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Expression, purification, and reconstitution of the Na^+/H^+ exchanger sod2 in *Saccharomyces cerevisiae*

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Abstract Sod2, is a Na^+/H^+ exchanger present on the cytoplasmic membrane of the fission yeast *Schizosaccharomyces pombe*. It expels toxic Na^+ from the cytosol. Sod2 was expressed in *Saccharomyces cerevisiae* with a C-terminal histidine tag under control of the GAL1 promoter. Western blots using anti-V5 antibodies identified the tagged protein. Solubilization of the protein was by *n*-dodecyl β-D-maltoside. Immobilized Ni-ion column affinity chromatography partially purified the protein at a yield of ~240 μg per liter of culture. Sod2 was present as a 40-kDa and an 80-kDa protein, however, it co-purified with a number of other proteins. Cross linking of sod2 with *N,N′*-(*o*-phenylene)dimaleimide showed that sod2 was present in association with a number of other proteins as a larger molecular weight complex. Partially purified sod2 protein was reconstituted in proteoliposomes and functionally active. Our results suggest that the sod2 protein associates with a number of other proteins and can be expressed in *S. cerevisiae* in active form.

Keywords Dodecyl maltoside · His-tag purification · Na^+/H^+ exchanger · Reconstitution · sod2 · Solubilization

Abbreviations used

DDM	*n*-dodecyl β-D-maltoside
DSS	Disuccinimidyl suberate
o-PDM	*N,N′*-(*o*-phenylene)dimaleimide

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Introduction

The Na^+/H^+ exchanger is a ubiquitous protein that transports Na^+ and H^+ in opposite directions across cell membranes. In mammals, Na^+/H^+ exchange is responsible for pH regulation, removing excess intracellular acid in exchange for extracellular sodium. In *E. coli*, plants and yeast, Na^+/H^+ exchange occurs principally in the opposite directions, using inward movement of protons from the proton motive force to remove excess intracellular sodium [1, 2]. Sod2 is a Na^+/H^+ exchanger of the fission yeast *Schizosaccharomyces pombe*. It is a plasma membrane protein that was determined to exchange sodium for protons and is responsible for most of sodium and lithium tolerance in this species. The *sod2* gene was cloned and characterized using selection for LiCl resistance [3] and its disruption results in an inability to extrude cytoplasmic Na^+ and to take up external protons in exchange for internal sodium ions. We have earlier examined several individual amino acids of this protein [4] and shown that they may be involved in Na^+/H^+ exchanger function [5].

While membrane proteins are vital in signal transduction, transport energy conversion and ion conductance, and account for approximately 25% of open reading frames in genomes [6], as a group they are difficult to over express and purify for structural analysis. Research in studying membrane protein structure has lagged greatly in comparison to that achieved with soluble proteins. Expression systems used for soluble proteins such as *E. coli*, often do not work well for eukaryotic membrane proteins with more than one transmembrane segment. Expression of these proteins in *E. coli* at high levels often impairs viability and the expressed proteins are often misfolded and in inclusion bodies [7, 8]. Mammalian Na^+/H^+ exchanger isoforms have been successfully expressed in *Saccharomyces cerevisiae,* although, only in

relatively small amounts with NHE1 expressed either as a mistargeted protein or being functionally inactive [9–11]. We were aware that one ion exchanger protein had been produced at high levels in *S. cerevisiae* [12]. We reasoned that this might be an excellent system in which to over express the *S. pombe* protein sod2. Our results show that sod2 can be successfully over expressed and enriched in functional form in this system and appears to exist as a protein complex.

