter principally functions in salt tolerance by removing Na^+ in exchange for H^+ (Figure 1B). Recently, the elegant study by Williams [42] examined the three-dimensional structure of the protein that is illustrated in Figure 3(a). NhaA was found to have 12 tilted, bilayer-spanning helices. A roughly linear arrangement of six helices is next to a compact bundle of

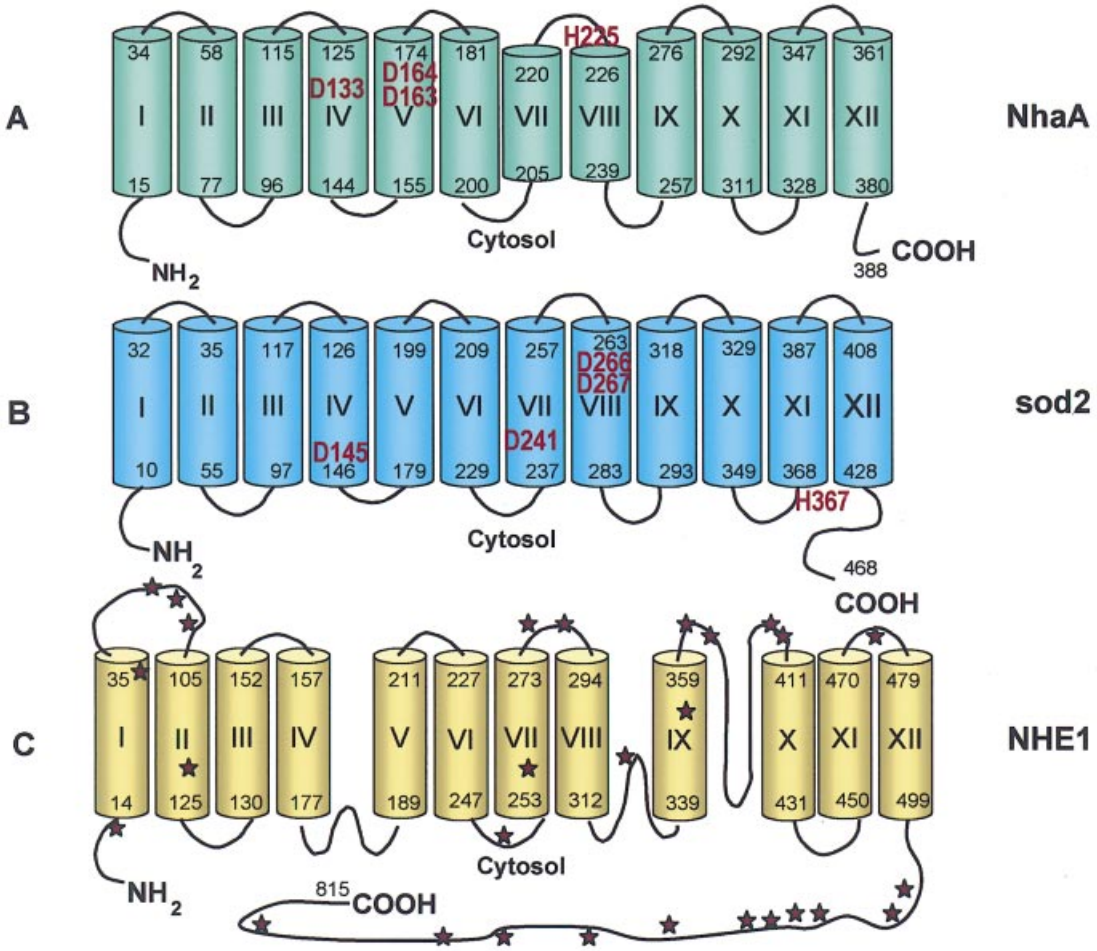

Figure 4 Models of the secondary structure of Na^+/H^+ exchangers

(**A**) Model of NhaA based on the findings by Rothman et al. [76]. The location of His^{225} is indicted. The locations of three aspartate ('D') residues important in activity are also indicated. (**B**) Model of sod2 based on predictions using the program TopPred II [86]. The position of His^{367}, and the positions of Asp^{145}, Asp^{241}, Asp^{266} and Asp^{267} that are important in transport ([47]; C. A. Wiebe and L. Fliegel, unpublished work), are indicated. (**C**) Model of mammalian, human NHE1 isoform of the Na^+/H^+ exchanger based on the findings by Wakabayashi et al. [53]. The positions of all histidine residues (at positions 13, 35, 76, 81, 98, 120, 250, 256, 275, 285, 325, 349, 373, 376, 407, 408, 473, 523, 529, 540, 543-545, 578, 610, 652, 689 and 801) are indicated by black-outlined red asterisks.

six helices. One helix in the bundle is not continuous throughout the membrane [42]. NhaA crystallizes as a dimer (Figure 3b). There is a relatively large distance between the two monomers, suggesting that the translocation pathway is not at the dimer interface (Figure 3b). A simplified schematic diagram of the *E. coli* protein is given in Figure 4(A), and is based on earlier studies [12], since it was not possible to identify individual helices in the initial three-dimensional structure of the protein.

The role of individual amino acids in the protein structure has been investigated by a variety of other techniques. Previous studies employed the histidine-reactive reagent diethyl pyrocarbonate (DEPC), which was found to inactivate NhaA [43]. This led to more recent studies by the research group lead by Etana Padan at the Hebrew University of Jerusalem. Initially each of the eight histidine residues of NhaA was mutated [44]. Since *E. coli* also has a second Na^+/H^+ exchanger (NhaB), the growth phenotype of the mutants in the presence of Na^+ was examined in *E. coli* lacking both the endogenous NhaA and NhaB genes. All the histidine mutants were able to restore growth in Na^+-containing medium. However, the His^{225} mutant was sensitive to external Na^+ at alkaline pH values (e.g. 8.5). The wild-type protein was activated between pH 7 and 8 and remained almost fully active to pH 8.5, whereas the H225R ($His^{225} \rightarrow Arg$) mutant was activated from pH 6.5 to 7.5. At pH values above 7.5 its activity drastically decreased [44]. Both the maximal activity and the degree of activation attained by the H225R mutant were similar to those of the wild-type. These results show that the H225R mutation significantly alters the profile of activation by pH.

