Minireview

Comparative molecular analysis of Na^+/H^+ exchangers: a unified model for Na^+/H^+ antiport?

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Received 19 January 1998

Abstract Despite 30 years of study on Na^+/H^+ exchange, the molecular mechanisms of antiport remain obscure. Most challenging, the identity of amino acids involved in binding transported cations is still unknown. We review data examining the identity of residues that are involved in cation binding and translocation of prokaryotic and eukaryotic Na^+/H^+ antiporters. Several polar residues specifically distributed within or immediately adjacent to membrane spanning regions are implicated as being important. These key amino acids are conserved in prokaryotes and in some lower eukaryotic forms of the Na^+/H^+ antiporter, despite their being dispersed throughout the protein and despite an overall low similarity in the linear sequence of these Na^+/H^+ antiporters. We suggest that this conservation of isolated residues (together with distances between them) reflects a general physicochemical mechanism of cation binding by exchangers. The binding could be based on coordination of the substrate cation by a crown ether-like cluster of polar atomic groups amino acids, as has been hypothesized by Boyer [1]. Traditional screening for the extended, highly conserved linear protein sequences might not be applicable when searching for functional domains of ion transporters. Three-dimensional constellations of polar residues (3D-motifs) may be evolutionary conserved rather than linear primary sequence.

Key words: Na^+/H^+ exchange; Cation binding; sod2

1. Introduction

Sodium-proton antiport, or Na^+/H^+ exchange, is a universal phenomenon in membrane energy conversion. In the most broad context, it is regarded as the process of coupling the H^+ cycle and the Na^+ cycle operating in energy-transducing membranes [2]. Three different types of Na^+/H^+ antiport can be distinguished: (1) simultaneous operation of a primary sodium pump and an artificial protonophore [3]; (2) simultaneous operation of a primary Na^+ pump and endogenous protonophore [4]; and (3) coupled antiport of Na^+ and H^+ by a specialized membrane protein, the Na^+/H^+ exchanger. In the present review, we focus on the latter type of sodium-proton antiport.

Na^+/H^+ exchangers are ubiquitous proteins present in virtually all cell types. Na^+/H^+ exchange fulfills different functions depending on the cell type. In higher eukaryotes the most common and important role is to regulate cytosolic pH. Mammalian cells maintain a physiological cytoplasmic pH of approximately 7.2. Shifts in intracellular pH initiate changes in the growth or functional state of the cell [5–7] and are often initiated by the Na^+/H^+ exchanger which normally functions to remove intracellular H^+ for extracellular Na^+ [8]. Na^+/H^+ exchange also protects cells from intracellular acidification, which is evident because mutant cell lines devoid of Na^+/H^+ exchanger are highly sensitive to acidosis [9,10]. In addition, Na^+/H^+ exchange participates in regulation of sodium fluxes and cell volume regulation after osmotic shrinkage [11]. Because it is vitally important for ionic homeostasis, Na^+/H^+ exchange is a subject of regulation by different mechanisms, including activation by growth factors [9].

The direction of Na^+/H^+ exchange is governed solely by the two ions' electrochemical gradients and requires no additional direct metabolic energy. All Na^+/H^+ exchangers also transport Li^+ in exchange for H^+. In bacteria, Na^+/H^+ antiporters normally function in the direction opposite to the mammalian antiporters and use a proton motive force to remove toxic Na^+ or Li^+ ions from the cytoplasm [12–15]. Prokaryotic Na^+/H^+ antiport also effectively increases the pH buffer capacity of the cytosol [16] and contributes to the buffering of the proton motive force [17]. In mammalian or fungal cells the stoichiometry of Na^+/H^+ exchange is 1:1 [18,19]. Bacterial exchange is electrogenic with an Na^+ to H^+ ratio of 1:2 [15,20]. In fission yeast excess levels of intracellular Na^+ are detrimental to these cells and the Na^+/H^+ antiporter (sod2) plays a major role in the regulation of internal sodium and pH. Disruption of the *sod2* gene results in an inability to extrude cytoplasmic Na^+ (or Li^+) and to take up external protons in exchange for internal sodium ions [19,21].