Materials and methods

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless stated otherwise. Yeast *S. cerevisiae* INVSc.1, pYesDest52 vector, pDonr21 vector, BP Clonase™ II enzyme mixture, and LR Clonase™ II enzyme mixture for BP were purchased from In Vitrogen (Burlington, Ont.). Ni-NTA agarose was purchased from Qiagen (Valencia, CA). Products for culture of *S. cerevisiae* were purchased from DIFCO Media Products. Taq DNA polymerase was purchased from Invitrogen life technologies. Restriction and modification enzymes were from New England BioLabs (Beverly, MA). Detergents were purchased from Sigma Co. (Sigma, MO, USA). L-α-lysophosphatidylcholine and egg-yolk L-α-phosphatidylcholine were from Sigma Co. (Sigma, MO, USA). Bio-Beads (SM-2) were from Bio-Rad (Hercules, CA, USA). His-probe mouse monoclonal antibody IgG was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Media and buffers

The following media were routinely used: YPD medium, 1% yeast extract, 2% peptone, 2% D-glucose; SC-U medium, 0.67% yeast nitrogen base without amino acids with ammonium sulfate, 2% glucose, 0.01% (adenine, arginine, cysteine, leucine, lysine, threonine, tryptophan), 0.005% (aspartic acid, histidine, isoleucine, methionine, phenylalanine, praline, serine, tyrosine, valine), 2% agar (for plates); SC-U induction medium, SC-U medium containing 2% galactose and 1% raffinose; Lysis Buffer, 1.85 M NaCl, 7.5% β-mercaptoethanol, 9.9% ethanol; Buffer 1, 25 mM HEPES pH 7.5, 2 M sorbitol, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, 1 mM PMSF, added by SL 0.5 µl/ml; Buffer2, 25 mM HEPES pH7.5, 20% Glycerol (V/V); Loading Buffer, 300 mM NaCl, 10 mM Tris–HCl pH 8.0, 10 mM imidazole, 0.1 mM PMSF, 0.1 mM Benzamidine, and a proteinase inhibitor cocktail (0.5 µl/ml buffer) [13]; Native Wash Buffer, 300 mM NaCl, 10 mM Tris–HCl pH8.0, 20 mM imidazole, 0.1 mM PMSF, 0.1 mM Benzamidine, and a proteinase inhibitor cocktail (0.5 µl/ml buffer) [13]; Elution Buffer, 300 mM NaCl, 10 mM Tris–HCl pH8.0, 100 mM imidazole. Reconstitution buffer, 20 mM BTP-MES, pH 7.5, 25 mM $(NH_4)_2SO_4$, 10% Glycerol.

Construction of a sod2 expression vector

Sod2 cDNA [4] was amplified by PCR using *Platinum Taq* DNA polymerase (In Vitrogen). The forward primer was 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTAATAATGGGCTGGAGACAACTTGATATAG-3′ and the reverse primer was 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCAACGTAATCTTCCTGTGACTTATC-3′. The primers had cloning sites: attB1 and attB2 (underlined) designed for use in the Gateway™ cloning system (Life Technologies, Inc (Rockville, MD)). The PCR product was cloned into the yeast expression vector pYesDest52, so as to add a 6 His tag at the C-terminus. DNA sequencing by the Department of Biochemistry (University of Alberta) core facility was used to confirm the fidelity of the product.

Expression and characterization of sod2 in yeast S. cerevisiae

The *S. cerevisiae* strain INVSc1 (Life Technologies, Inc (Rockville, MD)) was transformed via electroporation with the pYesDest52-*sod2* construct. After electric pulse, 1 ml 1 M ice-cold sorbitol was immediately added into the electroporation cuvette, as well as cells and medium were transferred to selective plates (SC-U medium selective plates). After 48–60-h of growth, transformant colonies were obtained. In the *S. cerevisiae* strain used (INVSc.1), transcription from the *GAL1* promoter is repressed in the presence of glucose. Removing glucose and adding galactose as a carbon source induces transcription. In order to induce expression of sod2 in our transformants, positive colonies were selected and grown to an $OD_{600\ nm}$ of ~1.5 and then induced by removing glucose and adding galactose to a final concentration of 2%. The detection of recombinant sod2 fusion protein was carried out by TCA precipitation of samples followed by Western blotting. For TCA precipitation, 1.5 ml culture ($OD_{600\ nm}$ ~1.5) was centrifuged at 6,000 × *g* for 5 min. The pellet was washed with 500 µl ddH_2O and centrifuged again. The pellet was resuspended in 120 µl lysis buffer at room temperature for 10 min. The lysed cells were made to a final concentration of TCA of 10%. The sample was spun down at 18,000 × *g* at 4°C for 15 min and the supernatant is discarded. About, 300 µl of cold acetone was added and the sample was spun down again at 4°C. The dried pellet is resuspended in 120 µl 1 × SDS PAGE loading buffer and heated at 100°C for 3–5 min. About 30 µl of sample was used for SDS

