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Functional analysis of amino acids of the Na^+/H^+ exchanger that are important for proton translocation

Christine A. Wiebe,[1] Carmen Rieder,[1] Paul G. Young,[2] Pavel Dibrov[3] and Larry Fliegel[1]

[1]*Department of Biochemistry, University of Alberta, Edmonton, Alberta;* [2]*Department of Biology, Queen's University, Kingston, Ontario;* [3]*Department of Microbiology, University of Manitoba, Winnipeg, Manitoba, Canada*

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Abstract

The Na^+/H^+ exchanger is an integral membrane protein found in the plasma membrane of eukaryotic and prokaryotic cells. In eukaryotes it functions to exchange one proton for a sodium ion. In mammals it removes intracellular protons while in plants and fungal cells the plasma membrane form removes intracellular sodium in exchange for extracellular protons. In this study we used the Na^+/H^+ exchanger of *Schizosaccharomyces pombe* (Sod2) as a model system to study amino acids critical for activity of the protein. Twelve mutant forms of the Na^+/H^+ exchanger were examined for their ability to translocate protons as assessed by a Cytosensor microphysiometer. Mutation of the amino acid Histidine 367 resulted in defective proton translocation. The acidic residues Asp145, Asp178, Asp266 and Asp267 were important in the proton translocation activity of the Na^+/H^+ exchanger. Mutation of amino acids His98, His233 and Asp241 did not significantly impair proton translocation by the Na^+/H^+ exchanger. These results confirm that polar amino acids are important in proton flux activity of Na^+/H^+ exchangers. (Mol Cell Biochem **254**: 117–124, 2003)

Key words: Na^+/H^+ exchanger, *Schizosaccharomyces pombe*, cation binding, proton translocation

Introduction

The Na^+/H^+ exchanger is an integral membrane protein that is responsible for the exchange of protons for Na^+ ions. It is found in almost all organisms occurring in mammals, bacteria, plants and fungal cells [1, 2]. In mammals there are numerous isoforms of the Na^+/H^+ exchanger at least eight of which have been identified and characterized to varying degrees. The NHE1 isoform is ubiquitous, plasma membrane bound, and catalyzes the removal of an intracellular proton in exchange for an extracellular sodium ion [1, 2]. Other mammalian Na^+/H^+ exchangers exist with different tissue locations and with varying intracellular localizations [1].

Fungal and plant Na^+/H^+ exchangers serve as model systems for the mammalian Na^+/H^+ exchangers. In plants, various plasma membrane Na^+/H^+ exchangers remove sodium in exchange for protons. The energy of sodium transport comes from the proton gradient generated by the plasma membrane H^+-ATPase. Sodium in the cytosol is also removed by vacuolar Na^+/H^+ exchangers that use the energy of the proton gradient generated by vacuolar H^+-ATPase and H^+-PPiase (reviewed in [3]). Salt tolerance in plants has very significant agricultural implications and improving salt tolerance in plants is one proposed method of increasing agricultural production [4].

In yeast, salt tolerance is mediated in a similar fashion to that of plants. In *Saccharomyces cerevisiae* the Na^+/H^+ exchanger Nhx1 [5] localizes to a late endosomal/prevacuolar compartment where it mediates sequestration of sodium coupled to the proton gradient established by the vacuolar H^+-

Address for offprints: L. Fliegel, Department of Biochemistry, 347 Medical Science Building, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7 (E-mail: lfliegel@gpu.srv.ualberta.ca)

ATPase. A Na^+-ATPase exists in the plasma membrane that removes Na^+ ions. In addition Nha1 in *S. cerevisiae* is a plasma membrane protein highly similar to Na^+/H^+ exchangers from *Schizosaccharomyces pombe* (Sod2). This Nha1-like protein mediates pH-dependent tolerance to sodium and lithium [6] and sodium efflux at the plasma membrane [7, 8]. Its disruption leads to an increased sensitivity towards sodium ions.

In the fission yeast *Schizosaccharomyces pombe* the Na^+/H^+ exchanger (Sod2) plays a major role in salt tolerance [9] and is an attractive model for the study of Na^+/H^+ exchangers. Unlike *S. cerevisiae,* fission yeast do not posses a Na^+-ATPase in the plasma membrane making the study of Na^+/H^+ exchange activity less complicated. The Na^+/H^+ exchanger Sod2 catalyzes the electroneutral removal of sodium in exchange for a proton. Ion exchange is electroneutral and bidirectional depending upon the ion concentration gradients [9, 10]. Sod2 has polar amino acids within membrane spanning regions that are conserved between other yeast Na^+/H^+ exchangers and mammalian Na^+/H^+ exchangers. This makes it an attractive model to study the mechanisms of ion transport by eukaryotic Na^+/H^+ exchangers. We have previously shown [10] that of the 8 histidines present in Sod2, only His367 was essential for Sod2 activity as measured by the ability to restore growth in the presence of salt to Sod2 deficient yeast. Similarly, *S. pombe* containing Sod2 with Asp241 $\rightarrow$ Asn and Asp266,267 $\rightarrow$ Asn mutations had impaired growth in LiCl containing medium. In our previous study we did not determine which of the Asp266 and Asp267 pair of amino acids was significant for activity nor did we examine other membrane associated amino acids that could be significant in ion transport.