2. Residues important in cation binding

Na^+/H^+ antiporters are present in all known species from higher eukaryotes to *E. coli*. There are many different isoforms even within the same species. A series of detailed reviews dealing with different types of antiporters has recently been published [15,18,21–24]. Though the sequences of closely related mammalian isoforms are highly conserved, there is no significant overall homology between more distantly related Na^+/H^+ antiporters. This fact impedes the search for functionally important amino acids among the proteins from different sources. There are two alternatives: (1) no *universal* Na^+ and H^+ binding sites exist; and (2) very few conserved residues may be adequate to carry out Na^+/H^+ antiport even though these are dispersed throughout the protein [15]. The first possibility suggests that different sequences can accomplish the same function. Experimentally this means that a major part of determining which residues are essential for

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PII S0014-5793(98)00119-7

S. entiritidis	60 LLWIN**D**ALMA(2)	127 AIPAAT**D**IAFA(4)	156 LMALAII**DD**L (5) [27]
V. alginolyticus	52 LLWIN**D**GLMA(3)	119 AIPAAT**D**IAFA(4)	148 LLALAII**DD**L (5) [25]
E. coli (NhaA)	60 LLWIN**D**ALMA(3)	127 AIPAAT**D**IAFA(4)	156 LMALAII**DD**L (5) [28]
Z. rouxii	171 ESGCN**D**GLAF(6)	234 EGKRII**D**RESFA(7)	258 FGSMLGV**DD**L (8) [30]
S. cerevisiae	174 ESGCN**D**GMAF(6)	237 EKKNII**D**RESFL(7)	261 FGSILGV**DD**L (8) [31]
S. pombe (sod2)	173 ESGCN**D**GMAV(5)	235 QKYRLI**D**RESFL(7)	259 IGTIIGV**DD**L (8) [19]

Fig. 1. Alignment of prokaryotic and eukaryotic Na^+/H^+ antiporters. Bold residues indicate conserved amino acids that may be important in cation binding. Numbers preceding sequences indicate the number of the first amino acid. Numbers in brackets following the sequence indicate in which transmembrane sequence the fragment is found. Assignment of transmembrane segment was according to the indicated reference. For *S. pombe* the assignment was made using the program Topopredict II [54]. The assignment of transmembrane sequences for sod2 varies in other algorithms [19]. Reference numbers are at the end of each line.

transport is through trial and error. In contrast, the second possibility can be investigated experimentally by comparing distinct regions of similarity found among different antiporters. Results of such analyses are presented below.

In *E. coli* NhaA and NhaB make up two examples of the Na^+/H^+ antiporter family which do not have strong overall homology to each other [15]. The marine bacterium *Vibrio alginolyticus* has an Na^+/H^+ antiporter that is highly similar to NhaA [25] as does the halophilic marine bacterium *Vibrio parahaemolyticus* [26] and *Salmonella enteritidis* [27]. Studies on residues important in the function of bacterial Na^+/H^+ antiport have been undertaken in the laboratory of E. Padan (reviewed in [13–15]). Overall similarity of NhaA and NhaB to the human antiporter is low but small specific regions are highly conserved [15,19]. NhaB is inhibited by amiloride [15] and has a domain similar to the mammalian amiloride binding domain of mammalian. In NhaA three aspartate residues (D 133, 163, 164) within transmembrane segments 3 and 4 have also been shown to be essential for activity. They were suggested to play a critical role in cation binding and transport [28].