PAGE. For Western blotting the anti-V5 body was used as the first antibody.

For large scale production of the sod2 protein, several transformants were screened and the colony with the highest level of expression was used. The colony was grown in 10 ml of SC-U media in 50 ml flasks at 30°C for 36 h. The 10 ml of yeast culture was then transferred to 290 ml of SC-U media in 1-l flasks and when the $OD_{600\ nm}$ of the growing yeast cells was $\sim$1.5, 700 ml of SC-U induction media was added. This 1-l of culture was transferred to a 6-l flask and grown for 8–10 h at 30°C until an $OD_{600\ nm}$ of approximately 1.4–1.5).

Solubilization, purification, and reconstitution of sod2 protein

The 1-l culture of yeast cells was centrifuged at 4°C at 4,000 × *g* for 8 min. The supernatant was decanted; the cells were resuspended in 500 ml H_2O, were centrifuged at 4°C at 4,000 × *g* for 8 min. The pellet containing yeast cells was resuspended in 40 ml buffer 1 with an added proteinase inhibitor cocktail [13]. The yeast cells were broken via mechanical disruption by passage of 6 times through a microfluidizer at 4°C at 20,000–25,000 psi. The crude extract from lysis of yeast cells was centrifuged at 4°C at 6,000 × *g* for 10 min, the pellet was discarded and the supernatant was centrifuged at 4°C at 15,000 × *g* for 10 min. This supernatant was diluted twofold with 0.6 M sorbitol and precipitated with NaCl (0.15 M final concentration) and PEG4000 (10% final concentration). The solution was put on ice for 30 min, and then centrifuged at 4°C at 12,000 × *g* for 20 min. The supernatant was decanted and the pellet containing microsomes was suspended in buffer 2 and then put on ice for 10 h. The suspension was diluted with buffer 2 and adjusted to 4 mg/ml. Subsequently, it was diluted by twofold with 10 mM Tris–HCl pH 8.0 to give a final concentration of suspension protein 2 mg/ml.

In order to solubilize sod2, the detergent DDM was added to the suspension for 8 h at 4°C. In order to determine the optimum ratio of detergent DDM to protein (W/W), a range of DDM/protein ratios were initially used—12:1, 9:1, 6:1, 3:1, and 1:1—for solubilization trials. The optimum ratio of 3:1 was routinely used. The solubilized suspension was centrifuged at 4°C at 100,000 × *g* for 60 min. The supernatant was used for purification of the protein via Ni-NTA affinity chromatography essentially as described by the manufacturer (Qiagen).

Ni-NTA affinity chromatography was performed at 4°C. A 50% Ni-NTA slurry was resuspended in 30% ethanol in 5.0 ml and was poured into a column. The column was equilibrated with six volumes (30 ml) of loading buffer. The solubilized protein (about 200 ml) was loaded onto the Ni-NTA column at a flow rate of 0.3 ml/min. The sample flow through was collected and re-run on the column. The second flow through was saved for SDS-PAGE and Western blotting analysis. The Ni-NTA column was washed with 40 ml of loading buffer and the wash was collected. The Ni-NTA column was then washed with 40 ml of native wash buffer. In order to elute the bound sod2 membrane protein, 16 ml of elution buffer was used and 200 µl fractions were collected.