In the present study we further characterize amino acids of the Na^+/H^+ exchanger of *S. pombe* and examine novel amino acids that are important in activity of the protein. We more directly measure the activity of the protein by measuring proton fluxes, as opposed to indirectly measuring activity by examining growth of yeast. We further demonstrate the efficacy of measuring proton fluxes by use of the Cytosensor microphysiometer [11]. Our results demonstrate that several polar, membrane-associated amino acids are important in the proton flux activity of the Na^+/H^+ exchanger. This finding further supports the hypothesis that the negatively charged side chains of membrane-associated amino acids are important in the binding and coordination of cations by Na^+/H^+ exchangers.

Materials and methods

Strains, media and growth conditions

S. pombe sod2::ura4 [9] that is deficient in endogenous Na^+/H^+ exchanger (Sod2), was used in this study for complementation and phenotype characterization. *S. pombe* were maintained on yeast extract adenine (YEA) or low sodium Edinburg Minimal medium adenine (EMMA), with appropriate supplements when necessary, using standard methods [12]. Low sodium minimal medium was made by replacing Na_2HPO_4 with K_2HPO_4 at the same concentration, and omitting the Na_2SO_4 from the 50X salts stock. The pH was adjusted to 5.5 with KOH. Cells were incubated at 30°C, and at 200 rpm for liquid cultures. Transformation of *S. pombe sod2::ura4* was performed using the lithium acetate method [12] or by electroporation [13].

Bacterial manipulation

E. coli DH5α were used for routine transformation and propagation of plasmid DNA. *E. coli* cells were cultured in LB media with appropriate antibiotic selection, at 37°C.

Recombinant DNA techniques

DNA manipulation was by standard protocols or, where appropriate according to manufacturer's instructions. DNA sequencing was performed by University of Alberta Department of Biochemistry Core Facility using a Beckman Coulter CEQ™ 2000XL DNA Analysis System.

Site-specific mutagenesis

Mutagenesis of amino acid residues His98, His233, His367, Asp145, Glu173, Asp178, Asp266, and Asp267 of Sod2 was performed using the Transformer Site-Directed Mutagenesis Kit (ClonTech, Version 2) as recommended by the manufacturer. The mutagenic primers that were used to create the desired gene product are summarized in Table 1. All mutations were designed to create a new restriction enzyme site that could easily be detected in subsequent analysis. For all mutagenesis reactions, the trans oligonucleotide ScaI/StuI (Clonetech) was used as the selection primer. The mutagenesis template was pSK-sod2, a pBluescript vector containing the Sod2 coding sequence along with the 187 nt upstream and 692 nt downstream flanking regions of the gene, as a 2.3 kb HindIII insert [9]. Following the polymerase and ligation reactions and subsequent selective digestion of the original template DNA by ScaI, the mutated DNA was transformed into a *mutS E. coli*, BMH 71-18. Plasmid DNA was isolated and subjected to a second selective digestion with ScaI, and transformed into *E. coli* DH5α. Restriction mapping using enzymes whose sites had been introduced by the mutagenic primers was performed to screen transformants for positive clones. Positive clones from the site-specific mutagenesis were confirmed by DNA sequencing.

Table 1. Oligonucleotides used for site-directed mutagenesis

Mutation	Oligonucleotide sequence	Restriction site
H98R	5′-GCATATTTTCA**g**CG**c**AATTTTCGAAGCATCATTG-3′	HhaI
H233Rr	5′-TTTCTGAGCGcGCTTTAAAATGAACG-3′	HhaI
H367Ar	5′-CCTATTGGTCCGAAAgcTCCAACGAAAAGGGC-3′	AluI
H367Rr	5′-TATTGGTCCGAAAcGgCCAACGAAAAGGGC-3′	HaeIII
H367Dr	5′-CCCTATTGGgCCGAAATcTCCAACGAAAAGGGC-3′	HaeIII
D145Nr	5′-TGATCGCAGGATGTATAACgTCgACTaATCCTGTTCTATCAGCATTG-3′	SalI
E173Qr	5′-CGGTCTTTATTGATCGCTcAGTCcGGATGTAATGATGGAATGGC-3′	BspEI
D178Nr	5′-TGATCGCTGAGTCcGGATGTAATaATGGAATGGCGGTTCCTTTT-3′	BspEI
D241Nr	5′-GCGGAAGGGAATAATAgCTAATAGCATtAATTAAACGGTAT-3′	AluI
D266,267Nr	5′-GGAACTATTATTGGAGTTaAcaACCTGTTGATGTCCTTTTTTGC-3′	HincII
D266Nr	5′-GGAACTATTATTGGAGTTaAcGACCTGTTGATGTCCTTTTTTGC-3′	Hinc II
D267Nr	5′-GGAACTATTATTGGAGTcGA**ca**ACCTGTTGATGTCCTTTTTTGC-3′	SalI/HincII/AccI

