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Functional role and analysis of cysteine residues of the salt tolerance protein Sod2

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Abstract Sod2 is the major salt tolerance plasma membrane protein of *Schizosaccharomyces pombe*. It functions to remove excess intracellular sodium (or lithium) in exchange for protons. We investigated the role of cysteine residues and created a cysteine-free Sod2 protein. Each cysteine residue of the ten present was individually mutated to serine and the different proteins expressed and characterized in *S. pombe*. Western blotting revealed that all the individual mutant proteins were expressed. We examined the ability of the mutant proteins to confer salt tolerance to *S. pombe* with the endogenous Sod2 protein deleted. Only proteins with C26S and C374S mutations were partially reduced in their ability to confer salt tolerance. Additionally, they showed a change in conformation in comparison to the wild-type protein, indicated by differential sensitivity to trypsin. Deletion of all the cysteine residues of Sod2 resulted in a functional protein that was expressed in *S. pombe* at levels similar to the wild type and also conferred salt tolerance. The conformation of the cysteine-free Sod2 protein was not altered relative to the wild-type protein. We examined the accessibility of amino acids of the cysteineless protein present on putative extracellular loop 2. A cysteine placed at position Ala119 was accessible to externally applied [2-(trimethylammonium)ethyl] methane thiosulfonate bromide. The results demonstrate that cysteines in the Sod2 protein can be changed to serine residues resulting in an expressed, functional protein. The utility of the cysteine-free Sod2 protein for determination of topology and amino acid accessibility is demonstrated.

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Introduction

Na^+/H^+ exchangers are a large superfamily of membrane proteins that function in the exchange of cations across lipid bilayers [1]. This superfamily includes eukaryotic and prokaryotic proteins. The mammalian NHE1 isoform is a well-studied member of the cation proton antiporter 1 family [2]. The cation proton antiporter 2 family includes Sod2, the *S. pombe* Na^+/H^+ exchanger which shares its origins with prokaryotic NhaA, the *E. coli* antiporter [1]. While a crystal structure has been deduced for NhaA [3], knowledge of the topology and structural analysis of all the other kinds of Na^+/H^+ exchangers has been lagging or is in dispute (reviewed in [4]). For example, for mammalian NHE1, there is discordance in models of the topology of NHE1 [5–7]. For the other members of the superfamily there is little or no experimental data on topology or structure aside from computer predictions based on hydrophobicity analysis, which still need to be confirmed.

Sod2 is a plasma membrane Na^+/H^+ exchanger, providing salt tolerance for *S. pombe* by removing excess intracellular sodium (or lithium) in exchange for protons [8]. Salt tolerance in eukaryotes is of enormous significance in agriculture [9] and Sod2 of *S. pombe* is a particularly useful model system in *S. pombe* to study mechanisms of salt transport as its knockout confers a salt-sensitive phenotype which can be complemented by return of the protein [10, 11].

In general, membrane proteins are particularly difficult to study, due to their low abundance and inherent

difficulties in overexpression and purification. One particularly useful technique to enhance understanding of membrane proteins is the construction of cysteineless membrane proteins. If the resultant cysteineless protein is active, the subsequent reintroduction of cysteine residues in particular locations can be used to determine membrane topology and to define pore lining residues of transport proteins. This approach has been used with success for mammalian Na^+/H^+ exchanger isoform 1 [12, 13]. However, caution must be used in construction and characterization of cysteine-free proteins. For example, it has been found earlier that derivatization of thiols affects the activity of Na^+/H^+ exchangers [14] and thiols are involved in dimer formation of the human placental Na^+/H^+ exchanger [15]. Further, mutation of individual amino acids of the NHE1 isoform of the Na^+/H^+ exchanger can sometimes cause mistargeting of the protein or decreased expression in some cases [12, 16].

Sod2 is considered to be a useful model of Na^+/H^+ exchangers mediating salt tolerance, but there is little empirical information on the structure and function of salt tolerance Na^+/H^+ exchangers. For this reason, we constructed and characterized a cysteine-free Sod2 protein. Our results suggest that cysteines 26 and 374 are significant in Sod2 function. However, the cysteine-free Sod2 protein was functional and conveyed salt tolerance. We further demonstrated the utility of the salt-tolerant cysteine-free Sod2 protein examining the accessibility of amino acids of the putative second extracellular loop of the protein.

Materials and methods

Materials

Restriction enzymes and DNA-modifying enzymes were obtained from New England Biolabs, Inc. (Mississauga ON, Canada) or from Roche Applied Science (Roche Molecular Biochemicals, Mannheim, Germany). Synthetic oligonucleotides were synthesized by Integrated DNA technologies.

Strains and media

For all transformations, we used *S. pombe* with the Sod2 gene disruption (sod2::ura4) and where indicated this strain was used as a control [11]. The sod2::ura4 strain was maintained on minimal KMA medium with low sodium or on yeast extract adenine (YEA) as described earlier [8, 11]. KMA medium contains: potassium hydrogen phthalate, 3 g; K_2HPO_4, 3 g; yeast nitrogen base without amino acids, 7 g; glucose, 20 g; and adenine, 200 mg (per 1 l). Leucine at a concentration of 200 mg/l was added to maintain the *sod2::ura4 leu1-32* strain whenever necessary and the media was buffered with 50 mM Mes/citrate. The pH was adjusted to 5.0 with KOH (rather than NaOH, to avoid introduction of sodium). Where indicted, NaCl or LiCl was added to the media and *S. pombe* growth was examined. Liquid cultures were grown at 30 °C with constant agitation using a rotary shaker. 2.5 ml of liquid media cultures were inoculated with 5×10^6 cells from an overnight exponentially growing culture and the absorbance at 600 nm was determined at the indicated times. Growth curves were determined at least three times and the results illustrated are mean ± SE. The plasmid pREP-41Sod2GFP was used for Sod2 expression experiments as described earlier [10, 17]. pREP-41Sod2GFP contains the entire Sod2 gene with a GFP tag at the C-terminal which is separated by a nine-amino acid Gly-Ala spacer. The GFP has the S65T mutation to enhance fluorescence and an NdeI site was also removed by silent mutation to assist in cloning of Sod2 into the plasmid. Growth on solid media was supplemented with NaCl or LiCl at the concentration indicated in KMA medium with leucine agar. The plasmid pREP-41Sod2GFP without any mutations [17] was used for control (wild type) transformations, while mutant Sod2 proteins were made as described below.

Site-directed mutagenesis

Mutations were made directly on the pREP-41Sod2GFP plasmid [10]. The mutations created or removed a restriction enzyme site that was used in screening mutants. Site-directed mutagenesis was performed using the Stratagene QuikChange™ mutagenesis kit (La Jolla, CA, USA) [18]. Table 1 summarizes the mutations made to the Sod2 gene. DNA sequencing was used to confirm the accuracy of the mutations and fidelity of the resultant plasmid.