Fractions enriched in sod2 protein were identified using Western blotting against an anti-V5 tag antibody. Sod2-enriched fractions were pooled and were concentrated using an Amicon ultrafiltration device at 4°C. About 50 µl of concentrated sample was taken for SDS-PAGE and silver stained.

Reconstitution of sod2 protein into proteoliposomes followed established protocols [14]. Briefly, 2.34 mg of egg yolk phosphatidyl choline was dried to a thin film under nitrogen gas, and lyophilized overnight. Dried lipids were mixed with a reconstitution buffer (20 mM BTP-MES pH 7.5, 25 mM $(NH_4)_2SO_4$, 10% glycerol, supplemented with the fluorescent pH indicator 2.5 mM pyranine), 20 µl of 20% *n*-octyl-D-glucoside and 10 µg of partially purified sod2 was added to a final volume of 220 µl. The solubilized protein/lipid/detergent mixture was applied to a 4 ml Sephadex G-50 column that was preloaded with reconstitution buffer containing pyranine. The column eluate fractions 3 and 4 (220 µl /fractions), were incubated for 30 min at room temperature with 100 mg of wet SM-2 Bio-Beads to remove excess detergent. The sample was again applied to a 4 ml Sephadex G-50 column that was preloaded with reconstitution buffer without pyranine. The resultant proteoliposomes containing sod2 were monitored for Na^+/H^+ exchanger activity via pyranine fluorescence using a PTI Deltascan spectrofluorometer with an excitation wavelength of 463 nm and an emission wavelength of 510 nm. Proteoliposomes containing sod2 (400 µl) and control liposomes (400 µl) were incubated at 25°C, followed by dilution into a 2-ml reaction cuvette containing ammonium free reconstitution buffer (20 mM BTP-MES pH 7.5, 10% glycerol) to generate a pH gradient. NaCl (135 mM) was added to initiate Na^+/H^+ exchange, which was monitored by the increase in pyranine fluorescence. Control proteoliposomes were made by the same procedures as described above with experimental extracts from yeast INVSc.1 that were not transformed with sod2 expression plasmid.

In order to check for the ability of sod2 to form oligomers, crude membranes containing sod2 were made as described above. These were incubated with the cross linkers DSS or *o*-PDM at the concentrations indicated for 30 min at room temperature. The cross linked products

were then subjected to SDS-PAGE and Western blot analysis using the anti-V5 monoclonal antibody.

Results

Expression and characterization of sod2 in yeast *S. cerevisiae*

The sod2 protein was cloned into the yeast expression vector pYesDest52 to create the construct pYesDest52-*sod2*. This expression vector utilizes the GAL1 promoter, allowing for relatively high levels of expression of sod2 by induction with galactose. Expression was designed such that a six histidine tag is at the C-terminus of the sod2 protein. The recombinant plasmid pYesDest52-*sod2* was transformed and expressed in yeast *S. cerevisiae* INVSc.1 as described above. DNA sequencing confirmed the identity and fidelity of the cloning (not shown).

In order to examine the expression of the sod2 protein, several colonies were picked and induced for different times. Whole cell lysates were made by TCA precipitation of the proteins and the expression levels from different colonies were examined by Western blotting against the anti-V5 tag engineered on the C-terminus of the protein. Figure 1a, b illustrates the results. Sod2 was present in the samples as two bands, one of ~40 kDa and a second of ~90 kDa, which was presumably a dimer. The sizes are roughly in agreement with the predicted size of the sod2 protein with the V5 and His tags (56.8 kDa). It should be noted that transmembrane proteins often run at slightly anomalous molecular weights on SDS-PAGE. There was noticeable variation in the expression levels of one colony versus another. Colony #3 expressed much better than colonies #1, 2 and 4. An examination of induction times showed that a shorter time of 8 h gave better expression as opposed to much longer times. For expression of the protein, shorter times of induction and colonies that expressed well were used.

