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# Proline 146 is critical to the structure, function and targeting of sod2, the $Na^+/H^+$ exchanger of *Schizosaccharomyces pombe*

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ABSTRACT

Sod2 is the $Na^+/H^+$ exchanger of the fission yeast *Schizosaccharomyces pombe* that is principally responsible for salt tolerance. We examined the role of nine polar, membrane associated amino acids in the ability of the protein to confer salt tolerance in *S. pombe*. Wild type sod2 protein with a C-terminal GFP tag effectively rescued salt tolerance in *S. pombe* with deleted endogenous sod2. Sod2 protein with the mutations P163A, P183A, D298N, D389N, E390Q, E392Q and E397Q also conveyed salt tolerance as effectively as the wild type sod2 protein. In contrast, the mutation P146A resulted in a protein that did not convey salt tolerance nearly as effectively as the wild type and did not extrude $Na^+$ as well as the wild type. Mutation of $Pro^{146}$ to Ser, Asp or Lys had an intermediate effect. Mutation of $Thr^{142}$ to Ser resulted in a slightly defective protein. Western blot analysis showed that all mutant proteins were expressed at similar levels as wild type sod2 protein. Examination of the localization of the proteins showed that wild type and most sod2 mutants were present in the plasma membrane while the P146A mutant had an intracellular localization. Limited tryptic digestion suggested that the P146A sod2 protein had a change in conformation in comparison to the wild type protein. The results suggest that $Pro^{146}$ is an amino acid critical to sod2 structure, function and localization.

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## 1. Introduction

The $Na^+/H^+$ exchanger is an ubiquitous protein that transports $Na^+$ and $H^+$ in opposite directions across cell membranes. In mammals the $Na^+/H^+$ exchanger is responsible for pH regulation, removing excess intracellular acid. The first known isoform of the $Na^+/H^+$ exchanger NHE1, has been implicated in different cardiac pathologies, including myocardial I/R injury [1] and cardiac hypertrophy [2]. Inhibition of NHE1 prevents I/R injury in various animal models [3–5] and the understanding and development of $Na^+/H^+$ exchanger inhibitors are of great clinical interest [6]. In *Escherichia coli*, plants and yeast, $Na^+/H^+$ exchange occurs in the reverse direction, using the proton motive force to remove excess intracellular sodium [7,8]. Sod2 is the $Na^+/H^+$ exchanger of the fission yeast *Schizosaccharomyces pombe*. It is a plasma membrane protein that exchanges sodium for protons and is responsible for sodium and lithium tolerance in this species. The *sod2* gene was cloned and characterized using selection for LiCl resistance and its disruption results in sensitivity to external $Na^+$ or $Li^+$ and in an inability to extrude cytoplasmic $Na^+$ and to take up external protons in exchange for internal sodium ions [9].

Membrane proteins such as the $Na^+/H^+$ exchanger have several types of amino acids important in transport. This includes amino acids critical to structure such as prolines in transmembrane segments and negatively charged membrane associated amino acids [8]. We have earlier suggested that a few critically conserved charged amino acids may be what is important in cation binding and coordination by $Na^+/H^+$ exchanger proteins [8,10] and our earlier experiments on sod2 [11] and by others on other $Na^+/H^+$ exchanger types [12] confirm the importance of some polar residues of transmembrane segments. In addition, we [13] have earlier shown that prolines are critical in transmembrane segment IV of NHE1 and others [14] have shown that prolines in transmembrane segments are critical for transport. An alignment of $Na^+/H^+$ exchangers and a putative topology model of sod2 are shown in Fig. 1. $Pro^{146}$ is in a conserved region in yeast and other $Na^+/H^+$ exchangers. In addition, a number of polar residues, amino acids $Thr^{142}$, $Pro^{163}$, $Pro^{183}$, $Asp^{298}$, $Asp^{389}$, $Glu^{390}$, $Glu^{392}$ and $Glu^{397}$ lie in membrane associated regions. In this study, we examined the importance of these amino acids in sod2 function. We demonstrate a critical role for $Pro^{146}$ in sod2 structure, function and localization.

## 2. Materials and methods

### 2.1. Strains and media

*S. pombe* bearing the sod2 gene disruption (*sod2::ura4*) was used as a host for all transformations and as a control where indicated [11]. It was maintained on low sodium minimal KMA medium or yeast extract adenine (YEA) using methods described earlier [9,11]. KMA medium contains (per 1 l): potassium hydrogen phthalate, 3 g; $K_2HPO_4$, 3 g; yeast nitrogen base without amino acids, 7 g; glucose,

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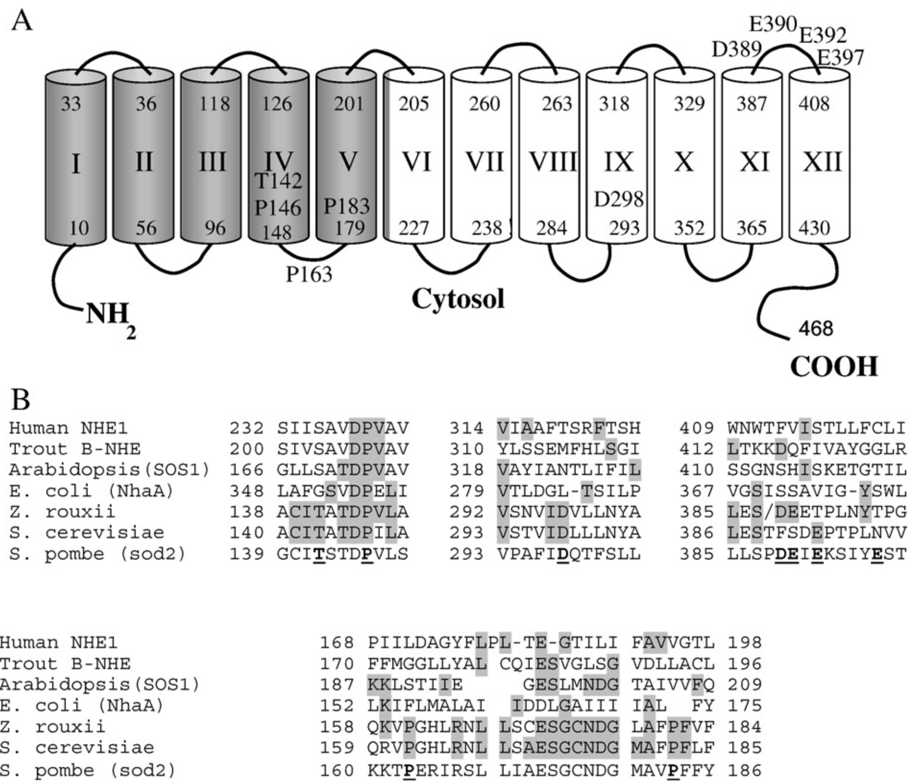


**Fig. 1.** Secondary structure and primary structure alignment of sod2. (A) Putative topology model of sod2 based on predictions of the program TopPred II [30]. The positions of several uncharacterized, membrane associated residues examined in this study are indicated. (B) Alignment of Prokaryotic and Eukaryotic $Na^+/H^+$ antiporters. Alignment of SOS1 is from [31], using the program Clustalw [32] (www.ebi.ac.uk/Tools/clustalw) or using DNA Strider. Numbers preceding sequences indicate the number of the first amino acid. "/" indicates 3 amino acid deletion to optimize alignment. "–" indicates 1–2 amino acid deletion to optimize alignment. Mutated residues of sod2 are bold and underlined. Gaps were introduced to optimize alignments as indicated by analyses. References are NHE1 [33]; Trout B-NHE [34]; *Arabidopsis* [31]; NhaA, [35]; *Z rouxii*, [36]; *S. cerevisiae*, [37]; sod2 [9].