Oligonucleotides that are suffixed with 'r' encode for the complementary DNA strand. Mutated amino acid residues are indicated using single letter notation. Mutated nucleotides are in lower case letters and boldfaced. New restriction sites are underlined.

Subcloning and plasmid construction

To avoid the possibility of introducing unwanted mutations produced by the mutagenesis procedure we excised a minimal fragment of Sod2 containing the sequenced and desired mutation and replaced it into a plasmid that had not undergone mutagenesis. Plasmid DNA from the mutagenized plasmid containing each mutation was digested with the following enzymes. NcoI/NheI were used to excise a 549 bp fragment containing the H98R, H233R, D145N, E173Q, and D178N mutations. NheI/BspEI were used to excise a 312 bp fragment containing the D241N, D266N, D266E, D267N, D267E, D266,267N, and D266,267E mutations. BsgI/BspEI digestion excised a 430 bp fragment containing the H367R and H367D mutations. These fragments were then used to replace the corresponding sequences in pSK-sod2 that had not been subjected to site-specific mutagenesis reactions. In parallel, the small internal fragments containing the specific mutations have been subcloned into pNICOL, replacing the corresponding wild type, non-mutated sequence. pNICOL is a pBluescript vector containing an HA tagged Sod2 sequence as a 2.4 kb HindIII insert. The construction of pNICOL is described previously [10]. Lastly, the various Sod2 mutants (± HA tag) were subcloned as 2.3/2.4 kb HindIII fragments into the *S. pombe* expression vector, pWH5 [9].

Measurement of proton uptake

For some experiments Na^+ dependent H^+ uptake of various *S. pombe* strains was measured using a pH meter as previously described [10] with minor modifications. Briefly, 250 ml cell cultures were grown overnight to mid-log phase (approximately 1×10^7 cells/ml). Cells were harvested and washed twice with Milli-Q H_2O, and twice with 50 mM MOPS pH 7.0. Cells were resuspended in 50 mM MOPS pH 7.0 to an OD_{600} = 14. Five hundred µl of cells were aliquoted to a new Eppendorf tube. Cells were incubated at room temperature for 2 h ± 200 mM NaCl on a rotary lab shaker. After sodium loading, cells were washed once with 1 mM citrate pH 6.6 and resuspended in 500 µl of 1 mM citrate pH 6.6 and added to 4.5 ml cuvette containing 1.5 ml, 1 mM citrate pH 6.6. The cell suspension was continually stirred and the pH was measured using a Fisher Scientific Accumet 925 pH meter interfaced with a Macintosh computer for data collection at 15 sec intervals.

Microphysiometry analysis of Sod2 activity

Sod2 activity was measured by assaying proton fluxes on a Cytosensor microphysiometer (Molecular Devices) using a method adapted from that described earlier [11]. For each sample, 4×10^6 cells were obtained from an exponentially growing culture in EMMA media. The cells were washed twice with water and once with 10 mM HEPES buffer (pH 6.3) to remove all media, and then resuspended in 5 µl, 10 mM HEPES buffer (pH 6.3). To immobilize the cells, 5 µl of warm 1.4% low melting point agarose was added to the cell suspension. The mixture was placed in the sensor chamber capsules and allowed to set before the capsules were loaded into the microphysiometer. The sensor chambers were maintained at 30°C throughout the experiment.