Solubilization of sod2 protein

Initial trials of solubilization of crude membranes was using the detergent DDM, at a protein final concentration of 2 mg/ml and with detergent/protein (W/W) ratios of from 12:1 to 1:1. Samples were solubilized as described in the "Materials and methods" and after centrifugation at 100,000 × *g*, the samples from the supernatant were then run on SDS-PAGE. Western blotting against the V5 tag was used to detect the sod2 protein. Figure 2 illustrates the results. Detergent to protein ratios of 12:1 to 3:1 were effective in solubilization of sod2 while a ratio of 1:1 was not as effective. A ratio of 3:1 was routinely used as a concentration that was effective yet minimized the use of expensive detergent.

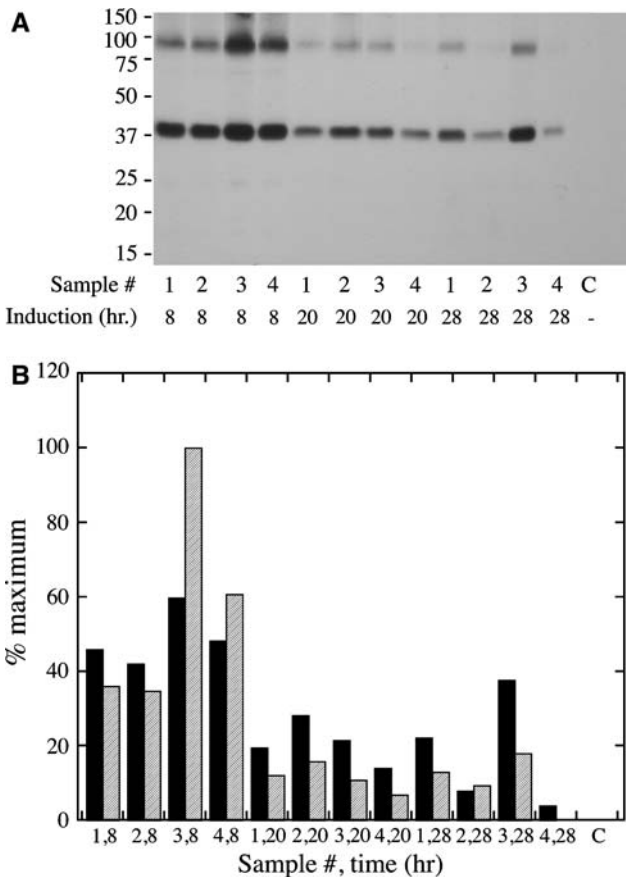

Fig. 1 Western blotting and analysis of sod2 expression in different yeast colonies at various times of induction: Yeast colonies were transformed with pYesDest52-sod2 and expression of sod2 was determined using an antibody against the V5 tag. (**a**) Example of Western blot. Various colonies of transformants (#1-4) were picked and induced for the indicated times. (**b**) Summary of levels of expression of tagged sod2 protein from quantification of Western blots as in (**a**). Notation indicates sample number followed by hours of induction, either 8, 20, or 28. "C" refers to a negative control which did not have the pYesDest52-sod2 plasmid and was induced for 8 h

Purification and reconstitution the of sod2 protein

Partial purification of the sod2 protein was through immobilized metal affinity chromatography. Figure 3a illustrates some typical results. A majority of the sod2 protein bound to the column. Some was removed by washing the column with loading buffer or low concentrations of imidazole, however, the majority remained on the column. Washing the column with elution buffer containing 100 mM imidazole resulted in removal of the protein from the column (Lanes 5–7). Further treatments of the column did not result in additional elution of the sod2 protein. Figure 3b illustrates a silver stain of the eluate of sod2 containing fractions. A 40 kDa band was apparent likely representing the sod2 protein. However,