This pattern of a pair of acidic transmembrane residues preceded by an earlier transmembrane acidic residue is conserved in bacterial proteins and has also been shown to be important for Na^+/H^+ antiport in *E. coli* [28] and *Vibrio alginolyticus* [29]. A comparison of the sequences is shown in Fig. 1. Even more distantly related bacteria possess the same pattern as do the eukaryotic yeast species despite the fact that surrounding residues are quite dissimilar. In mammalian antiporters there is no corresponding DD sequence within or adjacent to a transmembrane segment (see below).

The yeast *Saccharomyces cerevisiae* [31] has an Na^+/H^+ exchanger that is very similar to sod2 of *S. pombe* [19] and to the Na^+/H^+ antiporter of *Zygosaccharomyces rouxii* [30]. A number of hydrophobic putative membrane spanning regions are conserved among the three yeast species. The identity in the membrane spanning region is over 35% between the antiporters of *S. cerevisiae* and *S. pombe* and *Z. rouxii*. Recently, we have shown that mutation of membrane-located Asp^{241}, and Asp pair 266,267 are critical for proper function of the *S. pombe* antiporter, sod2 (Dibrov et al., submitted for publication). These acidic residues are similar in location to those of bacterial Na^+/H^+ antiporters described above, with an acidic transmembrane associated residue preceding a pair of acidic transmembrane amino acids (Fig. 1).

It is interesting that in all these examples there is an upstream Asp residue either within or near the surface of an upstream transmembrane region. Though in NhaA one such residue (residue #65) was not essential for function [28] the similarity in location in these antiporters is intriguing.

An important aspect of any Na^+/H^+ antiporter is the ability to detect changes in cytosolic pH. In *E. coli* His^{225} has been shown to function as a pH sensor in NhaA [32,33]. Substitution of His^{225} with an acidic residue (Asp) shifts the pH optimum toward a more alkaline pH. Substitution of His^{225} with a basic residue (Arg) results in a more acidic pH optimum of activity of the protein. The $H^{225}A$ mutation inactivates the antiporter almost completely at all pHs tested [32,33]. In recent experiments with sod2 we have mutated all the histidine residues and showed that H^{367} functions in a similar fashion to H^{225} of NhaA (Dibrov et al., submitted for publication). It is interesting that in both bacterial and yeast proteins the 'pH-sensitive' His is apparently located at the membrane surface. It is also curious that in both cases the only functional His is the first His residue located downstream of the 'three-Asp cluster'. It is not clear yet whether such a location is coincidental or it reflects a common mechanism involved in regulating cation binding and transport.

The mammalian Na^+/H^+ antiporters (NHE1–NHE5) have a hydrophobic N-terminal region of approximately 500 amino acids, and a cytoplasmic hydrophilic C-terminal region [23]. While the cytoplasmic regions of mammalian isoforms are very variable, the membrane-spanning segments have between 55% to 95% amino acid identity. The protein is believed to have 12 transmembrane segments that are responsible for Na^+/H^+ transport activity. The most conserved membrane segments are transmembrane segments 6 and 7, which suggests they might be involved in cation selective transport [18]. Though there are several polar residues scattered throughout the membrane region of these antiporters, there is no conserved pair of Asp residues within the transmembrane region. This suggests that there may be some fundamental difference in cation binding and transport between prokaryotes and yeast, and the higher eukaryotes.

Several other regions of the mammalian proteins have been suggested to be candidates for involvement in cation transport. Comparing the yeast sod2 with the human NHE1, Jia et al. [19] found substantial similarity in some restricted regions. For example the sequence ^{38}VGEAVLGSITG^{48} in sod2 aligned with the mammalian antiporter (NHE1, residues ^{138}VGLLVGGLIKG^{148}) [19]. It was once thought that amiloride and its analogs inhibit Na^+/H^+ exchange by competing with Na^+ for the binding site. In one study the sequence ^{164}VFFLFVLLPPI^{173} of transmembrane segment 4 of NHE1 has been shown to be involved in amiloride analog binding [34]. Another study showed that a segment between putative transmembrane regions 8 to 10 were shown to be involved in amiloride binding [34]. However, it is now thought that the amiloride binding site may not directly be involved in Na^+ binding and transport [35] and that other regions may be