20 g; and adenine, 200 mg. Leucine at 200 mg/l was added to maintain the *sod2::ura4 leu1-32* strain where appropriate and all media was buffered with 50 mM Mes/Citrate and adjusted to pH 5.0 with KOH. Wherever appropriate, NaCl or LiCl were added to the media at the indicated concentrations. For growth curves $2\times10^6$ cells from an overnight exponentially growing culture were used to inoculate 2.5 ml of fresh media liquid and cultures were grown at 30 °C in a rotary shaker. At the various times indicated aliquots of cells were harvested and the $A_{600}$ was determined. All growth curves were determined in triplicate a minimum of three times. The plasmid pREP-41sod2GFP has been described earlier [15]. It contains the full length sod2 gene with a C-terminal GFP tag separated by a nine amino acid Gly-Ala spacer. GFP has the Ser65Thr mutation and an NdeI site removed by silent mutation. *S. pombe* transformed with the pREP-41sod2GFP plasmid (and mutant derivatives) were routinely grown in medium in the absence of thiamine. The stably transfected *S. pombe* bearing a chromosomally integrated copy of the sod2 gene linked to GFP [15] was used for some experiments where indicated.

### 2.2. Site-directed mutagenesis

Mutations in sod2 were made to the pREP-41sod2GFP plasmid directly. Site-directed mutagenesis was performed using amplification with PWO DNA polymerase followed by the use of the Stratagene (La Jolla, CA, USA) QuikChange™ site directed mutagenesis kit. Mutations were designed to create a new restriction enzyme site for use in screening transformants. Table 1 summarizes the mutations made to the sod2 gene. DNA sequencing confirmed the accuracy of the mutations.

**Table 1**
Oligonucleotides used for site-directed mutagenesis of sod2

| Mutation | Oligos | Restriction site |
|---|---|---|
| T142S | GATCGCAGGATGTATc**tcg**agTACTGATCCTGTTC | XhoI |
| P146A | TAACTTCTACTGAT**gCa**GTaCTATCAGCATTGATTG | ScaI |
| P146S | TAACTTCTACTGAT**tca**GTaCTATCAGCATTG | ScaI |
| P146D | TAACTTCTACTGAT**gac**GTcCTATCAGCATTG | AatII |
| P146K | TAACTTCTACTGAT**aaa**GTaCTATCAGCATTG | ScaI |
| P163A | CCATTAGCTAAAAAGACT**gCa**GAACGGATTCGGTC | PstI |
| P183A | GGAATGGCGGTT**gCT**TTTTTCTATTTTGCTATCAA**g**CTTCTTACTGTTAAGC | HindIII |
| D298N | GTACCTGCTTTTATT**aaT**CAGACTTTTAGTTTAC | AseI |
| D389N | CATTTCTTGCAAAATTACTcTTaagCCCG**aAT**GAAATTGAAAAGAG | AflII |
| E390Q | GTTGTCCCCGGAT**cAA**ATTGAAAAatcgATTTATGAATCAAC | ClaI |
| E392Q | GTCCCCGGATGAAATT**cAA**AAatcgATTTATGAATCAACTACAG | ClaI |
| E397Q | GAAAAGAGTATTTAT**cAA**agtACTACAGTATTTTCAAC | ScaI |

Mutated nucleotides are in lowercase letters. Mutations resulting in changed amino acids have the codons indicated in boldface type. New restriction sites are underlined.

### 2.3. Atomic absorption spectrophotometry

To determine the ability of various strains of yeast to remove intracellular $Na^+$, strains were grown in KMA medium to an OD600 of ≈0.4. After harvest by centrifugation and washing, cells were incubated in KMA medium supplemented with 100 mM NaCl for 1 h to load the cells with $Na^+$. Cells were harvested and washed twice with 20 mM MES, pH 5.5 and resuspended in 10 ml incubation buffer containing 20 mM MES, pH 5.5, 0.1 mM $MgCl_2$, and 2% glucose. Cells were incubated at 30 °C and samples were taken at various times up to 120 min. Samples were washed with 20 mM $MgCl_2$ and acid extracted with $H_2SO_4$. $Na^+$ content was determined