Cells were initially perfused at a rate of 100 µl/min with a 300 mM tetramethylammonium (TMA) chloride solution (pH 6.3) supplemented with 10 mM HEPES, 0.1% BSA and 500 mM diethylstilbestrol, a H^+-ATPase inhibitor [14]. To measure extracellular acidification rates (EARs), fluid flow to cells was stopped for the last 40 sec of a 2 min pump cycle and the rates were quantified between 88–118 sec of the 2 min pump cycle. After 10' in the TMA-containing solution, a constant baseline extracellular acidification rate was estab-

lished and the 300 mM TMA in the perfusing solution was replaced with 300 mM NaCl to stimulate Sod2 activity. There was no extracellular acidification in the absence of immobilized cells. In the presence of immobilized yeast containing Sod2, the wild type Sod2 protein exhibited a maximum extracellular acidification rate after exposing cells to NaCl for 240 sec (EAR240). Consequently, the extracellular acidification rate of each mutant Sod2 protein was compared to the wild type protein at this time point. Some mutants (E173Q) were prepared with a hemagglutinin tag on the C-terminus and these were compared to the wild type Na^+/H^+ exchanger with the same tag. The EARs were normalized by subtracting the average baseline acidification rate in TMA (BEAR), and the EAR for each mutant Sod2 protein was expressed as a percentage of wild type Sod2. These calculations are described by the following equation:

$$\text{Relative extracellular acidification rate (\%)} = \frac{(\text{EAR240mutant} - \text{BEARmutant})}{(\text{EAR240wildtype} - \text{BEARwild type})} \times 100$$

Values indicated are mean relative acidification rates ± S.E.M. of at least 15 experiments. Data was statistically evaluated using the Mann Whitney U test and results with $p < 0.05$ were considered significant.

Results

To study the amino acids important in Sod2 function we mutated amino acid residues of putative transmembrane segments of the protein. The topology of Sod2 is not known with certainty but has been suggested from hydrophobicity analysis. Figure 1 illustrates a putative model of the protein. It is proposed to have 12 transmembrane segments with 5 intracellular loops connecting the integral membrane segments. We mutated several amino acids within two putative intracellular transmembrane loops. In addition, we examined the effect of mutation of some novel, conserved amino acids of putative integral membrane segments. Sod2 normally functions to remove internal sodium ions in exchange for extracellular protons. In so doing it can alkalinize the external medium while in reverse mode it can acidify the external medium. Initially we used a crude procedure to examine the ability of yeast to alkalinize the external medium while removing an internal load of sodium. Yeast were Na^+-loaded by incubation with high concentrations of sodium for two hours. Then they were washed and suspended in weakly buffered Na^+-free solution and their ability to alkalinize the external medium was recorded. The results are shown in Fig. 2. Control yeast were transformed with wild type Na^+/H^+ exchanger. Sodium loaded cells were removed to Na^+-free medium, the yeast then removed protons from the extracellular medium causing a rise in external pH. When the yeast were not Na^+-loaded, the extracellular solution was not significantly alkalinized. As expected, the *sod2::ura4* disruption strain did not alkalinize the extracellular medium subsequent to Na^+-loading.

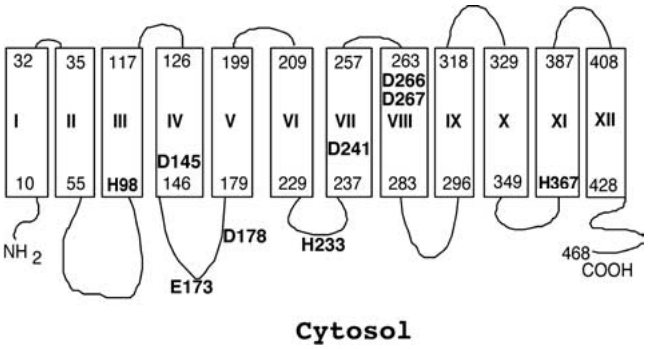

Fig. 1. Schematic model of Sod2 based on hydrophobicity analysis [23] as described earlier [1]. The approximate position of amino acid residues that were mutated and measured in this study is indicated.

While it was possible to obtain a rough idea of activity of the Na^+/H^+ exchanger using this procedure, the results were somewhat variable and not very quantitative. It was not possible to measure rates of activity and the technique required quantification of changes in pH of as little as 0.02 pH units with a pH meter and electrode. To characterize Na^+/H^+ ex-

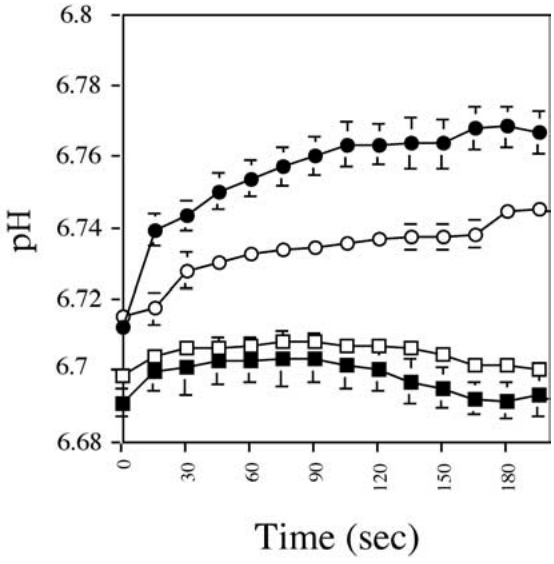

Fig. 2. Na^+ dependent H^+ fluxes mediated by *S. pombe* containing the Na^+/H^+ exchanger. Cells carried either the wild-type Na^+/H^+ exchanger (circles) or were the *sod2::ura4* strain (squares) and were either loaded with 200 mM NaCl (filled symbols) or not (open symbols). Cells were then washed and added to the experimental buffer; H^+ influx was then monitored as described in 'Materials and Methods'. Results are mean ± S.E. of at least 3 separate experiments.