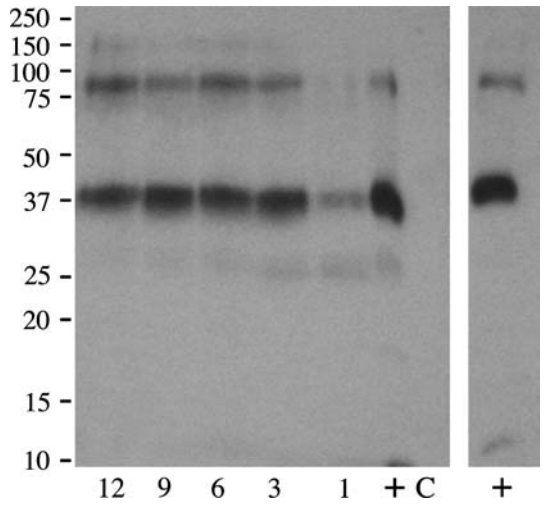

Fig. 2 Western blot analysis of sod2 solubilization with DDM: Samples of sod2 expressed in *S. cerevisiae* were treated with varying detergent to protein ratios as described in the "Materials and methods." Numbers underneath the lanes indicate the ratio of detergent to protein. "+" indicates positive controls, C indicates a negative control

a number of other protein bands were also apparent. Densitometric scans (not shown) suggested that the sod2 protein comprised ~15–20% of the protein present. The yield of partially purified sod2 protein was ~240 μg of sod2 per liter of culture.

In order to confirm that recombinant sod2 protein was functional and could conduct cation exchanger, the partially purified protein was reconstituted into phosphatidylcholine vesicles in the presence of ammonium (NH_4^+) and the fluorescent pH indicator pyranine. Pyranine fluorescence has been shown to directly reflect the intra-vesicular pH and has been used earlier for measurement of H^+ fluxes [15, 16]. Dilution of the proteoliposomes into ammonium free buffer results in acid loading of the vesicles due to outward diffusion of ammonia (NH_3). Efflux of intra-vesicular H^+ in exchange for extra-vesicular Na^+ was monitored by increased pyranine fluorescence upon addition of NaCl (Fig. 4a). The assays showed that sod2 containing proteoliposomes mediated rapid cation exchange. When a mock purification of sod2 protein was done and samples were used to the attempt for reconstituting Na^+/H^+ exchanger activity, there was no activity when NaCl was added (Fig. 4b).

In order to examine if the sod2 protein was present as an oligomer, we used DSS and *o*-PDM to determine if it was in association with itself or another protein. Figure 5a demonstrates the effect of DSS on the apparent molecular weight of the tagged sod2 protein. For these experiments, a crude membrane fraction of the yeast cells (Lanes 1–5) was used. Lane 6 illustrates a partially purified protein for comparative purposes. In the crude membrane fractions a majority of the sod2 immunoreactive protein was present as an 80 kDa species, while there was always some relatively insoluble material of over 250 kDa in size present. DSS caused a decrease in the amount of 40 kDa sod2. Partially purified sod2 protein was principally present as a monomer. A similar experiment was done with *o*-PDM (Fig. 5b). Crude membranes contained principally the 80 kDa form of the sod2 protein and some larger