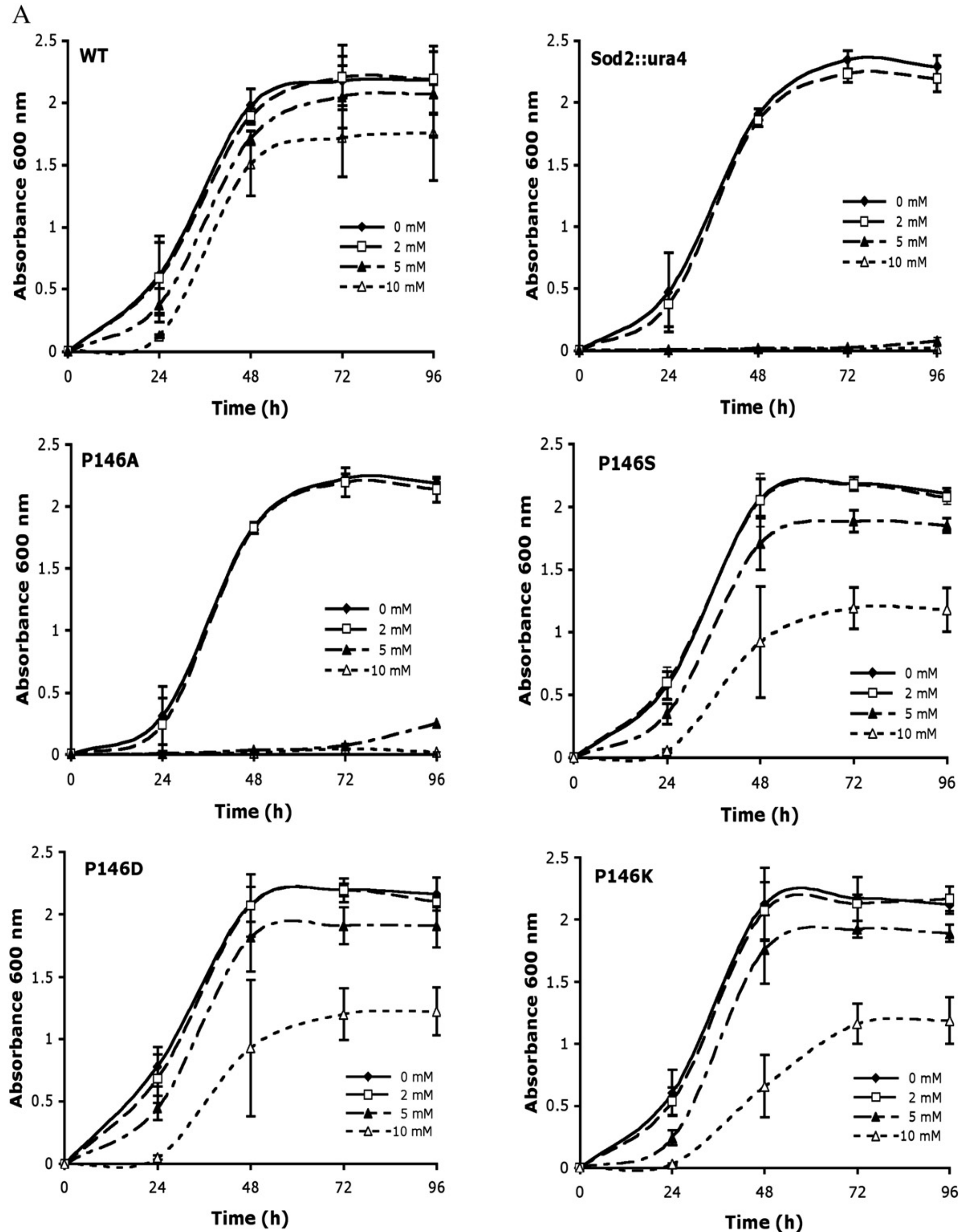


**Fig. 2.** Growth of *S. pombe* containing either wild type or mutant sod2. LiCl tolerance of strains was assessed by inoculating $2 \times 10^6$ cells into 2.5 ml of medium at 30 °C for up to 96 h. Growth was assessed by measuring the absorbance of the cell suspensions at 600 nm at the times indicated. Results are the mean ± SE of at least three determinations and were typical of at least two independent isolates of each mutant. *S. pombe* were grown in the presence of 0, 2, 5 or 10 mM LiCl. (A) mutants of P146 and controls. *Sod2::ura4* refers to *S. pombe* with the sod2 knockout described earlier [11]. WT refers to the *sod2::ura4* with the stable integrant of pREP-41sod2GFP described earlier [15]. (B) mutants of amino acids T142, P163, P183, D298 and D389, were examined for their ability to restore LiCl tolerance as described above. Sod2 refers to growth of *S. pombe* containing the sod2-GFP protein in *S. pombe* (not chromosomally integrated). (C) mutants of amino acids E390, E392 and E397 were examined for their ability to restore LiCl tolerance as described above.

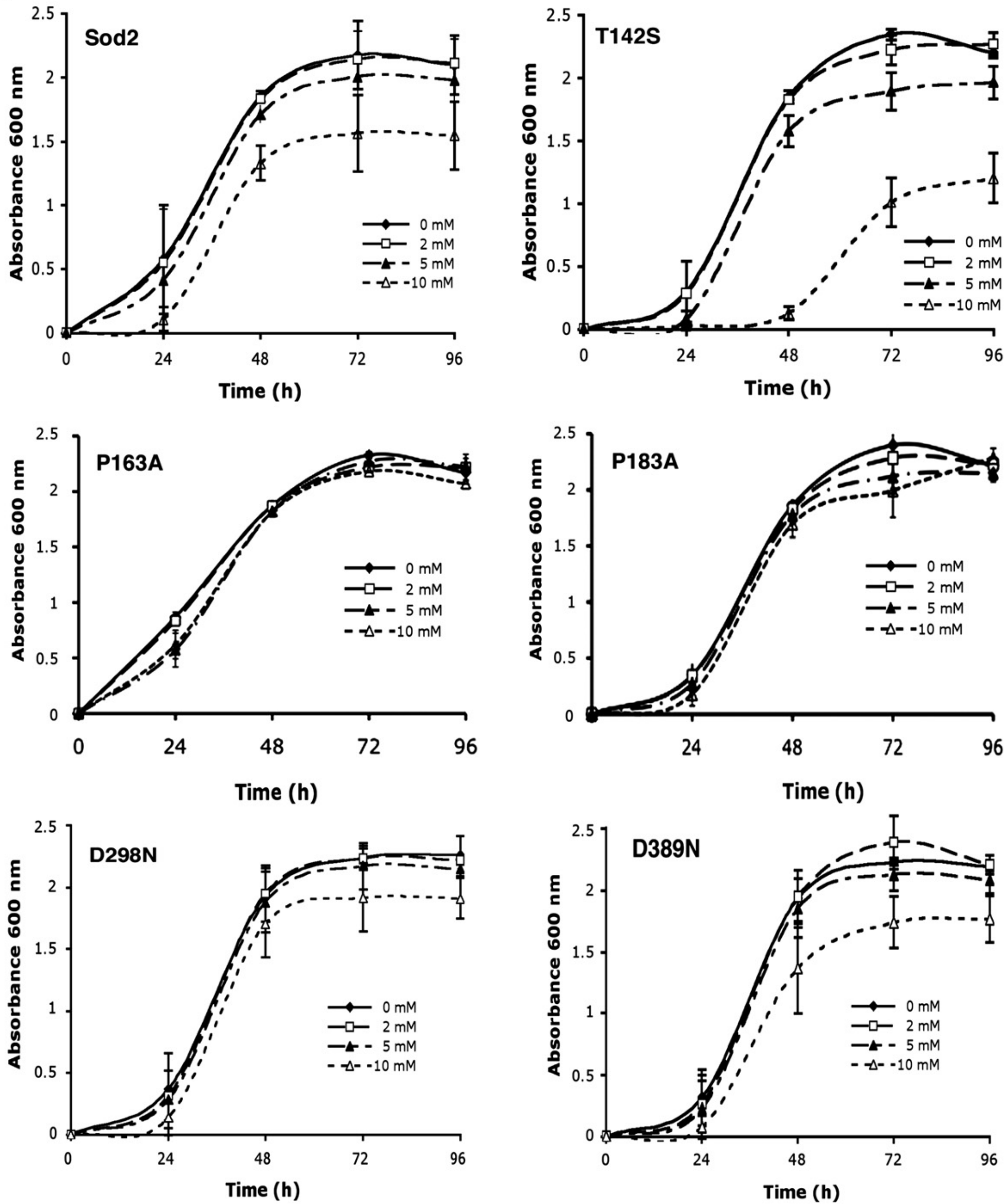


**Fig. 2** *(continued).*

by Atomic Absorption Spectrophotometry essentially as described by others [16]. Results were from at least three independent experiments. Absolute values of $Na^+$ were normalized to the amount of protein present in samples.

### *2.4. Microscopy and indirect immunofluorescence*

For fluorescence microscopy images were taken with a Lieca DM IRB microscope with a 100× objective equipped with a high performance CCD camera (Leica Microsystems, Wetzlar, Germany) and Image-Pro Plus analysis software.

Confocal imaging was performed on an Olympus IX81 microscope equipped with a Nipkow spinning-disk optimized by Quorum Technologies (Guelph, ON, Canada). Images were acquired with the 100× objective on a Hamamatsu EM-CCD camera (Hamamatsu, Japan) using the software Volocity (Improvision Inc., Lexington, MA), and further processed in Adobe Photoshop. Yeast cells were fixed in 4% formaldehyde prior to imaging.

Indirect immunofluorescence was performed using an antibody against the plasma membrane $H^+$-ATPase. Cells were prepared for immunofluorescence by treatment with zymolase essentially as described earlier [17]. Labeling was with rabbit anti-$H^+$-ATPase antibody at a dilution of 1:500 (a generous gift from Dr. G. Eitzen, Department of Cell Biology, University of Alberta). Visualization was with Cy3 conjugated donkey anti rabbit antibody (Jackson Immunochemicals, West Grove PA, USA) at a dilution of 1:250.