It was therefore postulated that NhaA contains a pH-sensitive site that regulates its activity, and that His^{225} is part of this site. There are likely to be other amino acid residues involved, as the H225R mutation does not cause NhaA to completely lose its pH-sensor ability [44]. These suggestions made the assumption that His^{225} is located on the cytosolic face of the membrane, where it is able to sense changes in cytosolic pH. However, it has since been convincingly demonstrated that His^{225} is exposed and faces the cell exterior (periplasm). Thus, it is more likely that His^{225} functions to interact indirectly with the pH-sensor region of NhaA (Figure 4A) [12].

The role of His^{225} was further investigated by characterizing the effects of substitution of different amino acid residues [45]. Both the H225C ($His^{225} \rightarrow Cys$) and H225S ($His^{225} \rightarrow Ser$) mutants were found to exhibit growth phenotypes similar to the wild-type at both pH 7.5 and pH 8.5, although the maximal activity of these mutant proteins was lower than that of the wild-type. Since histidine, cysteine and serine are similar with respect to their polarity and ability to form hydrogen bonds, it may be hypothesized that the activation of NhaA by pH is dependent on polarity, the capacity to form hydrogen bonds, or both. Histidine, which is normally not charged at the basic end of the physiological pH range, allows NhaA to be activated from pH 7 to 8 when present at position 225. When histidine is replaced by cysteine or serine, these replacements have uncharged side chains that, like histidine, are polar and can form hydrogen bonds. With mutation of His^{225} to the negatively charged aspartate, the pH profile of this mutant was shifted towards alkaline pH. When His^{225} was replaced by positively charged arginine residue, the pH profile of NhaA was shifted towards acidic pH. When His^{225} is replaced with alanine, a residue that is non-polar and is not capable of forming hydrogen bonds, the activity of the antiporter is no longer dependent on pH. From these results, it was concluded that polarity, the ability to form hydrogen bonds, or both, must be essential for the activation of NhaA by pH, and that the presence of a charged group results in a shift of the pH profile [45].