changer mutants in more detail we used a Cytosensor microphysiometer, which is capable of detecting small changes in pH in small volumes of liquid and can rapidly record rates of change in pH. Figure 3 illustrates the procedures used to measure proton extrusion using the Cytosensor microphysiometer. For these experiments cells were initially maintained in sodium free medium and then changed to medium with a high concentration of Na^+. This results in proton extrusion by the Na^+/H^+ exchanger and acidification of the external medium. This gave a more reliable measurement of Na^+/H^+ exchange than the measurement of proton uptake and was not dependent on prior loading of the cells with sodium. Loading of cells with high levels of sodium could affect their physiological function. In addition though sodium loading can occur through non-selective channels [15], an impairment of Sod2 function could also affect loading of the cells through this transporter.

The results of the proton extrusion measurements are shown in Fig. 4. Figure 4A illustrates extracellular acidification rate data from a typical experiment. Upon switching to sodium containing medium, there is an immediate extracellular acidification. There was no such response in the absence of immobilized cells (not shown). The response from cells containing a wild type Na^+/H^+ exchanger was much greater than that of cells with the mutation of Asp 267 to Asn. Figure 4B summarizes the results of these experiments with 12 different mutant forms of the Sod2 protein. Proton extrusion rates in mutants of amino acids His98, Glu173, His233, and Asp241 were not significantly reduced compared to the wild type protein. There was a background rate of extracellular acidification that was approximately 50% of the rate of Sod2 activity. The mutants Asp145Asn, Asp178Asn, Asp266Asn, Asp267Asn, Asp266,267Asn, His367Ala, His367Asp, His367Arg and the *sod2::ura4* strain had significantly reduced extracellular acidification rates compared to the wild type Sod2 protein. Surprisingly, the Asp145Asn and the double mutant Asp266,267Asn have relative acidification rates that were substantially lower than that of the Sod2 disruption strain.

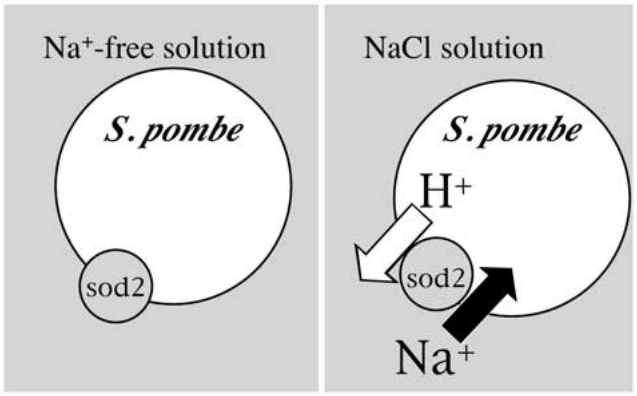

Fig. 3. Schematic diagram of measurement of proton fluxes measured using the Cytosensor microphysiometer. Left panel illustrates yeast and the Na^+/H^+ exchanger prior to sodium loading. The right panel illustrates the effect of adding external sodium and the resultant activity that was measured.