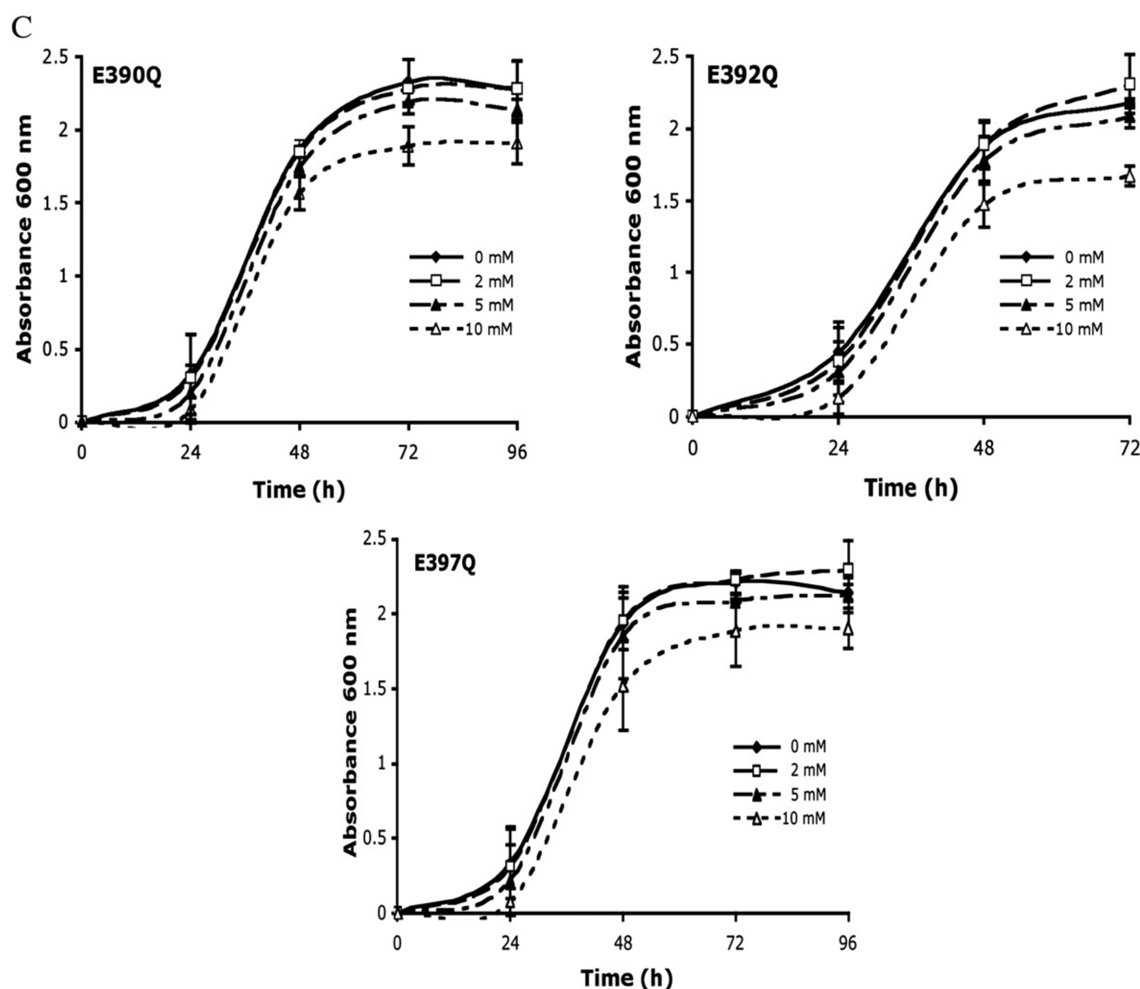


**Fig. 2** *(continued).*

## 2.5. SDS-PAGE and immunoblotting

Western blot analysis was used to confirm the level of sod2 expression [13]. Cell lysates were made from cultures of yeast transformed with wild type and mutant pREP41sod2GFP. Yeast cells grown in KMA medium to an OD600 of 2 at 30 °C. Cells were pelleted (3500×*g*, 10 min) and washed with double distilled water and resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 5 mM EDTA, protease inhibitor cocktail, and 1 mM dithiothreitol). They were then passed through an emulsiflex homogenizer at a pressure of 25 000 psi. Unbroken cells were pelleted by centrifugation at 3500×*g* for 5 min, and the supernatant was centrifuged at 14 000×*g* for 10 min. Enriched membranes of the supernatant were then pelleted at 100 000×*g* for 1 h, and resuspended in a small volume of the same buffer. They were quickly frozen in liquid nitrogen and stored at −80 °C. Equal amounts of up to 100 μg of each sample were resolved on a 10% SDS/polyacrylamide gel. Nitrocellulose transfers were immunostained using a primary antibody of anti-GFP polyclonal antibody (a generous gift from Dr. Luc Berthiaume, Department of Cell Biology, University of Alberta). The secondary antibody was IRDye 680-conjugated goat anti-rabbit polyclonal antibody (Bio/Can, Mississauga, ON, Canada). The Odyssey scanning system was used for Western detection (LI-COR Biosciences, USA).

## 2.6. Trypsin treatment

Yeast membranes were prepared as described above and diluted to a protein concentration of 2 mg/ml in 1 mM EDTA, adjusted to pH 7.4 with Tris HCl (with no protease inhibitors). Trypsin (phenylalanyl chloromethyl ketone-trypsin, Sigma, St., Louis, MO) was added and incubated at 37 °C, to give a desired trypsin:protein ratio (1:20). The reaction was terminated at various times by addition of sample buffer for SDS-PAGE. The samples were resolved on 10% SDS-polyacrylamide gels followed by transfer onto nitrocellulose membranes. Western blotting against the GFP tag was performed as described above. It should be noted that using this type of technique, we have been able to detect changes in protein conformation caused by site-specific mutagenesis of other amino acids of the mammalian $Na^+/H^+$ exchanger [18].

# 3. Results

## 3.1. Effect of mutation of sod2 on S. pombe salt tolerance

We examined a number of selected amino acids that were thought to be important in the function of sod2, the $Na^+/H^+$ exchanger of the fission yeast *S. pombe*. Fig. 1A illustrates a putative topological model of sod2 and the location of the amino acids that were examined in this study. Fig. 1B shows an alignment of the amino acids in comparison to other $Na^+/H^+$ exchanger isoforms. The amino acids were most conserved between the yeasts species. The sequence DPV, containing $Pro^{146}$ was present in most $Na^+/H^+$ exchanger isoforms. $Pro^{163}$ and $Pro^{183}$ were not well conserved outside the yeast species and were used as controls for $Pro^{146}$. A series of mutations were made to a number of polar amino acids and prolines, to test if they were important in sod2 activity. Mutants were introduced into the *sod2::ura4* strain and we examined their ability to allow growth in LiCl