Western blotting of Sod2

To compare the level of wild-type and mutant Sod2 expression, Western blot analysis was used [19]. Cell lysates were made from wild-type and mutant 50 ml cultures of yeast. Yeast cells were grown in KMA medium at 30 °C as described above to an OD600 of 2. Cells were pelleted (3,500×*g*, 10 min), washed with water and resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 1 mM dithiothreitol, and protease inhibitor cocktail [20]). Following this, cells were lysed using a Bullet Blender® using 1 mm zirconium oxide beads at speed 8 for 50 min. Alternatively, they were lysed using an Emulsiflex homogenizer at a pressure of 25,000 psi. Unbroken cells were removed by centrifugation at 3,500×*g* for 5 min, and then the supernatant was centrifuged at 14,000×*g* for 10 min. A membrane fraction of the

Table 1 Oligonucleotides used for site-directed mutagenesis of sod2

Mutation	Oligos	Site
C26S	GGATTTATAACgTTTTTC**aGt**TATTTTTCAGAAG	Acl1
C76S	CTTGACAGTAGAGATT**TcT**AGAATCGTACTTGATG	XbaI
C140S	GCTGATCGCAGGA**Tcc**ATAACTTCTACTG	BamHI
C176S	GCTGAGTCTGGA**Tcc**AATGATGGAATGGC	BamHI
C213S	GTGTTGTATGAA**agc**GCtTTTGGTATATTTTTTGG	AfeI
C221S	GGTATATTTTTTGGa**Tcc**GTAATAGGGTATC	BamHI
C256S	GCGATACCTTTATTc**Tcg**agTGGGATAGGAACTATTATTG	XhoI
C291S	CCAAAAATATATCTGC**ctcg**agTGTACCTGCTTTTATTG	XhoI
C340S	CATATTGACTCTAGTT**agT**CGaCGATTACCGGTTGTATTTTC	SalI
C374S	GACCAATAGGGGTTT**ctG**CAGTTTATATGGC	PstI
A119C	GTGGTTAGTTACg**tgc**GGATTTGCATATGCATTG	BsaA1
G120C	GCTTACGGGTGGTTAGTTACAGCA**tGc**TTTGCATATGCATTG	Sph1

Mutated nucleotides are indicated in lowercase. Changed amino acids have codons indicated in boldface type. Restriction sites that were introduced are underlined, and wherever indicated (−) a site was removed. The forward oligonucleotide of the pair used for mutagenesis is shown. Last two entries indicate oligonucelotides used to replace other residues with cysteine residues

supernatant was then pelleted at 100,000×*g* for 1 h and was then resuspended in a small volume of the same buffer. For Western blotting 25 μg of each sample was routinely resolved on 10 % SDS/polyacrylamide gels. Nitrocellulose transfers were immuno-stained using a primary antibody of anti-GFP polyclonal antibody which was present on the C-terminus of the Sod2 protein (the antibody was a generous gift of Dr. Luc Berthiaume, Department of Cell Biology, University of Alberta). Secondary antibody was goat anti-rabbit polyclonal antibody IRDye 680-conjugated (Bio/Can, Mississauga, ON, Canada). For Western detection, the Odyssey scanning system was used (LI-COR Biosciences, USA).

Trypsin treatment of microsomal membranes

Yeast cell membranes were prepared as above. The final concentration of sample was adjusted to 2 mg/ml in 1 mM EDTA, (pH 7.4). Trypsin (phenylalanyl chloromethyl ketone-trypsin, Sigma, St., Louis, MO) was added to give a trypsin:protein ratio of 1:300 and samples were incubated at 30 °C for the indicated times. The reaction was stopped by addition of SDS-PAGE sample buffer and then samples were resolved on 10 % SDS–polyacrylamide gels. Western blotting was against the GFP tag to examine protein fragmentation [10].

Cysteine accessibility of Sod2 proteins

To examine the applicability of the Sod2 protein for the determination of the topology and accessibility of regions of the Sod2 protein, cysteine mutations were introduced into the putative second extracellular loop of the protein (Fig. 1) at amino acids A119 and G120. The accessibility of these residues was determined using a modified procedure we

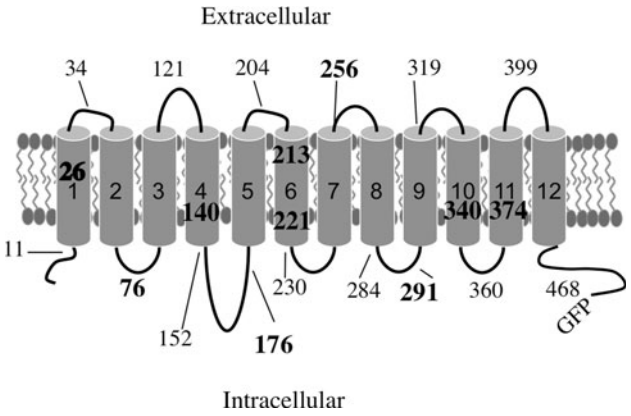
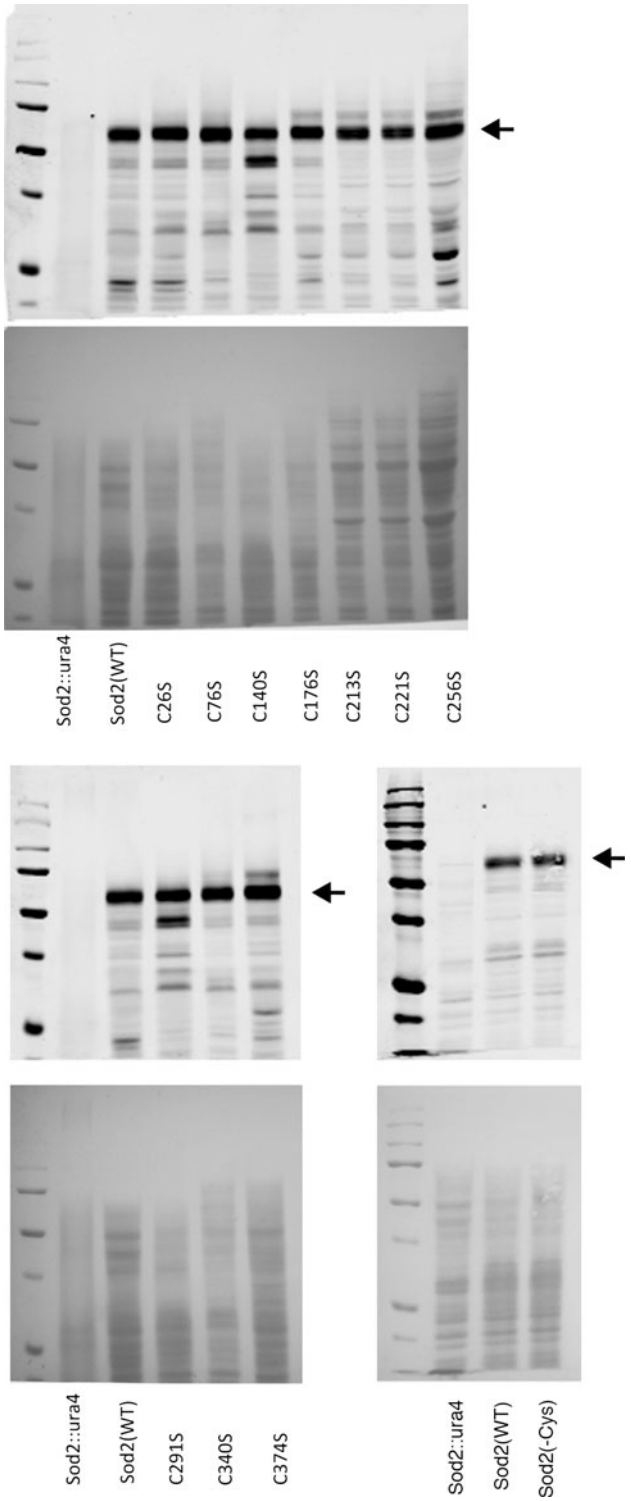