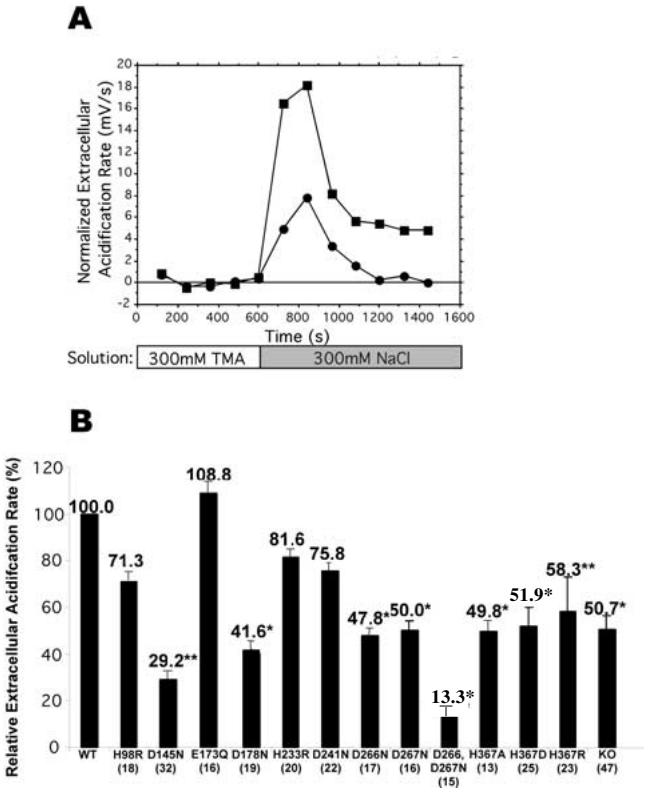

Fig. 4. Microphysiometry of *Schizosaccharomyces pombe sod2::ura4* transformed with pWH5-sod2 (WT), various mutants of Sod2 as indicated or completely lacking functional Sod2 protein (KO). (A) Extracellular acidification rate data from a typical microphysiometry experiment. Cells were perfused with 300 mM TMA chloride, 10 mM HEPES, 0.1% BSA, 500 µM DES at pH 6.3 for 600 sec, followed by a switch to a buffer containing 300 mM NaCl, 10 mM HEPES, 0.1% BSA and 500 µM DES at pH 6.3, as indicated by bars below the graph. Normalized extracellular acidification rates (mV/s) are plotted vs. time (s). Filled circles, D267N Sod2 mutant; filled squares, wild type Sod2. (B) Average Na^+-stimulated extracellular acidification rates for all Sod2 mutations examined by microphysiometry. Rates for each mutation were calculated as described in 'Materials and methods' and are expressed as a percentage of the wild type Sod2 rate. The number of experiments represented by each bar is indicated in parenthesis below respective mutation names. Asterisks indicate that the relative extracellular acidification rate is significantly less than wild type Sod2 (*$p < 0.01$; **$p < 0.05$).

Discussion

The yeast Na^+/H^+ exchanger Sod2 catalyzes the electroneutral exchange of sodium ions for protons, similar to the mamma-

lian Na^+/H^+ exchangers [16]. The molecular mechanisms of Na^+/H^+ exchange remain obscure; how the cations are coordinated and transported through the membrane still remains to be deduced. We have previously [17] hypothesized that polar amino acids distributed within or adjacent to membrane spanning regions could be important in transport. Some key amino acids are conserved between eukaryotic species suggesting that they may be involved in protein function. Earlier [10] we examined the functional importance of some polar conserved amino acids and these observations were extended in the present study. We had earlier shown that His367, Asp241 and the acid pair D266,267 were important for growth in media that contained external sodium. The D241N mutant was unusual in that when mutated, it was defective in supporting growth of the yeast in salt containing medium, but was normal in sodium transport. Point mutations in Sod2 did not affect the expression level of the protein [10].

In this study we examined several new mutants of the yeast Na^+/H^+ exchanger Sod2. In addition, we characterized some of the previous mutants in more detail and more directly examined Na^+/H^+ exchanger activity by examining proton fluxes. We initially showed that we could measure proton uptake by the wild type Na^+/H^+ exchanger when cells had been previously loaded with Na^+. While this method was feasible it has several disadvantages. Firstly, it was not very quantitative and the results were somewhat variable. It was not possible to rapidly change the media of the yeast. This required centrifugation steps that delayed the time of measurement greatly from when the medium was changed. Secondly the method was dependent on prior loading of yeast with sodium. Some of this is likely through the Sod2 protein so that it is probable that mutants defective in Sod2 activity are defective in sodium loading.

To characterize mutants of the Sod2 protein in more detail we examined proton extrusion using the cytosensor microphysiometer. This had several advantages over the previous method. Firstly, the yeast were immobilized. This allows the medium to be changed rapidly and without the delays involved in centrifugation. In addition, we could change the external medium to high salt medium rapidly, allowing us to measure activity of the Sod2 protein in reverse mode. This eliminated the necessity for prior sodium loading of the yeast that might vary between mutant and wild type strains. Further, sodium loaded yeast might also be abnormal in their activity due to either osmotic effects or activation of stress induced protein kinases that respond to sodium. We therefore examined the reverse mode of Na^+/H^+ exchanger activity in the cytosensor microphysiometer with the yeast immobilized as described earlier [11]. We found that several specific amino acids were important in proton efflux activity of Sod2. Amino acids 145 and 178 were important in transport. It had previously been shown that the acidic pair of residues Asp266 and Asp267 were important in activity of the protein. In this study we examined which of the pair of amino acids was important by mutating them individually. Our results show that both of these amino acids are important in Sod2 proton efflux activity. Mutation of either amino acid significantly inhibited Sod2 activity.

