Functional Analysis of Polar Residues Important for Activity of Na^+/H^+ Exchangers

LARRY FLIEGEL, CHRISTINE WIEBE, RAKHILYA MURTAZINA,
PAVEL DIBROV, BRENDA J. BOOTH, BONNIE L. BULLIS,
EMILY DIBATTISTA, AND DYAL N. SINGH

Department of Biochemistry, Faculty of Medicine, CIHR Membrane Protein Group, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

KEYWORDS: cation coordination; cation transport; Na^+/H^+ exchanger; membrane proteins; pH regulation

INTRODUCTION

Na^+/H^+ exchangers are a family of integral membrane proteins that exchange protons for Na^+. They exist in yeast, *Escherichia coli,* and vertebrates. In mammals, seven isoforms of the Na^+/H^+ exchanger are known. The first isoform to be discovered, the NHE1 isoform, is ubiquitous and transports one intracellular proton out of the cell in exchange for one extracellular Na^+. Another isoform of the Na^+/H^+ exchanger is the yeast protein *Sod2* that catalyzes the reverse process in *Schizosaccharomyces pombe,* the removal of an intracellular Na^+ in exchange for an extracellular proton.[1]

Little is known about the amino acids involved in cation coordination and transport by the eukaryotic Na^+/H^+ exchangers. Examination of the amino acid sequence of the Na^+/H^+ exchangers revealed conserved polar amino acids in some transmembrane segments. Polar amino acids of membrane proteins have been involved in the cation binding and transport of a number of ion pumps and transporters. We therefore examined the effect of mutation of several amino acids of conserved transmembrane segments of the exchanger. Our specific hypothesis was that specific polar amino acids that are within or are associated with specific transmembrane segments of the membrane domains are responsible for cation binding and translocation of Na^+ and H^+. To test this hypothesis we used site-specific mutagenesis to examine the role of specific amino acids that could be involved in the transport of cations. The human Na^+/H^+ exchanger NHE1 isoform was mutated and stably transfected into AP-1 cells, as described earlier.[2,3] Na^+/H^+ exchanger activity including V_{max} and K_m was analyzed after ammonium chloride-induced prepulse, as we described earlier.[4] For the yeast Na^+/H^+ exchanger sod2, mutagenesis and activity measurements were as described earlier.[5]

Address for correspondence: Dr. Larry Fliegel, Department of Biochemistry, Faculty of Medicine, CIHR Membrane Protein Group, University of Alberta, 347 Medical Science Building, Edmonton, Alberta, Canada, T6G 2H7.
lfliegel@gpu.srv.ualberta.ca

Ann. N.Y. Acad. Sci. 976: 117–120 (2002). © 2002 New York Academy of Sciences.

In the case of the NHE1 isoform of the human Na^+/H^+ exchanger we added a hemagglutinin tag to the C-terminus of the protein,[3] so that we could easily detect the amount of protein in Western blots and check the localization by immunocytochemistry. The mutations made were D238N, P239A, E262Q, E262D, S263A, N266A, D267N, D267E, S359A, SS387, 388AA, S390A, E391Q, E391D, T392V, S401A, T402V, and S406A. All of the mutant proteins expressed at similar levels, and all were targeted to the plasma membrane.

When we examined the activity of the control and mutant proteins, only mutation of Glu262, Asp267, and E391 affected Na^+/H^+ exchanger activity. The E262Q and D267N mutants showed no activity equivalent to the mock transfected AP-1 cells. The E391Q mutant showed greatly reduced activity compared to that of the controls. The conservative substitutions of E262D, D267E, and E391D all restored activity, although the E262D mutant had slightly depressed activity relative to the wild type. We found that the E262D mutant also had an increase in the K_m for Li^+ (but not for Na^+). This result suggested that a change had occurred in the relative affinity for the two cations, possibly indicating an alteration in the coordination site of the protein.

Other conserved amino acids were tested for their contribution to Na^+/H^+ exchanger activity. Ser359 of transmembrane segment IX was mutated to Ala; however, this had no effect on activity. Similarly, mutation of Pro239 and Asn266 did not affect activity. It is surprising that mutation of Pro239 did not affect activity, because prolines usually function as helix breakers within the membrane,[6] and we hypothesized that such a large change in conformation could disrupt the protein. However, this residue proved to be unimportant to the function of the protein. Asp238 together with Pro239 comprise a well-conserved motif in almost all the mammalian Na^+/H^+ exchangers and in the yeast Na^+/H^+ exchanger sod2. Thus, it was surprising that this residue was not important to the function of the mammalian Na^+/H^+ exchanger.

To determine if polar amino acids are important in different Na^+/H^+ exchangers similar experiments were done with the yeast Na^+/H^+ exchanger sod2. All eight His residues were mutated to Arg, and the conserved amino acids Asp145, Asp241, and Asp266,267 were mutated to Asn residues. We characterized the ability of the sod2 protein to allow growth of *S. pombe* in LiCl-containing medium. In addition, we examined the ability to expel sodium in acid medium and the ability to carry out sodium-dependent proton influx at pH 6.1, as described earlier.[5] Of the His residues, only His367 was essential for activity. In addition, mutation of amino acids Asp145, Asp241, and Asp266,267 all impaired, at least partially, sod2 activity.