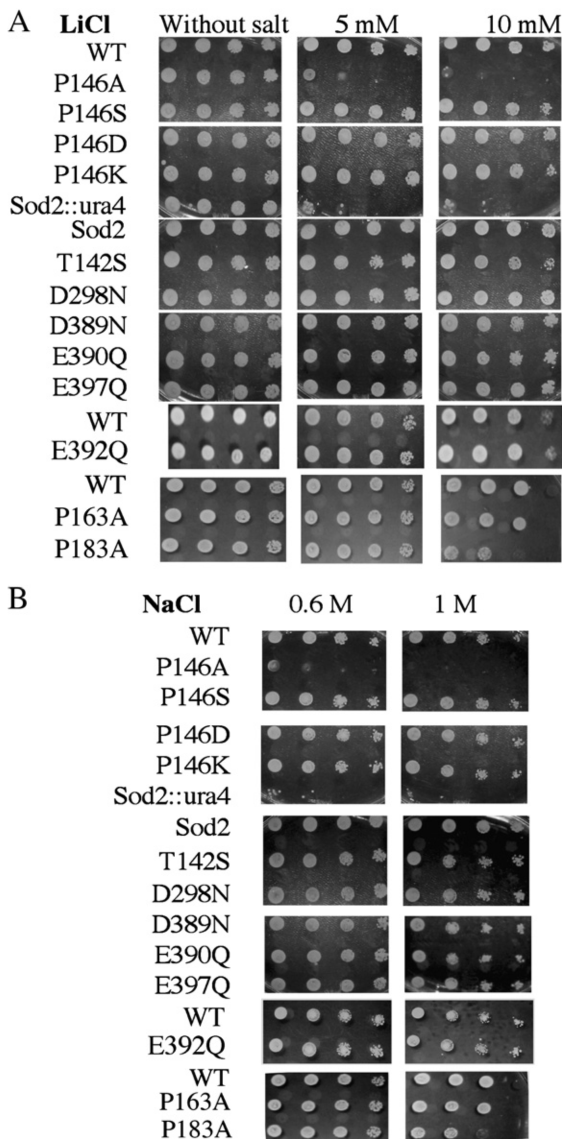


**Fig. 3.** Growth of wild type and mutant sod2 containing *S. pombe* transformants on solid media. Samples of the various yeast strains were taken from stationary phase cultures and were serially diluted 10-fold and spotted onto minimal media plates supplemented with NaCl and LiCl at the concentrations indicated. Plates were incubated for 3 days at 30 °C. (A) series of panels illustrating growth on plates supplemented with LiCl at the indicated concentrations. (B) series of panels illustrating growth on plates supplemented with NaCl at the indicated concentrations. *Sod2::ura4* refers to *S. pombe* with the sod2 knockout described earlier [11]. WT refers to the *sod2::ura4* with the stable integrant of pREP-41sod2GFP described earlier [15], sod2 refers to *S. pombe* transformed with pREP-41sod2GFP without any mutations. Other designations refer to *sod2::ura4* transformed with pREP-41sod2GFP with the indicated mutation. Results are typical of 3 experiments.

or NaCl containing medium. Both LiCl and NaCl are transported by sod2 and other $Na^+/H^+$ exchangers [11]. LiCl was used for assays in liquid media since it is toxic at lower concentrations, avoiding osmotic challenge of high concentrations of NaCl. Fig. 2A illustrates the effect of mutation of $Pro^{146}$. The *sod2::ura4* strain that was not transformed with the sod2-GFP plasmid was very sensitive to LiCl. Either 5 mM or 10 mM LiCl completely eliminated growth. Normal growth was observed in the absence of LiCl or in the presence of 2 mM LiCl. The *sod2::ura4* stably transformed with sod2-GFP, grew in all LiCl concentrations including 10 mM. Growth was only slightly reduced in 10 mM NaCl but occurred rapidly, with significant growth 48 h after inoculation. The same growth characteristics were shown by the sod2-GFP protein that was not stably integrated (Fig. 2B). $Pro^{146}$ was mutated to four different amino acids either alanine, serine, aspartate or lysine. In all cases there was a defect in the ability of the sod2-GFP protein to restore LiCl tolerance, though the effect varied. The proline to alanine mutant was very defective in restoring salt tolerance. In contrast, mutation to serine, aspartate or lysine had an intermediate effect. All of these mutants displayed slightly reduced growth in 5 mM LiCl, and there was a more pronounced effect with 10 mM LiCl where growth still occurred, but was reduced in both amplitude and onset.

Eight other amino acids were examined for their importance in the ability of sod2 to restore salt tolerance (Fig. 2B, C). The Thr142Ser mutant was partially defective. Growth in 5 mM LiCl was very slightly reduced, while there was a larger effect in medium containing 10 mM LiCl. There was almost no restoration of growth until after 40 h and at later times growth was reduced in amplitude. Mutation to amino acids $Pro^{163}$, $Pro^{183}$, $Asp^{298}$, $Pro^{163}$, $Pro^{183}$, $Asp^{389}$, $Glu^{390}$, $Glu^{392}$ and $Glu^{397}$ did not affect their ability to restore LiCl tolerance.

We also examined the yeast strains growth on solid medium. Fig. 3 illustrates the effects of both LiCl and NaCl on the growth of these cells. Cells containing wild type sod2 grew well in LiCl containing media while the *sod2::ura4* knockout strain only showed limited growth when the highest amount of cells were inoculated. Similar to the findings in liquid medium, growth by *S. pombe* containing the P146A mutant was greatly impaired (Fig. 3A, B). There was sometimes a slight impairment of growth of the other $Pro^{146}$ mutants that was sometimes evident at the highest dilutions of these cells. Similar results were found with NaCl containing medium (Fig. 3B). Growth by the P146A containing mutant cells was greatly impaired. There was no apparent difference between the other $Pro^{146}$ containing mutants and the wild type sod2. The $Thr^{142}$ mutation caused a slight decline in growth in 0.6 M NaCl or 1 M NaCl containing medium, relative to the wild type (Fig. 3B). Mutations to $Pro^{163}$, $Pro^{183}$, $Asp^{298}$, $Asp^{389}$, $Glu^{390}$, $Glu^{392}$ and $Glu^{397}$ had no effect on growth of these mutants except a slight defect in growth of P183A in very high salt concentrations on solid medium (Fig. 2B), which did not occur in liquid medium (Fig. 3A, B).

### 3.2. Sodium transport of S. pombe containing sod2

We also examined sod2 activity in a different manner, using expulsion of intracellular $Na^+$ as an indication of sod2 activity. We [11]

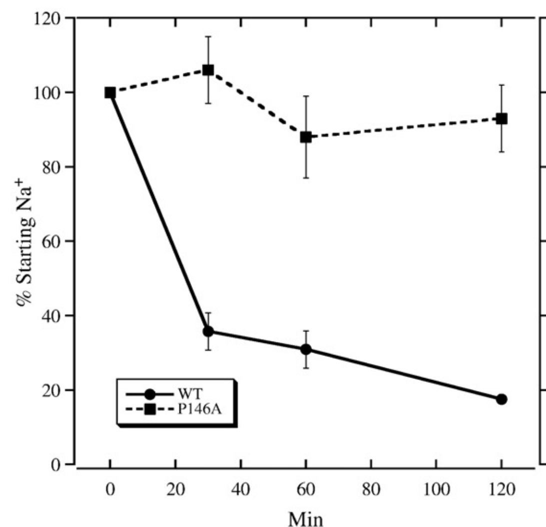


**Fig. 4.** Loss of $Na^+$ from *S. pombe* containing either wild type (WT) or P146A mutant sod2. Cells expressing sod2 were incubated in high NaCl containing medium for 1 h and after washing and resuspension, $Na^+$ content was determined as described in "Materials and methods". Results are the mean of three independent determinations. Error bars represent ±SEM.