We further characterized mutant Sod2 proteins that had changes in the histidine residues either within or adjacent to the lipid bilayer. Histidine 98 and Histidine 233 were not significantly different from the control Na^+/H^+ exchanger in their proton efflux activity. In contrast all three mutants of Histidine 367 had impaired proton flux ability. This agrees with earlier findings [10] that showed that His 98 and 233 support growth in Sod2 deficient yeast. All three His367 mutants were previously shown to be unable to support growth of *S. pombe* in salt containing media.

There was significant proton efflux activity above background in the *sod2::ura4* disruption strain. This occurred even in the absence of external glucose that normally activates the H^+-ATPase and even in the presence of diethylstilbestrol a H^+-ATPase inhibitor [14]. The cause of the background activity is not known at this time however there are several possibilities. There may be other related Na^+/H^+ exchanger – like proteins that have some proton efflux activity that is activated by sodium. Searches of the *S. pombe* database (not shown) have indicated that there are some Sod2 homologs present though they may be more homologous to vacuolar forms of the Na^+/H^+ exchanger [18] than to Sod2. Another possibility is that the endogenous plasma membrane Na^+/H^+ exchanger Sod2 has some residual activity. The initial disruption made in the *sod2::ura4* strain was an insertion in the distal third of the gene [*9*] that removes the last 4 transmembrane domains but leaves the first 8. Though the *sod2::ura4* disrupted strain has been shown to be defective in Na^+ translocation [*9*] the initial part of the protein would still likely be synthesized and could have some residual proton translocation activity. In this regard it was interesting that when in *S. pombe* some of the mutant forms of Sod2 seemed to have lower activity than the knockout (i.e. D266,267N). Both the mammalian [19] and the *E. coli* [20] Na^+/H^+ exchanger have been shown to exist as dimers. Coupling a partially active truncated Na^+/H^+ exchanger with a totally defective mutant could cause a dominant negative effect with a resultant lowering of the activity. Further experiments are necessary to discern if this is the present scenario.

In this study, we examined the effect of mutating of Asp241 to Asn. Previously [10], we had shown that this mutant was impaired in supporting growth in salt containing media, plus it had impaired proton uptake. However it did demonstrate sodium transport in a medium of external pH 4.0 and the impairment of growth was not as severe as with some other mutants. We therefore re-examined this mutant for its ability to acidify the external medium upon addition of sodium. Using the cytosensor microphysiometer we were able to

clarify the physiological effect of this mutant. We found that this mutant possessed significant activity above background. It is thus clear that mutation of this amino acid has an intermediate effect on activity of the yeast Na^+/H^+ exchanger. It is partially supportive of growth, supports sodium flux and as shown in this work, supports proton efflux.

Our results have several implications for the structure and function of Na^+/H^+ exchangers. Transmembrane segment IV of the mammalian Na^+/H^+ exchanger (NHE1 isoform) has been shown to be important in the function of that protein [21]. Similarly, transmembrane segment IV of the *E. coli* Na^+/H^+ exchanger NhaA [22] is important in activity of this isoform of the exchanger. In this study, we also demonstrated that transmembrane segment IV of Sod2 is also important in the function of this protein. Further, mutation of amino acid 178 eliminated H^+ translocation activity of the Sod2 protein. This amino acid is located on the second intracellular loop that immediately follows transmembrane segment IV. In the mammalian NHE1 isoform of the protein, this loop is believed to form a segment that partially reenters the membrane. Our results suggest that in Sod2, this segment is also important for activity. It is interesting to note that amino acid E173 was not critical for H^+ translocation activity of the protein. Clearly only some amino acids of this putative intracellular loop are important in function of the protein. It is interesting to note that residues D145, H367 and D178 were important for activity and are hypothesized to be immediately adjacent to the membrane aqueous interface. The membrane helices of this Na^+/H^+ exchanger are very likely to be folded adjacent to one another as is typical for a membrane protein and has been demonstrated for the NhaA isoform [20]. Therefore it is quite possible that within the membrane these residues and transmembrane segments IV, V and XI are close to each other. They could form part of either a cation binding pocket, or selectivity filter. In this regard, it is interesting to note that we previously demonstrated [10] that the His367 mutant retained some partial proton uptake activity when it was mutated to an acidic residue, though the mutant could not support growth in salt containing medium. This might suggest that this amino acid has proton sensing or binding activity. A histidine residue of NhaA determines the pH profile of activity of the protein. We suggest that His367 of Sod2 might have a similar function. It was surprising however that we obtained extracellular acidification above background with this mutant. Possibly it may have partial activity that is more detectable or active with proton uptake as opposed to proton extrusion.

Overall it clearly appears that polar residues located either within transmembrane segments or adjacent to transmembrane segments are important in Na^+/H^+ exchanger function. Future studies will further examine these residues in more detail and examine other residues of other transmembrane segments of the protein.