Fig. 1 Models of Sod2. Topological model of Sod2 based on hydrophobicity analysis [24]. *Larger*, *bold numbers* indicate putative positions of cysteine residues. *Other numbers* indicate approximate position of amino acids relative to the membrane, or transmembrane segment number. *GFP* indicates green fluorescent protein that was attached to the C-terminus of the protein

have used earlier [21]. Yeasts containing the cysteineless Sod2, or Sod2 with cysteine introduced at amino acid 119 or 120, were grown to an OD600 nm of 3–4 (∼1 day) in KMAL medium. 1 ml of the previous culture for each sample was used to start a fresh culture of 500 ml in KMAL until the OD600 nm was 2.5. For each strain, 500 ml of grown culture was divided into two portions and cells were pelleted by centrifugation at 3,800g_{max} × 20 min. Cells were washed two times in 10 ml of phosphate-buffered saline and stored at −80°. For pre-extraction, 0.3 ml of frozen membranes was thawed on ice. Samples were made to 4 ml in cold membrane buffer [5 mM EDTA; 1 mM EGTA; 10 mM Tris, pH 8.0 with protease inhibitors PMSF (4 mM) and benzamidine (4 mM)]. Where indicted,



◀**Fig. 2** Western blot analysis of comparative expression of Sod2-GFP containing *S. pombe*. Cell extracts were made from *S. pombe* strains and Western blots were probed with anti-GFP antibody. Molecular weight markers are indicated. *Notations* indicate sample is from cell extracts of *sod2::ura4*: untransformed (sod2::ura4); transformed with wild-type (WT) Sod2 protein; or transformed with the Sod2 DNA with the mutation indicated. *–Cys* indicates the cysteine-free sod2 protein. Cell extracts were prepared as described in "Materials and methods". *Upper panel*, Western blot; *lower panel*, Ponceau S staining of blot prior to reaction with antibody. *Arrow* indicates Sod2–GFP fusion protein

[2-(trimethylammonium)ethyl]methane thiosulfonate bromide, MTSET, was added to a final concentration of 10 mM to one of the pair of samples. Samples were incubated at 30 °C for 30 min, washed five times and finally pelleted. After this treatment, samples were disrupted using a Bullet Blender® essentially as described earlier [22]. Unbroken cells were pelleted by centrifugation (3,500g × 5 min). The sample was centrifuged at 14,000g × 20 min and the supernatant was pelleted at 100,000g × 1 h to collect crude membranes. The pellet was suspended in 300–600 µl of membrane buffer. The samples were solubilized using dodecylmaltoside essentially as described earlier [23]. Briefly, 84 µl of a 10 % solution of dodecylmaltoside was added to each 600 µl of sample. This was maintained at 0 °C and mixed by vortexing every 5 min for 45 min. Samples were centrifuged at 164,000g_{max} and the supernatant of solubilized protein was used for further analysis.

Immunoprecipitation of the Sod2 protein was done using 2 µl of rabbit polyclonal anti-GFP antibody coupled to beads using the Pierce® Crosslink Immunoprecipitation Kit according to the manufacturer's protocol. Coupling the antibody to the beads avoided contamination of the eluate with the immunoprecipitating antibody. The solubilized protein was pre-cleared by addition of control agarose resin as described in the manufacturer's protocol. Immunoprecipitation of Sod2 was by addition of the antibody cross-linked resin. After overnight incubation, Sod2 was eluted and divided into two portions. Part of the eluate was run on SDS-PAGE. Nitrocellulose transfers were immunostained with chicken anti-GFP antibody and a secondary antibody coupled to IRDye680. Another part of the eluate was probed with IRDye800-maleimide (LI-COR) to determine the reactivity of free sulfhydryls. Immunoreactive fluorescence of samples was examined using the LI-COR system. MTSET accessibility = 100-(fluorescence in the presence of MTSET/fluorescence in the absence of MTSET).

Results

In this study, we examined the effect of mutation and removal of cysteine residues from the *S. pombe* protein Sod2, with the ultimate goal of producing a functional cysteineless protein. Figure 1 illustrates a topological model of the Sod2 protein, based on hydrophobicity analysis as reported earlier [24]. The approximate positions of the ten cysteine residues present are indicated. Site-specific mutagenesis was used to delete all of the cysteine residues individually, and then to create a protein that was entirely cysteine free by successive mutation of all of the cysteine