important for Na^+ affinity, possibly between transmembrane segments 6–7 and 10–12, [36]. It should be added also that bacterial NhaA [37] as well as yeast [38] antiporters are insensitive to amiloride and its analogs. A recent report [39] has suggested that Gly^{174} of transmembrane segment 4 may be important in transport of cations. Mutation of this residue to Ser resulted in a 2-fold decreased affinity for Na^+. However, it seems unlikely that this residue is involved in coordination of cations since its sidegroup is uncharged (see below). In addition a change to a serine residue would be expected to cause a much greater decrease in affinity for Na^+ if this amino acid were directly involved in cation coordination. Nevertheless, this result suggests that residues of transmembrane segment 4 may be involved in cation coordination or associated with other amino acids coordinating cations. It also seems clear that higher eukaryotes may differ from prokaryotes and lower eukaryotes in the coordination of cations. However, the possibility is open that a closely related or similar structure is involved.

3. Cation binding sites of ion transporters

P. Boyer was the first to postulate a unified mechanism for the translocation of Na^+, Li^+, and H_3O^+ by various membrane transporters [1]. According to Boyer's hypothesis, cations can be coordinated by electronegative atomic groups forming a crown ether-like structure. Modest conformational shifts in protein structure could be responsible for the translocation of the coordinated cation from one side of the membrane to another one [1]. Appropriately placed oxygens and/or possibly nitrogens could form such a coordination cluster [1].

Evidence presented in the above section has suggested that acidic residues within transmembrane regions may be important in activity of the Na^+/H^+ exchangers. A number of other membrane proteins which transport cations have polar residues within the membrane that are known to be important in function. The melibiose permease of *E. coli* catalyzes accumulation of this disaccharide by cation-coupled cotransport. In this carrier several Asp residues from different transmembrane segments are thought to act as a cation binding pocket, possibly in a structure similar to that suggested by Boyer [40]. Mutagenesis of the Asp residues to Glu reduced transporter efficiency and the binding affinity for Na^+. Mutations removing the carboxyl eliminated activity (reviewed in Ref. [40]).

Mutations in several polar residues of the mammalian Na^+-Ca^{2+} exchangers result in reduced exchanger activity. The important amino acid residues included Asp, Ser, Thr and Asn. It was suggested that the ion conduction pathway is lined with acidic and hydroxyl containing residues, mostly in the conserved regions of the proteins [41]. 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 of the Ca^{2+}-ATPase of the sarcoplasmic reticulum [42]. In addition a number of other polar amino acids in the transmembrane segments of the Ca^{2+}-ATPase are believed to interact with the Ca^{2+} ions during transport [43].

A similar situation occurs with Na^+-K^+ ATPase. A number of conserved acidic residues are essential for Na^+ and K^+ binding and transport [44–48]. Several of these residues are conserved and are important in cation translocation by the Ca^{2+}-ATPase [49]. Other proteins also have acidic and other polar residues important in cation translocation. For example Asp^{285} is thought to be important in binding of a cationic substrate to the metal-tetracycline/H^+ antiporter of *E. coli* [49]. A conserved Asp residue is also important in proton translocation in the F_1F_0-ATPase from *E. coli* [50] and a conserved Asp residue is important in proton translocation in the B subunit of *E. coli* nicotinamide nucleotide transhydrogenase [51].

To summarize, clearly a number of conserved polar residues are important in cation translocation in a wide variety of integral membrane proteins. This appears to be true for eukaryotic and prokaryotic proteins. Polar amino acids are commonly involved, in particular those with carboxyl groups. Often they are not only conserved within a protein type but across proteins with different functions. It is important to note that often key residues are conserved in particular transmembrane locations, even though overall linear similarity between the proteins may be low. The key residues are not necessarily in the same transmembrane segments of the proteins (see Fig. 1 and above). In other words, it looks that certain three-dimensional clusters of coordinating atomic groups (3D-motifs) may be conserved in ion translocating proteins rather than specific primary sequences. If so, the search for functionally important domains of ion transporters cannot be based on traditional sequence alignments. It should rather be focused on isolated, conserved polar residues which are characteristically distributed among transmembrane segments.