FIGURE 1 illustrates the relative location of the mutated amino acids in human NHE1 and sod2. The topology of NHE1 is based on recent analysis by cysteine scanning mutagenesis.[7] That of sod2 is theoretical and is based on hydrophobicity analysis.[1] Whereas a complete comparison still awaits further analysis of the protein structure, it is of interest that in both cases amino acid D267 is important in function. In addition, surrounding amino acids of this region are also significant in function. It was of particular interest that conservation of the side chains in NHE1 conserved activity. We hypothesized earlier that Na^+/H^+ exchangers may act by coordination of substrate cations through a crown ether-like cluster of polar amino acids.[8] Our current results support the hypothesis that the oxygen in the side chains of E262, D267, and E391 could serve this role for the mammalian Na^+/H^+ exchanger. Although it is impossible at this stage to determine if the amino acids are influencing transport through effects on structure, experiments using limited trypsinolysis (not shown)

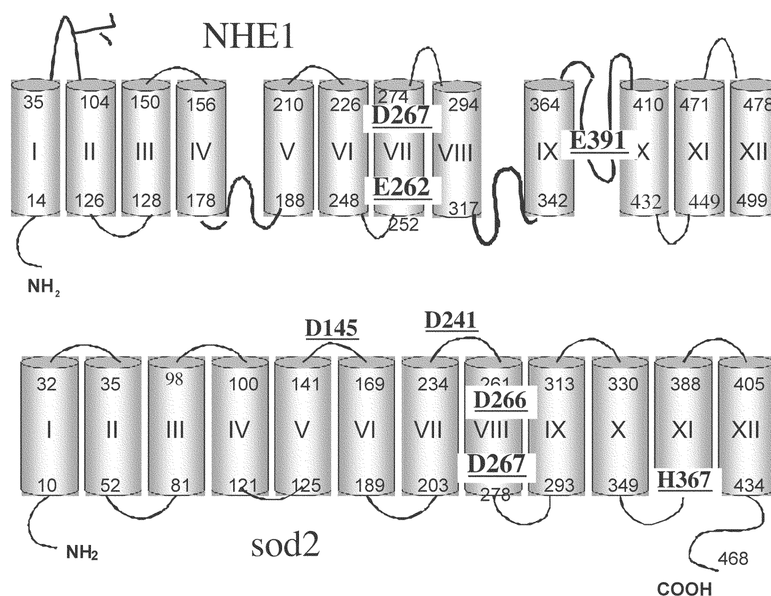

FIGURE 1. Topological models of the transmembrane region of the NHE1 isoform of the human Na^+/H^+ exchanger and the yeast Na^+/H^+ exchanger sod2. Residues that are important in activity are underlined.

suggest that this may not be the case. Future experiments are necessary to determine the exact conformation of these parts of the protein and their role in cation binding and transport.

SUMMARY

Na^+/H^+ exchangers are a family of ubiquitous membrane proteins. In mammals the NHE1 isoform of the protein is widely distributed through all tissues and regulates cytosolic pH by removing an intracellular H^+ in exchange for an extracellular Na^+. In fission yeast, excess levels of intracellular Na^+ are detrimental to these cells, and the Na^+/H^+ exchanger, sod2, plays a major role in the regulation of internal sodium concentration. We examined the functional role of conserved, polar, amino acid residues occurring in membrane-associated segments of the Na^+/H^+ exchanger proteins. For the mammalian Na^+/H^+ exchanger, mutant proteins of transmembrane segments VI and VII and the membrane-associated segment from amino acids 387 to 406 were assessed by characterization of intracellular pH changes in stably transfected cells that lacked an endogenous Na^+/H^+ exchanger. All of the mutant proteins were expressed and were targeted properly to the plasma membrane. Mutation of amino acid residues Glu262, Asp267, and E391 affected the Na^+/H^+ exchanger. Conservative substitutions with alternative acidic residues restored Na^+/H^+ exchanger activity. The Glu262Asp mutant had a decreased affinity for Li^+. For the yeast

Na^+/H^+ exchanger, similar experiments examined the effect of mutagenesis of several conserved polar amino acids. Of all Histidine residues, only His 367 was significant for activity. The Asp266,267 pair were mutated simultaneously, and sod2 function was found to be significantly impaired. Results also indicated that residues Asp145 and Asp241 are important for proper function of sod2. The results support the hypothesis that side chain oxygen atoms in a few, critically placed amino acids are important in various kinds of Na^+/H^+ exchanger activity.

REFERENCES

1. WIEBE, C.A., E.R. DIBATTISTA & L. FLIEGEL. 2001. Functional role of amino acid residues in Na^+/H^+ exchangers. Biochem. J. **357:** 1–10.
2. MURTAZINA, B.R., B.J. BOOTH, B.L. BULLIS, *et al.* 2001. Functional analysis of polar amino acid residues in membrane associated regions of the NHE1 isoform of the Na^+/H^+exchanger. Eur. J. Biochem. **268:** 1–13.
3. WANG, H., D. SINGH & L. FLIEGEL. 1998. Functional role of cysteine residues in the Na^+/H^+exchanger. Arch. Biochem. Biophys. **358:** 116–124.
4. SILVA, N.L.C.L., H. WANG, C.V. HARRIS, *et al.* 1997. Characterization of the Na^+/H^+ exchanger in human choriocarcinoma (BeWo) cells. Eur. J. Physiol. **433:** 792–802.
5. DIBROV, P., P.G. YOUNG & L. FLIEGEL. 1998. Functional analysis of amino acid residues essential for activity in the Na^+/H^+ exchanger of fission yeast. Biochemistry **36:** 8282–8288.
6. VON HEIJNE, G. 1991. Proline kinks in transmembrane alpha-helices. J. Mol. Biol. **218:** 499–503.
7. WAKABAYASHI, S., T. PANG, X. SU & M. SHIGEKAWA. 2000. A novel topology model of the human Na^+/H^+ exchanger isoform 1. J. Biol. Chem. **275:** 7942–7949.
8. DIBROV, P. & L. FLIEGEL. 1998. Comparative molecular analysis of Na^+/H^+ exchangers: a unified model for Na^+/H^+ antiport? FEBS Lett. **424:** 1–5.