To examine the accessibility and location of His^{225}, it was replaced with a cysteine residue (Cys^{225}) in the cysteine-less protein. The Cys^{225} residue was readily accessible on the periplasmic surface to membrane-impermeant reagents [12]. In addition, Cys^{225} was more readily alkylated by *N*-ethylmaleimide than native cysteine residues, demonstrating that this amino acid is much more exposed. This result suggests that it is unlikely that His^{225} is directly involved in sensing changes in cytosolic pH. Although it is known that His^{225} affects the pH sensitivity of NhaA, it is likely that His^{225} somehow interacts with or influences the pH sensor region of NhaA, which is composed of other residues. It is noteworthy that a region of loop VIII–IX has recently been implicated in the pH response of the protein (Figure 4A) [46]. It is involved in a pH-induced conformational change that leads to activation of NhaA at alkaline pH.

2.3 *Schizosaccharomyces pombe* Sod2

Although the most intense research regarding the role of histidine in the activity of the Na^+/H^+ antiporter has focused on NhaA, this is not the only Na^+/H^+ antiporter in which the role of histidine has been investigated. Eukaryotic membrane proteins are of great interest and are of higher physiological relevance to humans. Therefore some groups have studied these proteins despite the greater technical difficulties involved in their expression and characterization. In the case of sod2, the Na^+/H^+ exchanger of *S. pombe*, there is not a large overall sequence similarity between the yeast protein and NhaA. However, there are several isolated conserved groups of residues that occur between these proteins (Figure 2, left-hand panel).

To examine the role of the histidines in sod2, we mutated each of the histidine residues of sod2 to arginine [47]. To assay function, the Li^+ resistance of the sod2 mutants was observed at various external pH values. As Li^+ is even more toxic than Na^+ to most cells, it can be used to test for salt tolerance at lower concentrations than Na^+, therefore causing little or no osmotic effects. Since sod2 represents the only major Na^+-extrusion pathway for this yeast species [48], growth in Li^+- or Na^+-containing medium was a reasonable assay of sod2 function. Growth of a *S. pombe* sod2 disruption strain was fully suppressed in the presence of 2 mM LiCl from pH 3.5 to 6.5. When this strain was transformed with a plasmid harbouring wild-type sod2 or any of the sod2 histidine mutants other than H367R, growth was observed in up to 10 mM LiCl over the same pH range. However, the H367R mutant was as sensitive to LiCl as the sod2 disruption strain. Further mutational analysis demonstrated that H367A and H367D were also sensitive to external Li^+ at pH 3.5–6.5. These mutant proteins were found to be expressed and correctly targeted to the plasma membrane, thus confirming that the salt-sensitivity of the His^{367} mutants was due to a defect in the protein itself (Figure 4B) [47].

Antiport activity of the His^{367} mutants was also directly assayed by measuring $^{22}Na^+$ efflux by whole cells. All three mutants – H367R, H367A and H367D – showed only background Na^+ efflux, indicating that the sod2 protein with this mutation was not active. In addition Na^+-dependent H^+ uptake was measured by incubating cells in buffer containing high NaCl and subsequently monitoring the pH change caused by the cells in a weakly buffered NaCl-free external medium. Cells expressing wild-type sod2 exhibited Na^+-dependent H^+ uptake, thus increasing the pH of the medium by about 0.15 pH unit in approx. 4 min. Cells expressing the H367R and H367A mutants were incapable of alkalinizing the external medium. The H367D mutant caused the pH of the medium to increase to an extent similar to that seen with the wild-type sod2, showing that an acidic residue can substitute for the histidine residue, while a basic and neutral residue cannot.