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References

1. Wiebe CA, DiBattista ER, Fliegel L: Functional role of amino acid residues in Na^+/H^+ exchangers. Biochem J 357: 1–10, 2001
2. Slepkov E, Fliegel L: Structure and function of the NHE1 isoform of the Na^+/H^+ exchanger. Biochem Cell Biol 80: 499–508, 2002
3. Blumwald E: Sodium transport and salt tolerance in plants. Curr Opin Cell Biol 12: 431–434, 2000
4. Apse MP, Aharon GS, Snedden WA, Blumwald E: Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in Arabidopsis. Science 285: 1256–1258, 1999
5. Nass R, Rao R: Novel localization of a Na^+/H^+ exchanger in a late endosomal compartment of yeast. J Biol Chem 273: 21054–21060, 1998
6. Prior C, Potier S, Souciet J-L, Sychrova H: Characterization of the NHA1 gene encoding a Na^+/H^+-antiporter of the yeast *Saccharomyces cerevisiae*. FEBS Lett 387: 89–93, 1996
7. Banuelos MA, Sychrova H, Bleykasten-Grosshans C, Souciet JL, Potier S: The Nha1 antiporter of *Saccharomyces cerevisiae* mediates sodium and potassium efflux. Microbiology 144: 2749–2758, 1998
8. Sychrova H, Ramirez J, Pena A: Involvement of Nha1 antiporter in regulation of intracellular pH in *Saccharomyces cerevisiae*. FEMS Microbiol Lett 171: 167–172, 1999
9. Jia Z-P, McCullough N, Martel R, Hemmingsen S, Young PG: Gene amplification at a locus encoding a putative Na^+/H^+ antiporter confers sodium and lithium tolerance in fission yeast. EMBO J 11: 1631–1640, 1992
10. Dibrov P, Young PG, Fliegel L: Functional analysis of amino acid residues essential for activity in the Na^+/H^+ exchanger of fission yeast. Biochemistry 36: 8282–8288, 1998
11. Hahnenberger KM, Jia Z, Young PG: Functional expression of the *Schizosaccharomyces pombe* Na^+/H^+ antiporter gene, sod2, in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 93: 5031–5036, 1996
12. Moreno S, Klar A, Nurse P: Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. Meth Enzymol 194: 795–823, 1991
13. Toone WM, Kuge S, Samuels M, Morgan BA, Toda T, Jones N: Regulation of the fission yeast transcription factor Pap1 by oxidative stress: Requirement for the nuclear export factor Crm1 (Exportin) and the stress-activated MAP kinase Sty1/Spc1. Genes Dev 12: 1453–1463, 1998
14. Serrano R, Villalba JM, Palmgren MG, Portillo F, Parets-Soler A, Roldan M, Ferguson C, Montesinos C: Studies of the plasma membrane H(+)-ATPase of yeast and plants. Biochem Soc Trans 20: 562–566, 1992
15. Serrano R, Rodriguez-Navarro A: Ion homeostasis during salt stress in plants. Curr Opin Cell Biol 13: 399–404, 2001
16. Dibrov P, Smith JJ, Young P, Fliegel L: Identification and localization of the sod2 gene product in fission yeast. FEBS Lett 405: 119–124, 1997
17. Dibrov P, Fliegel L: Comparative molecular analysis of Na^+/H^+ exchangers: A unified model for Na^+/H^+ antiport? FEBS Lett 424: 1–5, 1998
18. Brett CL, Wei Y, Donowitz M, Rao R: Human Na(+)/H(+) exchanger isoform 6 is found in recycling endosomes of cells, not in mitochondria. Am J Physiol Cell Physiol 282: C1031–C1041, 2002

19. Fliegel L, Haworth RS, Dyck JRB: Characterization of the placental brush border membrane Na^+/H^+ exchanger: Identification of thiol-dependent transitions in apparent molecular size. Biochem J. 289: 101–107, 1993
20. Williams KA: Three-dimensional structure of the ion-coupled transport protein NhaA. Nature 403: 112–115, 2000
21. Touret N, Poujeol P, Counillon L: Second-site revertants of a low-sodium-affinity mutant of the Na^+/H^+ exchanger reveal the participation of TM4 into a highly constrained sodium-binding site. Biochemistry 40: 5095–5101, 2001
22. Galili L, Rothman A, Kozachkov L, Rimon A, Padan E: Trans membrane domain IV is involved in ion transport activity and pH regulation of the NhaA-Na(+)/H(+) antiporter of *Escherichia coli*. Biochemistry 41: 609–17, 2002
23. Claros M, von Heijne G: TopPredII: An improved software for membrane protein structure predictions. Comp Appl Biosci 10: 685–686, 1994