4. Problems and prospects

The hypothesis outlined above suggests that conserved, negative intramembrane amino acids are important in ion exchange providing oxygen atoms for coordination of the transported cation. To test this hypothesis further, it is necessary to check a number of polar amino acid residues within the transmembrane segments that may be involved in cation binding and translocation. We have already mutated $D^{241}N$ as well as $D^{266,267}N$ in the yeast protein, sod2. In both cases, mutants were unable to grow in the presence of LiCl; both Na-dependent proton translocation and Na^+ efflux were impaired in those cells (Dibrov et al., submitted for publication). These observations are of considerable interest because they suggest functional analogy between bacterial and at least some eukaryotic antiporters. The same type of mutation (change of Asp to Asn to remove the carboxyl group) of analogous residues arrested Na-dependent H^+ transport in *E. coli* [28] and *V. alginolyticus* [29]. Other potential targets for mutation in sod2 are D^{178} and E^{173} because of their similarity with the sequence of the other antiporters (see Fig. 1). The mammalian antiporters will require a great deal of study and mutation of a number of residues in order to identify amino acids involved in cation binding and transport. Because of the nature of mutations which abolish activity it is impossible to screen transfectants using normal procedures which are based on activity of the protein.

Once the amino acids important in cation binding have been elucidated, the next goal is to modify the residues further to understand details of the binding/translocation process. For example, besides the $D \rightarrow N$ mutations it would be interesting to do $D \rightarrow E$ mutations of functional Asps. The larger size of the E residue may shift the coordination of a cation so that a smaller cation is favored. This could result in an in-

crease in the relative affinity for Li^+ as compared to Na^+ (the ionic radii of Na^+ and Li^+ are 0.95 and 0.65 Å, respectively [40]). Modification of the affinity of cation binding would be an ideal way to show that given residues are indeed involved in binding/transport. Such an approach allows exclusion of the possibility that a given mutation simply disrupted the gross overall structure of the protein in the membrane. A similar approach has been used earlier for modifying the Na^+ affinity of the melibiose permease (see [40]) and of the F_1F_0-ATPase of *E. coli* [52]. A related type of analysis is to shift the position of important amino acids within the membrane by insertion of an additional amino acid. This may disrupt cation binding by shifting the position of critical amino acids away from the cation binding site.

Another perspective line of research is evaluation of the membrane topology of Na^+/H^+ antiporters. At the present moment, NhaA from *E. coli* is a rare example of Na^+/H^+ antiporter whose topology has been systematically investigated by a series of gene fusions [53]. However, in nearly all other cases hypothetical predictions of topology are based only on hydrophobicity plots and have not been proven experimentally. Hence, there has been no real evidence to show the genuine topology of any eukaryotic Na^+/H^+ antiporter. In addition the predicted topology varies depending on the algorithm used. To analyze systematically the amino acids involved in cation transport it is necessary to have topological data concerning the topography of the protein in the membrane. For example, one could try to determine possible 'active' residues in NhaB, which has no 'three-Asp motif' preserved. This task would be much easier if topological characteristics of NhaB were available. The topological information is also of great importance for engineering of mutant proteins with altered cation specificity. Future studies may address the topology of Na^+/H^+ antiporters and specific amino acids involved in cation binding and transport of mammalian Na^+/H^+ exchangers.

Acknowledgements: Related research in the laboratory of L.F. was supported by a Collaborative Research Grant from the Natural Sciences and Engineering Research Council of Canada and by MRC Program Grant #PG-11440.

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