Taken together, it can be concluded that the H367R and H367A mutations completely inactivate sod2. Although the H367D mutant prohibits cells from extruding Na^+ when Na^+-loaded, cells continue to demonstrate Na^+-dependent H^+ uptake. Although these results initially appear contradictory, they are put into perspective when one takes into account the external pH at which the experiments were performed. The $^{22}Na^+$ efflux was measured at pH 4.0, while the Na^+-dependent H^+ uptake was measured at pH 6.1. The H367D mutant appears to shift the pH optimum of the antiporter to a more alkaline pH, thus allowing it to be active at pH 6.1, but rendering it inactive at pH 4.

Results indicate that His^{367} of sod2 may indeed be a functional analogue of His^{225} of NhaA, also acting as a pH sensor. This is supported by the fact that both the H367D and the H225D mutations shift the pH optima of their respective antiporters towards alkaline pH. However, the results for the H367R and H225R mutations do not correlate as well. The H225R mutant of NhaA was found to shift the pH optimum of NhaA towards acidic pH [45], but the H367R mutant of sod2 was found to be inactive at both pH 6.1 and 4.0, indicating that the pH optimum was almost certainly not shifted towards acidic pH. This difference may reflect the functional differences between sod2 and NhaA. As previously mentioned, NhaA is electrogenic, exchanging 2 H^+ per 1 Na^+, whereas sod2 is electroneutral, exchanging 1 H^+ per 1 Na^+ [12]. Furthermore, NhaA is active from pH 7 to 9, whereas sod2 is active under more acidic pH conditions. Overall the results suggest that His^{367} of sod2 plays a role similar to, though not identical with, His^{225} of NhaA. It is noteworthy that the position of the two residues within the membrane proteins is similar (Figure 4). His^{225} of NhaA is downstream of a paired double aspartate motif that is important in activity [49]. Similarly, His^{367} of sod2 is downstream of a paired aspartate motif that is important in activity of sod2 (see below) [47]. It is possible that these amino acids are part of a conserved structure involved in co-ordinating transport of cations or regulation of activity of these proteins. It must be noted, however, that His^{367} of sod2 is predicted to be cytosolic, while

His^{225} is known to be periplasmic (Figures 4A and 4B). This difference could account for the differences in function that occurs with mutation of these residues. In addition, the topology of sod2 is not really known, and the models present so far rely only on analysis of hydrophobicity. Initial models of NhaA based on hydrophobicity also placed His^{225} in the cytosol until they were later disproved [12]. Therefore it may be that His^{367} is indeed also present in the periplasm. It is also noteworthy that the linear spacing between the 'sensor' histidine and aspartate pairs is different between sod2 and NhaA (61 amino acids in NhaA versus 100 in sod2). However, this is not a critical factor in cation co-ordination, as a more downstream transmembrane segment can easily associate with an upstream one. Further experiments are necessary to determine if it is not the precise homology of primary and secondary structure of the exchangers that is critical, but rather the conservation of the three-dimensional organization involved in cation co-ordination.

2.4 The mammalian Na^+/H^+ exchanger

The mammalian Na^+/H^+ exchangers comprise a family of at least six isoforms, namely NHE1–NHE6 [2]. The first isoform cloned, NHE1, has been the most extensively studied and is the most widely distributed. It is the isoform discussed here in detail. Early studies, looking at chemical modification of the Na^+/H^+ exchanger from rabbit renal-cortex brush-border-membrane vesicles [50] and the Na^+/H^+ exchanger of thymic lymphocytes [51], demonstrated that a significant inhibition of Na^+/H^+ exchanger activity was observed on treatment with DEPC. This inactivation was direct and not due to vesicle disruption, nor the collapse of transmembrane H^+ gradients. Furthermore, inactivation of exchange was reflected by a decreased V_{max} with no change in the K_m for Na^+, a dependence on external pH but not internal pH, and blockage by amiloride. Inactivation by DEPC was specifically due to modification of histidine residues, as inactivation of the exchanger was reversed by hydroxylamine [which removes the ethoxyformyl group from DEPC-modified histidine, but does not reverse the DEPC modification of other possible amino groups (e.g. those of lysine, tyrosine or cysteine)]. DEPC inhibition has also been demonstrated for the human, amiloride-sensitive NHE1 in stably transfected fibroblasts. Kinetic analysis of this DEPC inhibition suggests that there are two critical histidine residues that are involved in the transport activity of NHE1 [52].