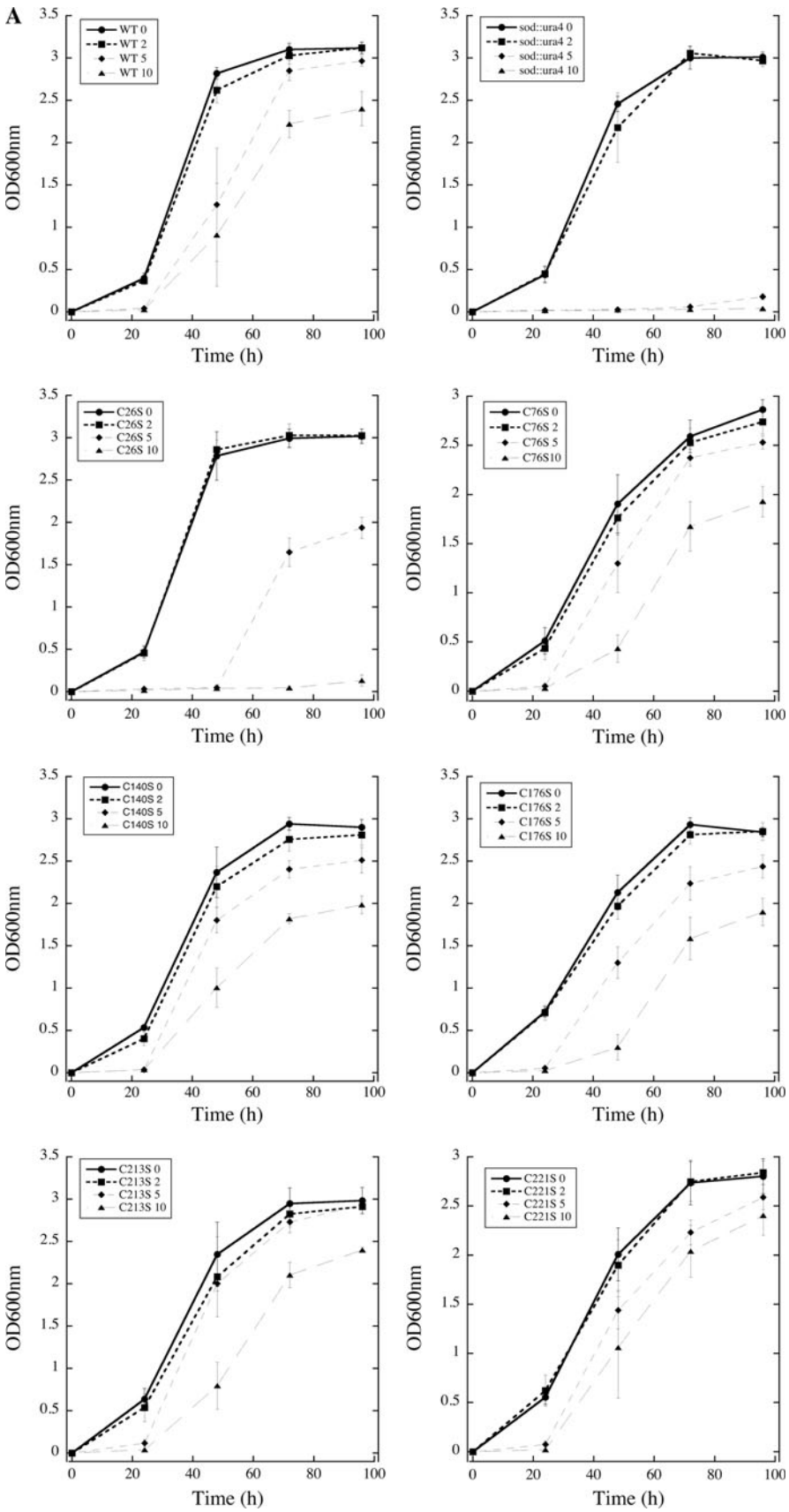
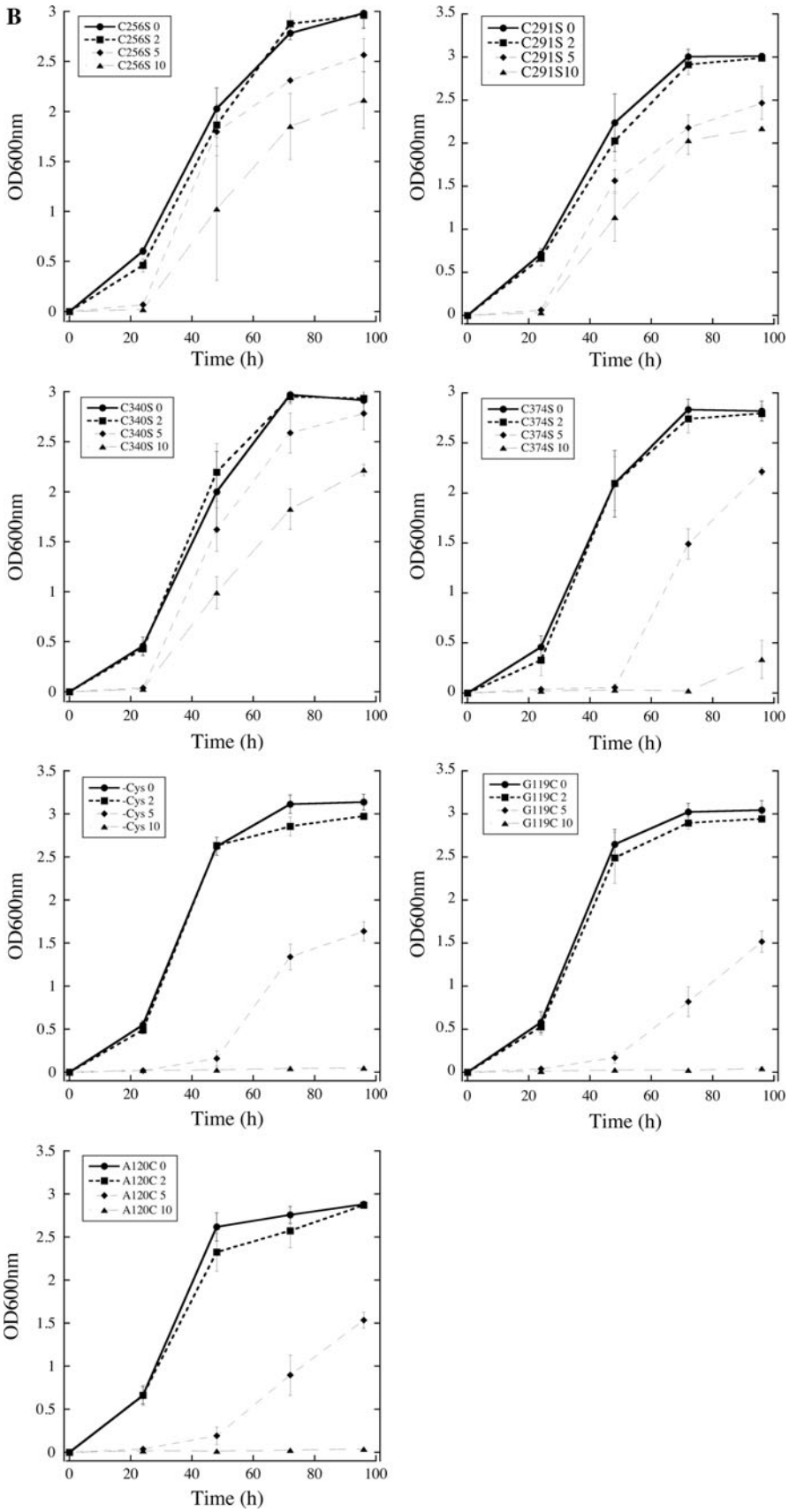


Fig. 3 Growth of *S. pombe* in liquid media with various concentrations of LiCl. *S. pombe* contained either wild-type or cysteine-free mutant Sod2 proteins [or did not contain Sod2 (*sod2::ura4)*]. LiCl tolerance was assessed by inoculating 2×10^6 cells into 2.5 ml of medium at 30 °C and allowing growth for up to 72 h. *S. pombe* were grown in the presence of 0, 2, 5 or 10 mM LiCl. Absorbance of the cell suspensions at 600 nm was measured at the indicated times. Results are the mean ± SE of at least three experiments. Sod2 refers to *sod2::ura4* containing the wild-type Sod2 protein [17]. A, wild type (WT), sod2::ura4 (sod2 knock out), and sod2 with the individual cysteine mutations of C26S, C76S, C140S, C176S, C213S, C221S. B, C256S, C291S, C340S, C374S, −Cys (cysteineless) and G119C (cysteineless with G119C mutation) and A120C (cysteineless with A120C mutation). C, *S. pombe* strains (WT, sod2::ura4, C26S, C347S and −Cys) were grown in the presence of 0, 2, 3, 4, 5, 6, 7, 8, 9 or 10 mM LiCl