and others [9] previously demonstrated that sod2 is the major mechanism of $Na^+$ expulsion in *S. pombe* and its absence results in an inability to extrude intracellular $Na^+$. We examined $Na^+$ content of the P146A mutant after a period of sodium loading. The results are shown in Fig. 4. $Na^+$ content of yeast with the wild type sod2 decreased relatively rapidly and after 120 min, was less than 20% of the initial value obtained after incubation in NaCl containing medium. In contrast, there was only a slight and insignificant decline in the NaCl content of yeast containing the mutant sod2 with the P146A mutation. These results again demonstrate that the activity of the P146A mutant protein is impaired. After incubation in NaCl containing medium and before recovery was allowed, the absolute values of the $Na^+$ present in the mutant sod2 containing yeast were elevated almost two fold ($1.9 \pm 0.4$) compared to the *S. pombe* containing wild type sod2. The difference could be the result of activity of the sod2 protein activity during the incubation period with NaCl. This result indicates that the subsequent lack of a decrease in $Na^+$ content in the mutant containing cells, was not due to the presence of less intracellular $Na^+$ and less availability of substrate for transport by sod2 protein.

### 3.3. Expression levels of mutant sod2 proteins

To determine if the mutant sod2 proteins were expressed we used Western blotting against the GFP tag on the C-terminus of the control and mutant proteins. The results are shown in Fig. 5. Panel A shows the results with extracts of the mutants of $Pro^{146}$. Panel B and C illustrate the results with the balance of the mutants. Anti-GFP antibody reacted with a protein of approximately 80 kDa in size in all *S. pombe* with wild type and mutant sod2. *S. pombe* not containing sod2-GFP protein did not show immunoreactivity with this protein. The relative levels of the sod2-GFP protein present in each yeast type were measured and are shown in Fig. 5D. Though levels varied slightly, in all cases the level of sod2-GFP protein expressed was similar to that of controls.

### 3.4. Localization of sod2 proteins

To examine if the mutations to the sod2 protein influenced cellular localization of the protein we examined localization of the protein using confocal microscopy. Initial experiments examined localization of the protein using immunofluorescence [15] microscopy (not shown). The chromosomally integrated sod2 had localization similar to what we reported earlier, on the surface of the cell plus in some intracellular organelles, possibly with a perinuclear localization [15]. In the cells containing the sod2 plasmid, the expression and localization was somewhat more irregular. In general there appeared to be an irregular distribution on the plasma membrane with some parts of the cell containing higher levels of

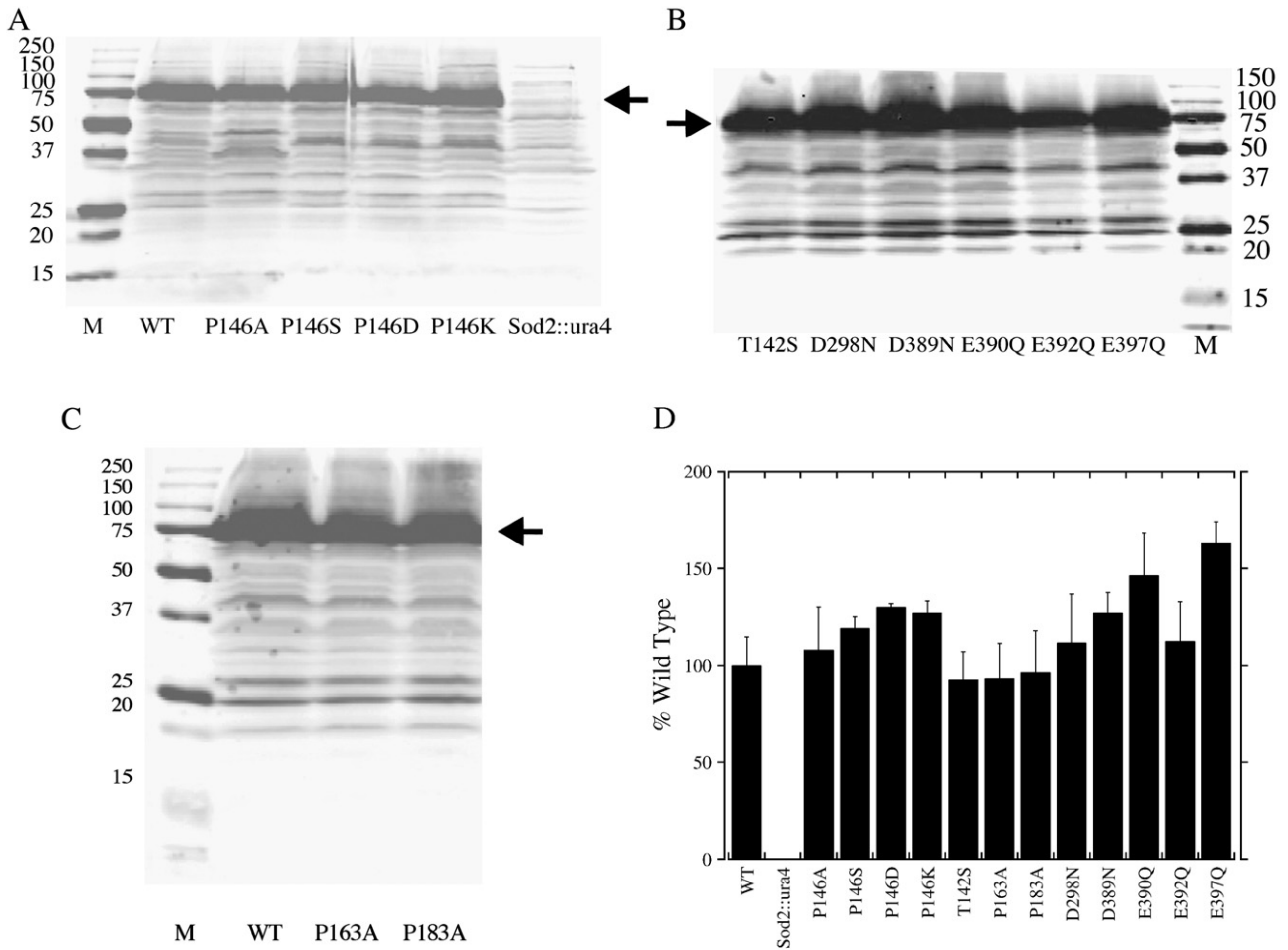


**Fig. 5.** Western blot analysis of expression levels of wild type and sod2 mutant containing transformants. Cell extracts are from *S. pombe* strains and were blotted with anti-GFP antibody. (A) Lane 1, molecular weight markers, M, Lane 2 wild type sod2-GFP (WT) expressed from a plasmid. The mutants P146A, P146S, P146D and P146K are indicated. *Sod2::ura4* indicates the sod2 knock out strain. Arrow indicates location of sod2-GFP fusion. (B) Cells extracts from *S. pombe* were made and examined for sod2-GFP expression as in "A". M = molecular weight markers. Mutants are as indicated below the appropriate lane. Arrow indicates location of sod2-GFP fusion. (C) Cell extracts from *S. pombe* made and examined for sod2-GFP expression as in "A". Extracts were made from wild type and from P163A and P183A mutants. Arrow indicates location of sod2-GFP fusion. (D). Summary of expression levels of wild type and mutant sod2 proteins. Cell extracts were made as described in "Materials and methods" and were blotted with antibody against the GFP tag. WT, wild type sod2-GFP protein. Quantification was with the Odyssey V3.0 analysis software (LI-COR Biosciences, USA).