Recently a new model for the topology of human NHE1 has been proposed. This study determined the accessibility of 83 cysteine residues to cysteine-directed reagents introduced into a cysteine-less form of NHE1. The novel topology model is significantly different from that derived from hydropathy analysis [53]. In this model the 28 histidine residues present in NHE1 can be roughly classified into three groups: 1, transmembrane or integral membrane histidine residues; 2, extra-/intra-cellular loop residues; and 3, residues of the cytoplasmic C-terminal tail (Figure 4C). These amino acids are discussed below.

2.4.1 Transmembrane histidine residues

The most up-to-date model of NHE1 topology [53] places His^{35}, His^{120}, His^{256}, His^{325} and His^{349} embedded at least partially within the lipid bilayer (Figure 4C). Although evidence supports the theory that external histidine residues are important for the function of the Na^+/H^+ exchanger [50] (see below), it has also been suggested that external histidine residues alone are unable to mediate the transfer of H^+ across the plasma membrane. Rather, Wang et al. [52] suggest that a series of putative transmembrane histidine residues in NHE1 may be involved in H^+ translocation, functioning as a H^+-relay system. To test this hypothesis, site-directed mutagenesis was employed, replacing three histidine residues with glycine residues in the putative transmembrane segments (TMSs) according to the topology model of NHE1 as follows: His^{35} of TMS1, His^{120} of TMS2 and His^{349} of TMS9 (Figure 4C) [52]. NHE1 activity and amiloride-sensitivity were measured for the histidine mutants H120G, H349G, H120,349G and H35,120,349G. Na^+/H^+ exchanger activity was shown to be the same for the wild-type and all of the histidine mutant exchangers. However, the H349G mutation appeared to decrease the sensitivity of the exchanger to inhibition by amiloride, 5-(*N*-ethyl)-*N*-isopropylamiloride and cimetidine, without affecting the affinity of NHE1 for Na^+ or Li^+. This suggests that this site may interact directly with NHE inhibitors or may possibly have an allosteric effect at a more distant inhibitor binding site. The H35G mutation alone was not examined. Demonstrating the result from the single mutation would have made the interpretation of the triple, His^{35}-containing, mutation more complete.

The exchanger activity of the H35,120,349G mutant remained sensitive to DEPC inhibition, demonstrating that other histidine residues are necessary for NHE1 function. However, these results do not exclude the possibility that other putative transmembrane histidine residues that were not mutated in this study (e.g. His^{256}, located within TMS7) participate in H^+ translocation. Recent work by Wakabayashi et al. has suggested that His^{325} may fold into the membrane and somehow contribute to a channel or pore-lining region. However, it should be noted that, when His^{325} was mutated to cysteine, it was possible to obtain mutant cells lines with this active Na^+/H^+ exchanger mutant [53]. Therefore His^{325}, along with His^{35}, His^{120} and His^{349}, are not essential for transport and are unlikely to be essential contributors to a H^+-relay system. In the case of His^{325}, the sensitivity of the mutant to various concentrations of H^+ was not examined.