Fig. 3 continued



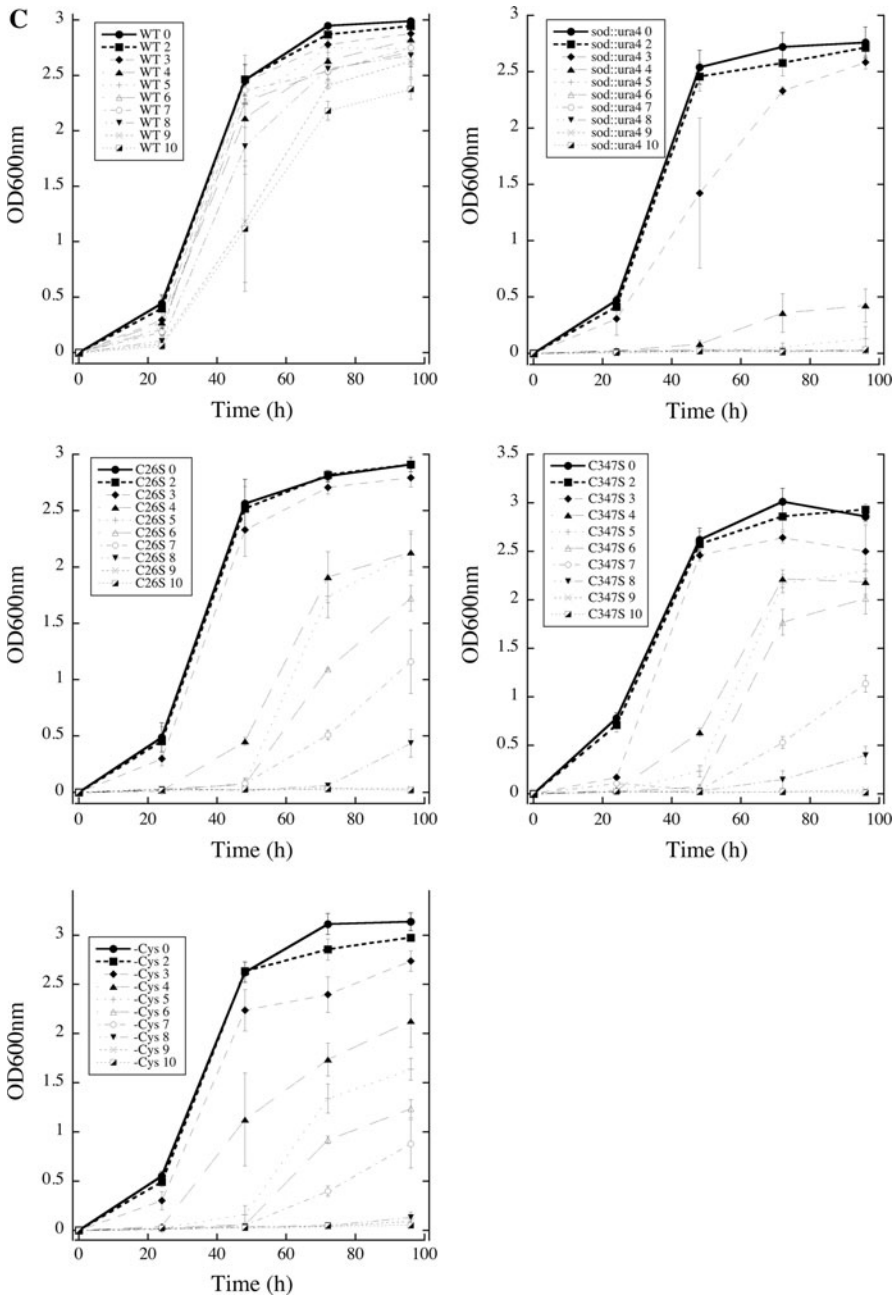


Fig. 3 continued

residues. Initially, to learn about the protein's structure and function, we examined the effect of deletion of individual cysteine residues, and then the effect of removal of all cysteine on expression of the Sod2 protein in *S. pombe*. Crude membrane preparations were made essentially as described earlier [23] and Western blotting was with antibodies against the GFP tag that was present on the C-terminal of the Sod2 protein. Western transfers of proteins were stained with Ponceau S prior to immunoblotting to ensure that the proteins were roughly equivalent in transferred amounts. The results are shown in Fig. 2. The sod2::ura4 knockout strain, which was not transfected with Sod2-containing plasmid, did not contain protein that was immunoreactive with anti-GFP antibody. In contrast, all the mutants expressed the full-length Sod2 protein. The full-length cysteine-free Sod2 protein was also expressed in similar quantities to the wild-type protein.

With the knowledge that all the mutant proteins were expressed, we examined their functional properties, using an assay described earlier [23], assay of their ability to rescue salt sensitivity in the sod2::ura4 strain. Figure 3a–c illustrates the results for LiCl tolerance in liquid media. The sod2::ura4 knockout strain was unable to grow in LiCl-containing media at concentrations of 5 mM or