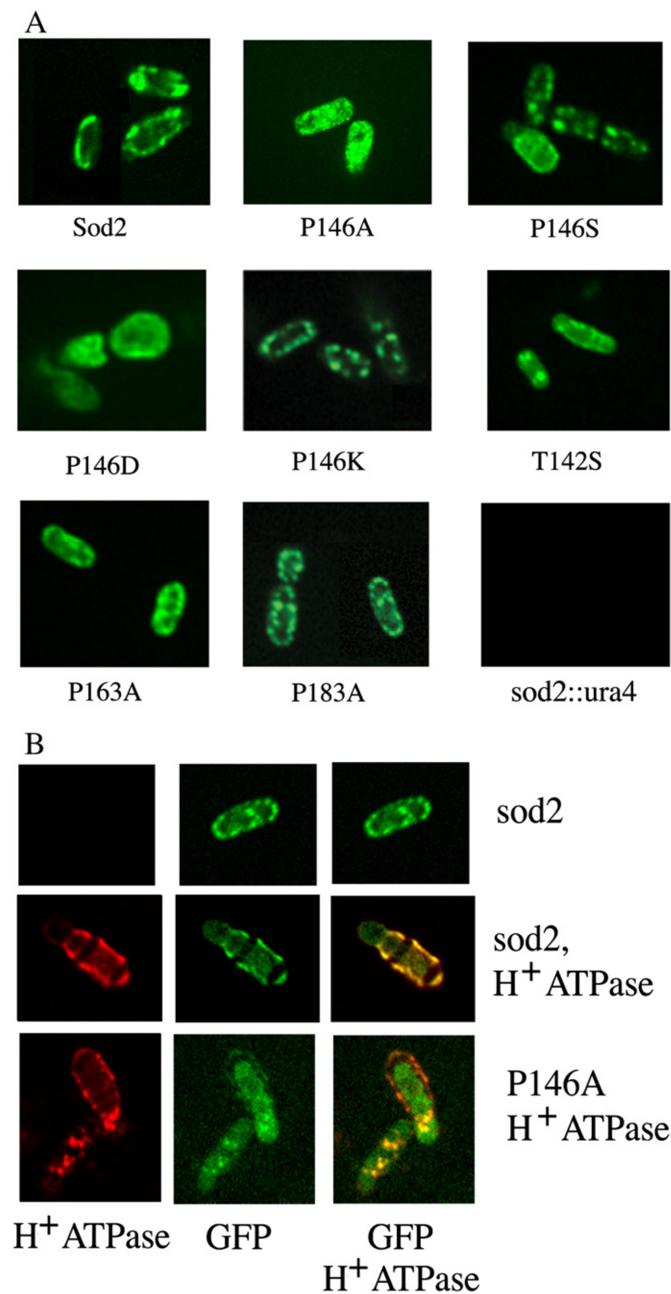


**Fig. 6.** Spinning-disk confocal microscopy of *S. pombe* expressing wild type sod2-GFP and sod-GFP mutants. (A) Exponentially growing cells were harvested and fixed in 4% formaldehyde, washed and mounted on coverslips before imaging. Sod2 refers to the wild type sod2 protein expressed without mutations. *Sod2::ura4* indicates *S. pombe* without the sod2 protein. Mutant sod2 proteins expressed are indicated. (B) Immunolocalization of wild type and mutant Sod2-GFP P146A in comparison with plasma membrane $H^+$-ATPase. Confocal microscopy was used to compare indirect immunofluorescence of the plasma membrane $H^+$-ATPase with that of wild type or mutant sod2 as in "A". Top row illustrates wild type sod2 not immunostained for $H^+$-ATPase for comparative purposes. Middle row illustrates wild type sod2 immunostained with $H^+$-ATPase antibody. Bottom row illustrates P146A mutant sod2. Left panels illustrate Cy3 (red) fluorescence ($H^+$-ATPase). Middle panel illustrates GFP fluorescence and right panel illustrates both Cy3 and GFP fluorescence.

sod2-GFP and other lower levels. There was also usually a small and variable amount of perinuclear sod-GFP protein. The notable exception to this type of distribution was the P146A mutant, which had very little plasma membrane sod2-GFP protein apparent but had some intracellular protein present. There was no fluorescence in the *sod2::ura4* strain that had not contained and added sod2-GFP protein.

More detailed examination of the localization of sod2 and the sod2 mutant proteins was done using confocal microscopy and is presented in Fig. 6A. Expression of wild type sod2 without any mutations resulted in a localization that was mostly at the plasma membrane. Though the distribution throughout the plasma membrane was not even, and there were significant amounts of intracellular protein. In contrast, the fluorescent signal from P146A was almost completely intracellular. It appeared to be contained in discrete structures distributed within and throughout the cell. The expression of the other Pro mutants and $Thr^{142}$ resulted in a localization similar to that of wild type sod2, mostly present on the cell surface (Fig. 6A). Fig. 6B shows the localization sod2 in comparison to that of the plasma membrane $H^+$-ATPase. The upper rows show a control not stained with $H^+$-ATPase for comparative purposes. Immunostaining of the wild type sod2 and of the P146A mutant for $H^+$-ATPase was principally confined to the cell surface, with some foci of present in some intracellular locations (left column). GFP fluorescence (middle column) was mostly at the cell surface for the wild type sod2. In the P146A the GFP fluorescence was mostly in an intracellular location, though a faint GFP signal was also present on the cell surface. The right column shows the co-localization of sod2 proteins and $H^+$-ATPase. The wild type sod2 was mostly co-localized with the $H^+$-ATPase on the cell surface with only a small amount of intracellular signal. The P146A mutant was mostly in an intracellular location with a small amount of co-localization on the cell surface.

### 3.5. Trypsinolysis of sod2 protein

To determine whether the mutant sod2 protein was properly folded we carried out limited trypsinolysis. This method examines the accessibility of Arg and Lys residues to proteolytic attack, and has been used earlier to examine the structure of membrane proteins such as the $H^+$-ATPase of yeast [19]. We have also used this procedure earlier to examine changes in the conformation of the mammalian $Na^+/H^+$ exchanger [18]. We compared the wild type sod2 with the sod2 mutant that had the most reduced activity, P146A. Fig. 7 shows an example of our results. The most striking difference that we found was that the sod2 protein was more rapidly degraded by trypsin treatment. In multiple repetitions of the experiment there was a more rapid disappearance of the full length sod2 protein, in comparison with the wild type protein. We could not distinguish any new particular degradation product that appeared in the pattern of digested proteins.