2.4.2 Extra-/intra-cellular-loop histidine residues

If histidine residues are involved in a 'charge-relay' system for H^+ translocation, then not only transmembrane histidine residues but also histidine residues at, or in the vicinity of, the membrane/aqueous interface may also participate. In addition, histidine residues at the membrane/aqueous interface could be involved in modulating the sensitivity to H^+, similarly to what occurs with sod2 and NhaA. On the basis of the newly proposed model, these would include histidine residues at positions 13, 76, 81, 98, 250, 275, 285, 373, 376, 407, 408 and 473 (Figure 4C). Preliminary data suggest that His^{250} is not involved in exchanger function [52]. However, most of the other residues have not been extensively tested. In their study on the topology of NHE1, Wakabayashi et al. [53] mutated histidine residues at positions 76, 81, 250, 285, 373, 376, 407, 408 and 473 to cysteine. It was possible to obtain cells with active Na^+/H^+ exchanger for all these residues. It should be noted, however, that since the cells in these experiments were selected for by acid selection, there might be a tendency for only overexpressing cells to be selected for. The protein-to-activity ratio and pH-sensitivity were not measured, so there was no certainty that the protein was functioning normally. The H373C mutant did show a slight decrease in overall activity when treated with externally applied 2-trimethylammoniumethyl methanethiosulphonate, a thiol-group-reactive compound. This suggested that this residue could be important in activity. However, there was no analysis of the pH-sensitivity of the mutant or of the activity relative to the amount of protein expressed, so it is not known whether

this amino acid could play a role similar to His^{225} of NhaA and H367 of sod2. A determination of whether this, or any, of these histidine residues are important for the 'pH-sensing' function of the exchanger would help elucidate their importance. It should be noted that early experiments demonstrated the presence of an external DEPC-sensitive histidine residue. The effect of DEPC was influenced by Na^+ and H^+ binding, suggesting that an external histidine residue was important in cation binding or activity of the Na^+/H^+ exchanger [50]. It is quite possible that His^{363} is the external histidine residue involved. However, this must be confirmed by further experiments. As mentioned above, the H225C mutation of NhaA functioned relatively normally, possibly because histidine and cysteine are similar with respect to their polarity and ability to form hydrogen bonds.

2.4.3 C-terminal histidine residues

Examination of the amino acid sequence of the intracellular C-terminal region of NHE1 shows that 11 histidine residues are present in the cytosolic tail (Figure 4C). Of particular interest is an unusual sequence of histidine residues, ^{540}HYGHHH^{545} (in the one-letter amino acid code), found relatively close to the membrane domain [54]. This HYGHHH sequence is conserved in a variety of NHE1 isoforms, including human, rabbit, rat, mouse, South African clawed toad (*Xenopus*), salamander (*Salamandra*), carp (*Cyprinus*) and flounder (*Pleuronectes*). This high level of conservation suggests an important role of the HYGHHH sequence in the activity of the exchanger. We found [54] that this sequence could function as an endogenous histidine tag and could be used to partially purify the expressed cytosolic domain of NHE1 by immobilized-metal-affinity chromatography. The sequence bound to Ni^{2+} or Co^{2+}, although with somewhat lower affinity than the typical histidine tag sequences consisting of six continuous histidine residues. The function of this heavy-metal binding *in vivo* is still unknown. It should also be noted that Ca^{2+} binding, which has been demonstrated for other proteins containing histidine-rich motifs, did not occur. Owing to the presence of this unusual sequence, and because previous results have demonstrated the importance of histidine residues in exchanger activity, the effect of mutations of this histidine-rich sequence on activity of the intact Na^+/H^+ exchanger was examined to determine its possible role [54]. Mutation to either HYGAAA or HYGRRR did not affect exchanger activity as measured by the cells' ability to recover from an induced acid load. Mutation to HHHHHH resulted in a decreased maximal velocity of the exchanger, but did not affect its H^+ activation. These results suggest that although this conserved histidine-rich sequence can influence the maximal activity of NHE1, it does not appear to participate in the H^+-sensing capability of the exchanger. Rather than playing a significant role in the H^+-sensing or translocation capability of the exchanger, this histidine-rich region may participate in 'fine-tuning' exchanger function, by stabilizing the inter- or intramolecular interactions that are strengthened by the H-bonding that the histidine residues provide. Though many different approaches have begun to investigate the histidine residues of NHE1, clearly further study is required to identify fully the role of histidine residues in exchanger function.