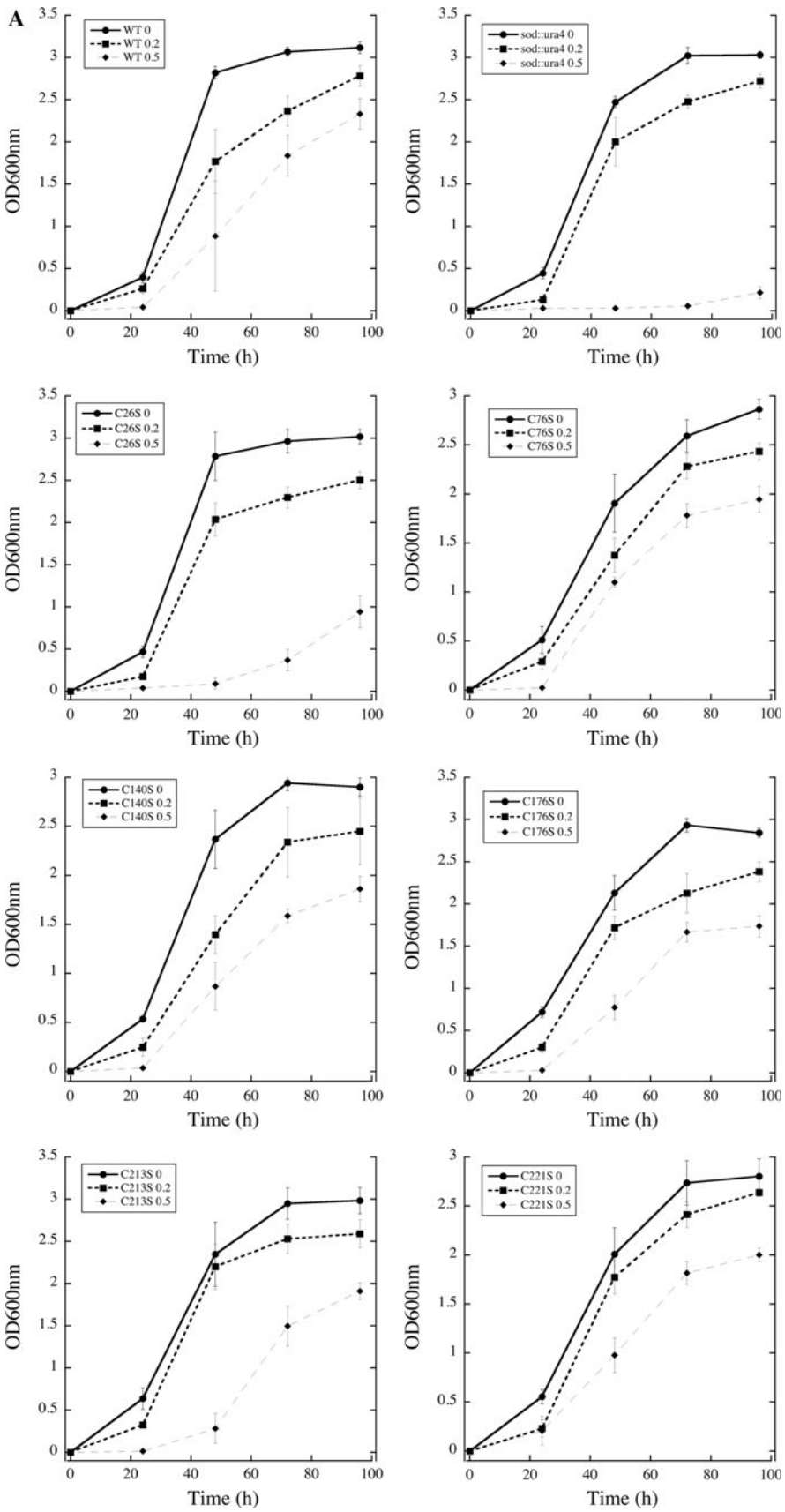
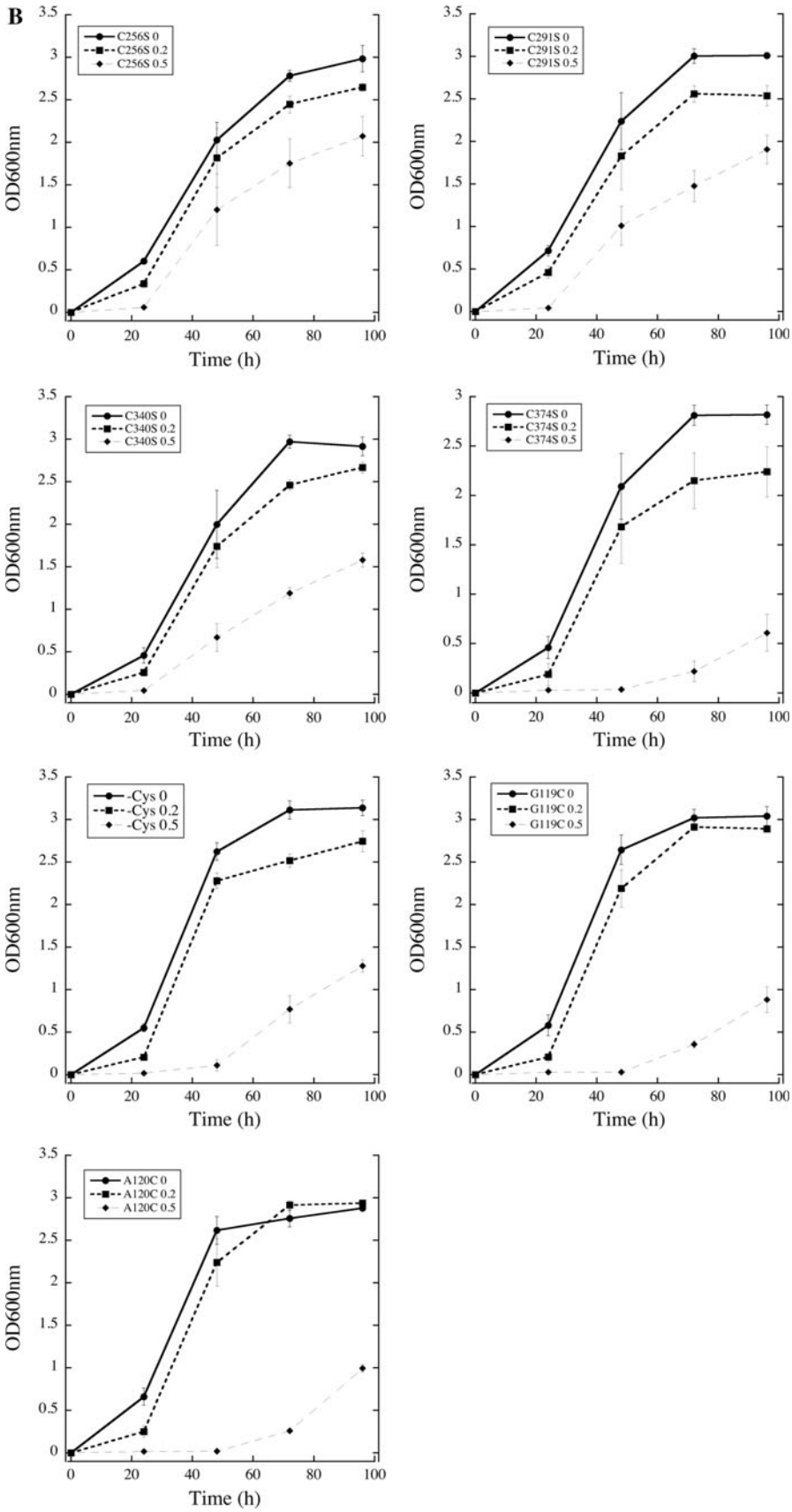


Fig. 4 Growth of *S. pombe* containing either wild-type or cysteine-free mutant Sod2 proteins in liquid media with various concentrations of NaCl, 0, 0.2 or 0.5 M as indicated. Results are the mean ± SE of at least three experiments. Growth determination was as described in Fig. 3