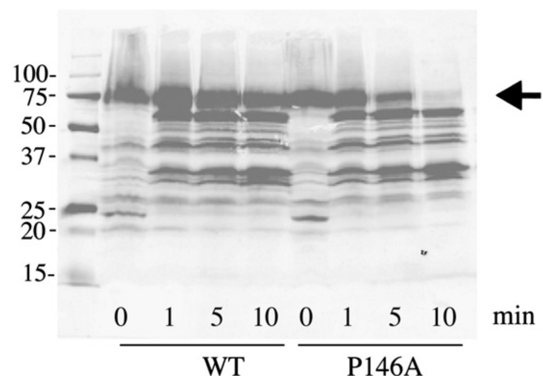


**Fig. 7.** Time course of trypsinolysis of wild type and mutant (P146A) sod2 protein. Yeast membrane fractions were incubated with trypsin for 0–10 min at 37 °C as described in "Materials and methods". Equal amounts of samples were then analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting with anti-GFP antibody (as with Fig. 5). Arrow denotes location of the full length sod2 protein product. Results are typical of three independent experiments.

## 4. Discussion

To identify amino acids that were important in the activity of the sod2 protein, we used site specific mutagenesis of several amino acids that were suspected of being important to function. We previously have hypothesized that polar negatively charged amino acids are important in cation coordination [10] and recent results with the $Na^+/H^+$ exchanger NhaA of *E. coli* have confirmed this suggestion [12,20]. We also previously demonstrated that prolines within TM IV of the mammalian $Na^+/H^+$ exchanger isoform 1 are important in activity of this protein [13]. While we have earlier examined several polar residues important in sod2 activity, [10,21] a number of others remained to be characterized. Fig. 1A illustrates a hypothetical topology of the sod2 protein based on hydropathy analysis which is similar to that published earlier [21]. The topology of sod2 is not known. Another model of sod2 topology [9] has earlier suggested that amino acids 125–141 are part of TMV and that those distals of $Ile^{141}$ are part of an extracellular loop. However, this makes for a short transmembrane segment atypical of a eukaryotic membrane [22]. While the topology of NHE1 has been examined by cysteine accessibility studies [23] a recent analysis of a NHE1 model was developed based on fold-recognition using the *E. coli* NhaA crystal structure as a template and this differed with the previous model significantly [24]. For purposes of the present study, the exact position of these amino acids within the membrane cannot be considered certain, though they are certainly associated with the membrane domain of the protein.

It is often thought that there is a relationship between conservation of amino acids throughout a gene type and importance to activity. In this regard we have previously noted the presence of a characteristic DPV sequence that was widespread throughout most $Na^+/H^+$ exchanger isoforms (Fig. 1B). We earlier demonstrated that $Asp^{145}$ of this sequence is critical to sod2 function [25] however $Asp^{238}$ and $Pro^{239}$ of mammalian NHE1 were not [18], suggesting that this particular sequence is not essential in all $Na^+/H^+$ exchangers despite its widespread occurrence. $Thr^{142}$ was well conserved in the yeast species and in other species was sometimes replaced with the conservative change to Ser. Mutation of both these residues influenced sod2 function.

Examination of the several proline residues of sod2 further suggested that well conserved residues are more likely to be important. While all the prolines we examined were conserved in yeast species, only $Pro^{146}$ was conserved across species and it was the only proline that was critical to function. Mutation of $Pro^{146}$ to Ala was much more efficacious than mutation to Ser, Asp or Lys. The reason for this is not yet certain but could be related to effects on the structure of the protein. Prolines are helix breakers, disrupting the structure of membrane alpha helices [26]. Alanine is a small, hydrophobic, helix forming amino acid that is relatively non-intrusive [27]. It may have a greater tendency to form an alpha helical structure than the other amino acids, which is more discordant with the structure of the transmembrane segment with proline present. This would be in agreement with our results that found that the P146A mutant was more sensitive to protease digestion and may be mis-folded. Since either Lys or Asp partially restored the activity of sod2, it is clear that the nature of the charge of the replacing residue is not critically supporting that idea that the P146A protein is aberrant in structure. The P146A mutant protein was also the only mutant protein that did not appear to target very well to the plasma membrane. This is in agreement with the idea that an alteration of protein structure results in a mis-folding that leads to a greater intracellular retention. Pathways have been reported earlier for intracellular retention and redirected targeting of mutant proteins in yeast [28]. We earlier found that prolines were critical for targeting of the mammalian $Na^+/H^+$ exchanger NHE1 [13].

As noted above, when we tested for mis-folding of the sod2 protein by limited trypsin treatment of the sod2 protein, we found that the P146A mutant was more sensitive to digestion with trypsin. This indicated that there was an alteration in the structure of the sod2 protein. We were not able to discern a particular protein band that was unique to the P146A sod2 protein. It could be that any new digestion products were smaller than what was retained in the SDS-PAGE that we used. Alternatively, a more likely explanation is that new digestion products lacked the GFP and thus were not visible in the Western blot after digestion. Nevertheless, clearly the mutant protein had some change in conformation that made it more accessible to digestion.

The effects we found on mutation of $Pro^{146}$ are partially similar to those reported earlier in *Zygosaccharomyces rouxii* [14]. In that case, the conserved $Pro^{146}$ was also important in function of the homologous protein ZrSod2-22. Mutation to Lys, Gly or Asp resulted in an inactive protein, but mutation to Ser resulted in a fully active protein. A major difference between their and our study is that they did not find that this mutation had an effect on targeting of the protein. In addition, we demonstrated that mutation to alanine affected the structure of the protein. The reason why they found no effect on targeting of the protein in contrast to our results, could be due to local differences in the character of the transmembrane segment and due to the different mutations that we made. Despite these variations, our present and earlier results clearly indicate that this region of the membrane domain is critical for the function of sod2.

Residue $Thr^{142}$ was of special interest as the residue in the same position in Sod2-22p was important in substrate specificity broadening the cation selectivity to include potassium [29]. We found that mutation of this residue to serine had a slight detrimental effect on the ability of sod2 to rescue salt tolerance. We were not able to test for an alteration in sensitivity to external potassium as the *sod2::ura4* strain was tolerant of high external potassium concentrations (not shown). Nevertheless, it was clear that this amino acid was significant in sod2 function.

It was surprising that the mutations P163A, P183A, D298N, D389N, E390Q, E392Q, and E397Q resulted in no major adverse effect on sod2 function in our assay. In our model of sod2, $Pro^{183}$ was predicted to be within the membrane but only had a very minor effect on function. D298 was predicted to be near the middle of a transmembrane segment of sod2 and seemed to be a good candidate for cation coordination. However we earlier found that some negatively charged residues in the middle of transmembrane segments of NHE1 were important in function, while others were not [18]. While amino acids $Asp^{389}$, $Glu^{390}$, $Glu^{392}$, and $Glu^{397}$ were predicted to be on an extracellular loop, charged amino acids of this type and location have been suggested to be important in other cation transport proteins, such as in funneling cations towards the opening of the *E. coli* $Na^+/H^+$ exchanger NhaA [20]. Nevertheless, it appears as though this group of polar amino acids is not critical to sod2 function. Likely other amino acids are involved in attraction of cations to the transport pore.

Overall our results have shown that the region encompassing amino acids 142–146 is critical to sod2 function. These results support the hypothesis that this conserved region is critical in the function of yeast $Na^+/H^+$ exchangers. This region is not important in the function of mammalian NHE1, therefore it appears as though sod2 and the yeast $Na^+/H^+$ exchangers are quite divergent from the mammalian $Na^+/H^+$ exchanger in this regard [25]. Future studies will explore these differences in structure and function in more detail.

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