3. OTHER AMINO ACIDS INVOLVED IN CATION BINDING AND TRANSPORT BY THE Na^+/H^+ EXCHANGERS

It is surprising that little is known about the specific amino acids involved in Na^+/H^+ exchange and their mechanism of operation, particularly for the mammalian Na^+/H^+ exchanger NHE1. For other membrane-transport proteins the side chains of polar amino acids have been suggested to be important in ion binding and transport. For example, the melibiose permease of *E. coli* catalyses the accumulation of this disaccharide by cation-coupled co-transport. In this cation carrier, several aspartate residues from different transmembrane-associated segments are thought to co-ordinate cation binding [55]. Mutagenesis of the aspartate residues to glutamate reduces transporter efficiency and the binding affinity for Na^+. Mutations removing the side-chain carboxy groups eliminate activity [55]. Other studies also demonstrated important roles for acidic residues within membrane proteins. Mutations of aspartate residues within TMSs of bacteriorhodopsin have shown that they are involved in H^+ translocation [56,57]. A number of other cation-transporting membrane proteins have important polar residues within the membrane. Mutations of polar residues (aspartate, serine, threonine and asparagine) of the mammalian Na^+/Ca^{2+} exchangers result in reduced exchanger activity [58]. A glutamate residue (Glu^{199}) was of importance in the Na^+/Ca^{2+} exchanger and an analogous residue is important in ion binding and translocation in the Ca^{2+}-ATPase of the sarcoplasmic reticulum [59]. In addition, a number of other polar amino acids in the TMSs of the Ca^{2+}-ATPase and Na^+,K^+- ATPase are believed to interact with their respective cations during transport [59–65].

3.1 NhaA

Studies on Na^+/H^+ exchangers are much more limited than those on other types of ion transporters. For NhaA a number of individual amino acid residues are involved in cation binding and translocation. Asp^{133}, Asp^{163} and Asp^{164} are essential for transport [49]. Their substitution to the amide derivative, asparagine, resulted in a complete loss of activity under all pH conditions examined, suggesting that they are involved in Na^+ recognition and binding. These residues are candidates for amino acids that could be involved in co-ordination of cations, as illustrated in Figure 2 (right-hand panel). The same conserved residues in NhaA of the marine bacterium *Vibrio alginolyticus* are also critical for activity [66]. However, with respect to NhaA of *E. coli*, there was no investigation as to whether glutamate residues could substitute for aspartate. A later study, however, showed that changing Asp^{133} to alanine resulted in only a partial loss of activity, suggesting that a negative charge at this position is not essential [67]. A number of other residues have been shown by random mutagenesis to result in loss of antiporter activity. G14R, G166R, F267S, L302P, G303R, C335R, S342P and S369P mutations all resulted in loss of activity [67]. Whether these effects were due to changes in stability, expression or activity of the protein was not clarified. Thus it is not known whether these residues are involved in cation binding or transport. A related study examined NhaA of *Vibrio parahaemolyticus* [68]. This Na^+/H^+ exchanger has an amiloride-binding-domain region (^{62}VFFL^{65}) that is also present in the mammalian Na^+/H^+ exchanger. Mutations in this region mainly caused minor changes in the K_m or V_{max} for Na^+ or Li^+. The F64Y mutation caused the greatest (10-fold) increase in the K_i for amiloride inhibition. It had no effect on the K_m for Na^+ and a 3-fold increase in the K_m for Li^+. These results suggest that the binding site for Na^+ is distinct from the amiloride-inhibition site, but may be closely related (see below). It is surprising that more detailed studies of the NhaA protein have not yet been undertaken in this area, especially considering the relative ease of expression of this protein in Na^+/H^+-exchanger-deficient *E. coli*. Future studies in this area should include the effects of substitution of different

polar amino acids on essential residues and their effects on Li^+- versus Na^+-mediated transport.