Fig. 4 continued



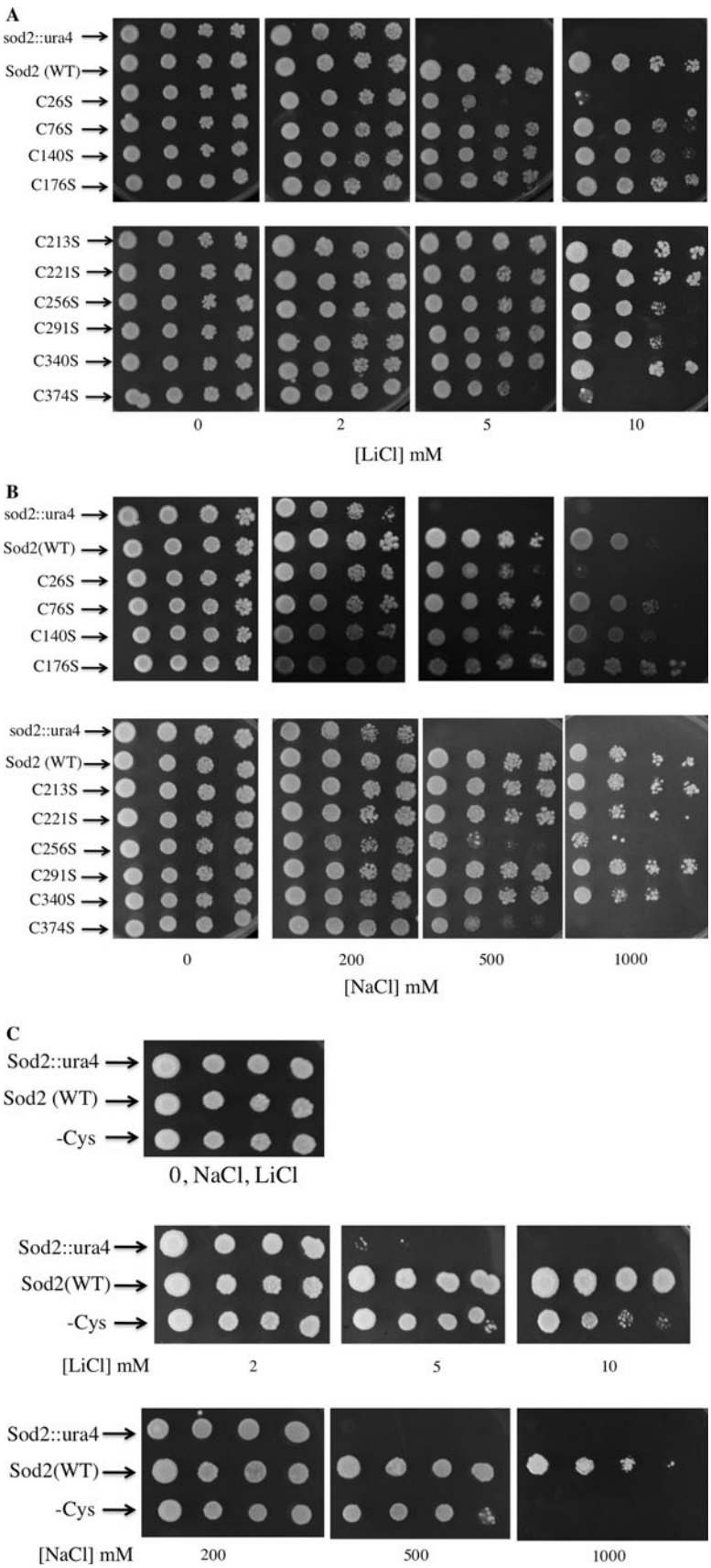


Fig. 5 Growth of *S. pombe* containing wild-type and mutant sod2 on solid media containing various concentrations of LiCl or NaCl as indicated. Samples of stationary phase cultures of the various yeast transformants were serially diluted tenfold and spotted onto minimal media plates supplemented with LiCl or NaCl at the indicated concentration. Plates were incubated for 4–5 days at 30 °C. Sod2 (WT) refers to *S. pombe* transformed with wild-type Sod2 without any mutations. *sod2::ura4* is *S. pombe* with the Sod2 knockout described earlier [11]. Other designations are the *sod2::ura4* yeast strain transformed with pREP-41sod2GFP with the indicated mutation in sod2 or cysteineless Sod2 (−Cys). Results are typical of at least three experiments. **a**, **b** Growth on LiCl or NaCl (respectively) of indicated mutants. **c** Growth of cysteineless (−Cys) relative to that of wild type containing Sod2 and *sod2::ura4* yeast strain

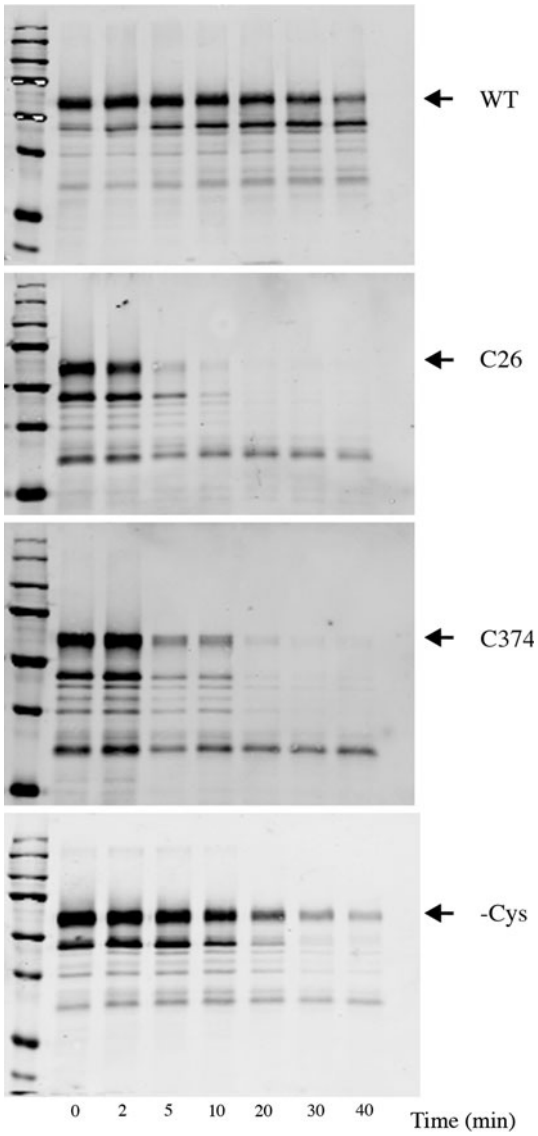


Fig. 6 Analysis of susceptibility of wild-type and mutant Sod2 proteins to trypsin digestion. Yeast cell membrane fractions were incubated with a 1:300 trypsin:protein ratio for 0–40 min at 30 °C as described in "Materials and methods". Samples were then analyzed by SDS-PAGE and Western blotting using anti-GFP antibody. Results are typical of three experiments

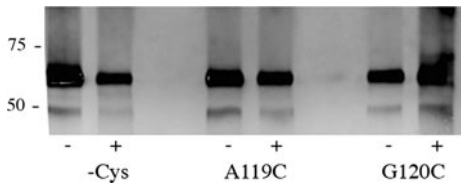


Fig. 7 Accessibility of residues of Sod2 to reaction with MTSET. The accessibility of the residues of Sod2-GFP protein was measured as described in "Materials and methods". For cysteine-free Sod2 protein the sample was solubilized prior to MTSET treatment. Other samples were treated with MTSET on intact cells. Immunoprecipitates were reacted with IRDye800-maleimide. A poorer reaction with MTSET indicates the cysteine residue reacted with MTSET prior to reaction with IRDye800-maleimide. + indicates sample was treated with MTSET

greater. *S. pombe* with wild-type Sod2 protein grew well in LiCl-containing media up to 10 mM. Mutant forms of Sod2 protein with single cysteine-to-serine substitutions all grew in media containing 10 mM LiCl with only little or no deterioration in their growth ability. The exceptions were Sod2 with the C26S and the C374S mutations, which did not grow in medium with 10 mM LiCl, but did grow reasonably well in media with 5 mM LiCl (with reasonable salt tolerance defined as OD600 >0.5 after 96 h).

Similar results were obtained when testing the resistance to growth in NaCl-containing medium. In this case, wild-type Sod2 and most of the mutants were completely resistant to external NaCl up to 500 mM. The *sod2::ura4* knockout strain did not grow in an external NaCl concentration of 500 mM. Yeast containing either of the C26S and the C374S mutants displayed some resistance to the addition of 500 mM NaCl, though they did not grow as well as cells with wild-type Sod2 (Fig. 4a, b).

We also characterized the effect of mutation of all the cysteine residues of Sod2 to serine. In liquid medium containing LiCl, the cysteineless Sod2 protein conveyed good resistance to 5 mM LiCl, but not to 10 mM LiCl. Two other mutants of Sod2 were also characterized. They were the cysteineless Sod2 with cysteine residues reintroduced at positions 119 and 120. Both conveyed good salt tolerance to 5 mM LiCl, but not to 10 mM LiCl. Similar results were obtained with NaCl. Cysteineless Sod2 conveyed good salt tolerance to 500 mM NaCl, and the G119C and A120C mutants also conveyed good salt tolerance to 500 mM NaCl.

Growth in intermediate concentrations of LiCl was characterized for wild-type Sod2, for the C26C and C347S and the cysteineless Sod2. Wild-type Sod2 protein conveyed salt tolerance in up to 10 mM LiCl. The Sod2 knockout strain (sod2::ura4) was greatly inhibited in growth above 3 mM LiCl. All of C26C, C347S and the cysteineless Sod2 rescued salt tolerance in up to 7 mM LiCl.

Complementary experiments (Fig. 5a–c) examined the growth of the mutant containing strains and the cysteineless containing strain on solid media containing either LiCl or NaCl. On LiCl-containing media (Fig. 5a), yeast containing wild-type Sod2 protein grew well in concentrations up to 10 mM, while the *sod2::ura4* strain did not grow in medium containing 5 or 10 mM LiCl. Single cysteine mutant containing strains all displayed improved salt tolerance relative to the *sod2::ura4* strain, though the C26S and C374S containing strains were not very tolerant of 10 mM LiCl. Results with NaCl (Fig. 5b) followed the same pattern. All single cysteine mutants were improved in

salt tolerance relative to the *sod2::ura4* knockout strain, though the C26S and C374S containing strains were not as tolerant as the others in the highest NaCl concentrations. The cysteineless Sod2-containing strain displayed LiCl tolerance nearly equivalent to the wild-type Sod2, though growth in the highest NaCl concentration was impaired.