3.2 Yeast Na^+/H^+ exchangers

For the fission yeast (*S. pombe*) Na^+/H^+ exchanger sod2, we have demonstrated that specific, conserved acidic residues are important in cation binding and transport. Mutation of Asp^{145}, Asp^{241} and the aspartate pair $Asp^{266,267}$ eliminated proper function of this Na^+/H^+ exchanger ([47]; C. A. Wiebe and L. Fliegel, unpublished work). These acidic residues are similar in location to those of NhaA that were important in function, suggesting they may serve an analogous function in cation binding and co-ordination (Figure 2, right-hand panel) [11]. Further investigations on the role of these particular amino acids are underway, including whether glutamate can functionally substitute for aspartate. It is clear, however, that there is a great deal yet to be learned from this model of Na^+/H^+ exchange.

Several related species have proteins similar in structure and function to sod2 of *S. pombe*. The yeast *Saccharomyces cerevisiae* [69] has a Na^+/H^+ exchanger that is very similar to sod2 of *S. pombe* and to the Na^+/H^+ antiporter of *Zygosaccharomyces rouxii* [70]. However, these and other related proteins have not been analysed in detail, and there is little information on the role of particular amino acids in the activity of the protein and in cation binding and transport.

3.3 Mammalian Na^+/H^+ exchangers

For the mammalian Na^+/H^+ exchanger (NHE1 isoform) the analysis of critical amino acids involved in cation transport is much more limited. Some studies have examined the amino acids and TMSs involved in amiloride inhibition of the protein. The premise has, in some cases, been that the Na^+-binding site and the amiloride-binding site are the same. In one study, the sequence ^{164}VFFLFLLPPI^{173} of TMS4 of NHE1 was shown to be involved in amiloride-analogue binding [71]. Another study showed that a region between TMS8 and TMS10 might be involved in amiloride binding [72]. However, it is now thought that the amiloride-binding site may not be directly involved in Na^+ binding and transport [73] and that other regions may be important for Na^+ affinity [72]. For example, it has been shown that, in NHE1 mutants with altered affinity for amiloride analogues, Na^+/H^+ exchange function is essentially normal (reviewed in [74]). We recently suggested that Na^+ and amiloride molecules interact at unique regions of the Na^+/H^+ exchanger, the two binding sites being on related, but distinct, regions of the protein [74]. One amino acid, Glu^{262}, may be important in NHE1 function. The mutation E262I has been shown to inactivate the mammalian NHE1 isoform. However, the nature of the defect was not investigated, nor was the ability of other amino acids to substitute for glutamate [75]. It is surprising that the location of individual amino acids involved in cation binding and transport of the mammalian Na^+/H^+ exchanger remains largely undetermined.

4. CONCLUDING REMARKS

In summary, research supports a significant role for histidine residues in the activity of several Na^+/H^+ antiporters. Specific residues have been identified as being important in activity of the yeast Na^+/H^+ exchanger sod2, and in the Na^+/H^+ exchanger of *E. coli* NhaA. Although less is known about the mammalian Na^+/H^+ exchanger, indications are that, in this protein too, histidine residues play an important role in transport. Histidine residues are ideal candidates for being involved in H^+ sensing, since they are the only amino acids with an imidazole side chain that is titratable within the physiological pH range. It is clear that they play a vital role in H^+ transport and pH sensing in a variety of proteins, though further studies are necessary to characterize their role in the mammalian Na^+/H^+ exchanger. It is noteworthy that there have been no detailed studies on the other isoforms (NHE2–NHE6) of the mammalian proteins in this area.

Polar amino acids clearly play a role in cation binding and transport in NhaA and sod2. However, there is only an early, emerging picture demonstrating which of these amino acids are involved and how they function in these two Na^+/H^+ exchanger isoforms. Surprisingly, there has been little study of the amino acids critical for transport in the mammalian Na^+/H^+ exchanger. Studies on other related transport proteins have indicated that it is likely that side chains of polar amino acids are critical in transport activity. Future studies will certainly examine the role of these, and other amino acids, in cation binding and ion transport across the membrane, and in the pH-sensitivity of the Na^+/H^+ exchangers.

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