We examined whether several of the Sod2 variants were properly folded using limited digestion with trypsin. We have earlier used this method for analysis of structural changes of membrane proteins including Sod2 [10, 25]. It is dependent on changes in the accessibility of arginine and lysine residues. We examined the wild-type Sod2 protein, the cysteineless protein and the mutants C26S and C374S that showed reductions in their ability to confer salt tolerance. The results are shown in Fig. 6. Mutants C26S and C374S showed increased sensitivity to trypsin and the primary Sod2 protein-immunoreactive species was mostly digested within 5 min. In contrast, the cysteineless Sod2 protein was more resistant to trypsin digestion. Its digestion pattern was closely similar to the wild-type protein.

Finally, after confirming that the cysteine-free sod2 protein was active, we examined two residues of putative extracellular loop 2 (Fig. 1) which were mutated to cysteine, to make the mutants A119C and G120C. As noted above, these proteins conveyed salt tolerance to the *sod2::ura4* strain indicating they were functional proteins. We tested the accessibility of these mutants using a procedure we established for the mammalian Na^+/H^+ exchanger protein [21]. For these experiments we used the cysteine-free Sod2 protein as a control. Though it had no cysteine residues remaining in sod2, there are two cysteines remaining in the intracellular GFP tag. For the cysteine-free Sod2, we solubilized the protein prior to MTSET addition, allowing accessibility to the intracellular cysteines remaining on the GFP tag. For mutants of Sod2 A119C and G120C, MTSET treatment was on the intact *S. pombe* cells. Examples of the results are shown in Fig. 7. MTSET treatment of the solubilized cys-less Sod2 protein resulted in decreased labeling by IRDye800-maleimide. Accessibility was 60.2 (±17.7 %). (Without prior solubilization of Sod2, MTSET treatment did not affect accessibility; not shown). For the A119C mutant, the treatment of intact cells reduced labeling by IRDye800-maleimide. The residue was 58.8 (±4 %) accessible. In contrast, the G120C mutant Sod2 labeling by IRDye800-maleimide was not reduced by treatment of intact cells with MTSET and the residue was completely inaccessible.

Discussion

Sod2 is the Na^+/H^+ exchanger of the plasma membrane of the fission yeast *S. pombe*. Families in its class of proteins provide salt tolerance to plants and yeast. While salt tolerance is important economically, little is known about the structure of this entire class of proteins. We have recently shown that TMIV is critical to Sod2 function and have examined the structure of TMIV and modeled the Sod2 protein [22]. Although this study represents the first such detailed results on this class of proteins, little other data for the balance of the Sod2 protein are available aside from studies of isolated amino acids that were suggested to be important because of their polar nature within a hydrophobic bilayer [10, 11, 26].

One way to learn about the topology and the pore of membrane proteins is to construct a cysteine-free protein and then to use cysteine accessibility or cysteine scanning mutagenesis. This approach has been used to study a number of membrane proteins including the mammalian Na^+/H^+ exchanger isoform one [13, 27], a Na^+/glucose co-transporter [28] and Cx46 hemichannels [29]. A prerequisite of this analysis is that after removal of the cysteine residues of the protein, a major portion of the activity of the protein remains, indicating that the protein maintains its structural integrity.

With this in mind, we set about examining the Sod2 and systematically eliminating cysteine residues of the protein both individually, and as whole, also characterizing a full-length cysteine-free protein. Individual elimination of most of the cysteine residues did not have an adverse effect on the protein, with the exception of an effect caused by C26S and C374S mutation. When either of these mutant proteins was expressed in sod2::ura4 *S. pombe*, they restored salt tolerance, though not quite as well as the other mutants. The reason for this effect is uncertain at this time; however, we suspected that it might be due to improper folding of the Sod2 protein, in the absence of these cysteines. This suggestion was supported by the results of limited trypsin digestion. They showed that these two proteins had undergone a change in conformation that allowed different access to the trypsin in comparison to the wild type. We have earlier constructed a cysteine-free mammalian Na^+/H^+ exchanger protein [16]. Surprisingly, we found a similar phenomenon in this protein. Mutation of a serine in N-terminal of the protein (amino acid number 9) and in much more distal region of the membrane domain of the protein (amino acid number 477) resulted in decreases in activity of the protein. Recently, we have suggested that Sod2 has a structure that is similar to NhaA [22]. Others [7] have suggested that the mammalian Na^+/H^+ exchanger NHE1 also has a structure similar to that of NhaA. If the mammalian Na^+/H^+ exchanger and Sod2 have a similar structure, it may be that there is a common folding pattern and a common folding defect related to the mutated cysteines. Future experiments could examine this hypothesis.

When all the cysteine residues of Sod2 were removed, the protein was functional. This was borne out by it conferring salt tolerance to the sod2::ura4 strain. There was a reduction in its effectiveness in conferring salt tolerance, though it clearly conveyed salt tolerance indicating that the protein was active. There was some indication that the cysteineless sod2 protein was in a more functional or improved conformation relative to the C26S and C374S mutant proteins. On solid media the cysteineless sod2 conveyed salt tolerance to 10 mM LiCl, which was better than the C26S and C374S mutant proteins. Additionally, limited tryptic digestion suggested that the conformation of the cysteine-free Sod2 protein was quite similar to the wild type, unlike the C26S and C374S proteins. Interestingly, we found a possibly related phenomenon also present in the mammalian NHE1 isoform of the Na^+/H^+ exchanger. Removal of all the cysteines resulted in a fully functional protein even though removal of some of the cysteines individually impaired protein function [21]. The presence of this phenomenon may be caused by the removal of the balance of the cysteines preventing stabilization of adverse folding through aberrant disulfide formation. That this occurs in both the mammalian Na^+/H^+ exchanger and in Sod2 of *S. pombe* could be indicative of a similarity in structure between the two proteins, as noted above.

Since the Sod2 cysteine-free protein was functional, we confirmed the utility of the protein for the determination of membrane topology and accessibility. We found that we were able to determine the accessibility of amino acid A119C to externally applied MTSET. This compound reacted with the cysteine placed at this position, confirming that this amino acid is externally accessible. The protein with a mutation of the adjacent amino acid (G120C) was not accessible to MTSET. We have earlier [21] found that adjacent amino acids of extracellular loops can vary greatly in their accessibility, likely due to local differences in structure, burying side chains. Some models have suggested that Ala119 is present on the extracellular loop two of Sod2 [24] (Fig. 1); however, further study of more surrounding amino acids are necessary to conclusively assign this position to an extracellular loop. It is also possible that Ala119 is an amino acid present within a transmembrane segment as suggested by computer modeling of sod2 [22]. Ala119 could be within the membrane, but accessible as part of the pore of the protein. Further study of surrounding residues is necessary to conclusively assign a location.

In summary, a functional cysteine-free Sod2 protein was created that could be used to study the structure of the protein. We demonstrate that Sod2 can have abnormal conformation and likely folding, with mutation of only C26 and C374. We also show that amino acid position 119 is accessible from the extracellular surface. Future studies will examine the topology and pore lining residues of Sod2 in detail.

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Conflict of interest None.

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