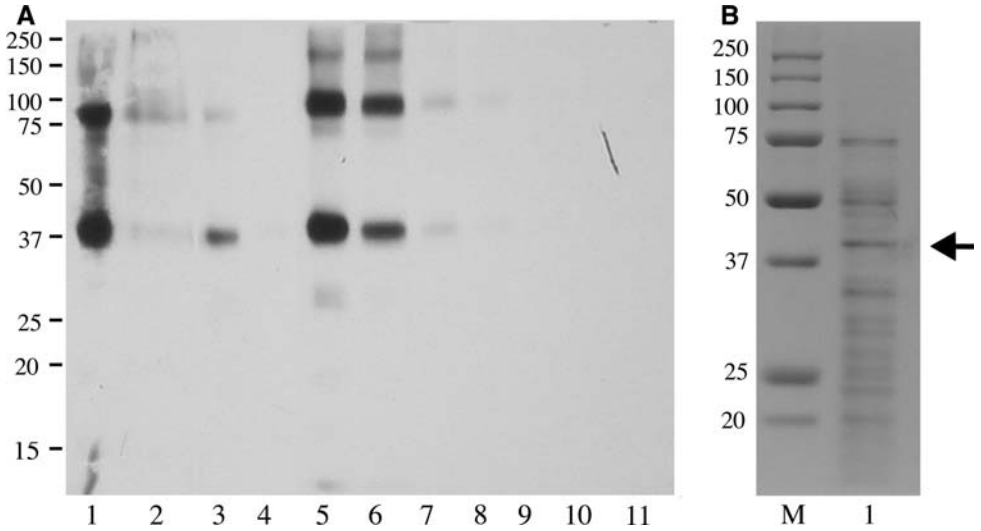

Fig. 3 Purification of the sod2 protein. (**a**) Western blot analysis of sod2 purification by immobilized metal affinity chromatography. Sod2 protein was expressed as described in the "Materials and methods" and was purified using Ni-NTA Agarose resin. Lane 1—positive control; Lane 2—flow through, sample after passing through column; Lane 3—sample of wash of column with loading buffer; Lane 4—sample of wash of column with native wash buffer (containing 20 mM imidazole); Lanes 5–11—elution of sample with elution buffer (containing 100 mM imidazole). (**b**) Silver stain of partially purified sod2 protein. M—molecular weight markers; Lane 1—sample of eluate from Ni-NTA Agarose resin. Arrow indicates position of the sod2 immunoreactive protein

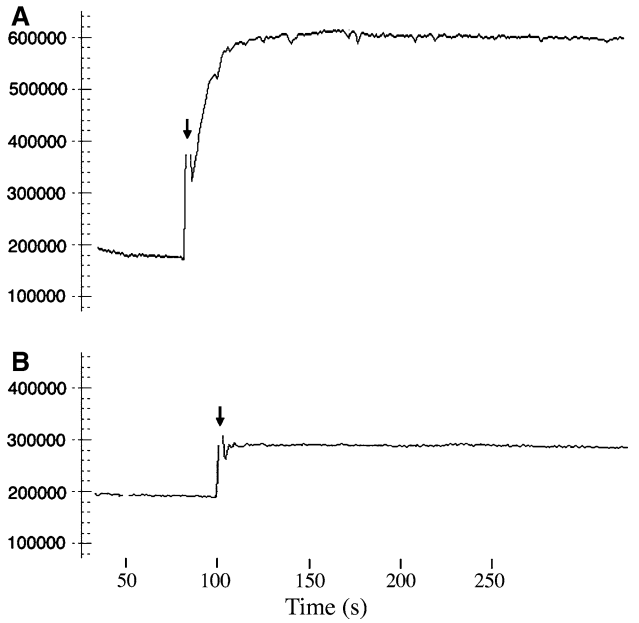

Fig. 4 Na^+/H^+ exchange activity of reconstituted sod2: Reconstitution into liposomes was carried out in the presence (**a**) and absence (**b**) of sod2, in a buffer containing ammonium at pH 7.5. An acid-inside pH gradient was created by dilution of proteoliposomes into reconstitution buffer without ammonium at pH 7.5. After 30–60 s Na^+/H^+ exchange was initiated by the addition of NaCl. Discontinuities, in the tracing prior to NaCl addition, were created by opening and closing the sample compartment of the spectrofluorophotometer. Arrow denotes point of addition of NaCl (135 mM). Measurement of Na^+/H^+ exchanger activity of reconstituted proteolipsomes was in the presence (**a**) or absence (**b**) of expressed sod2 protein. Results are typical of 3 independent measurements

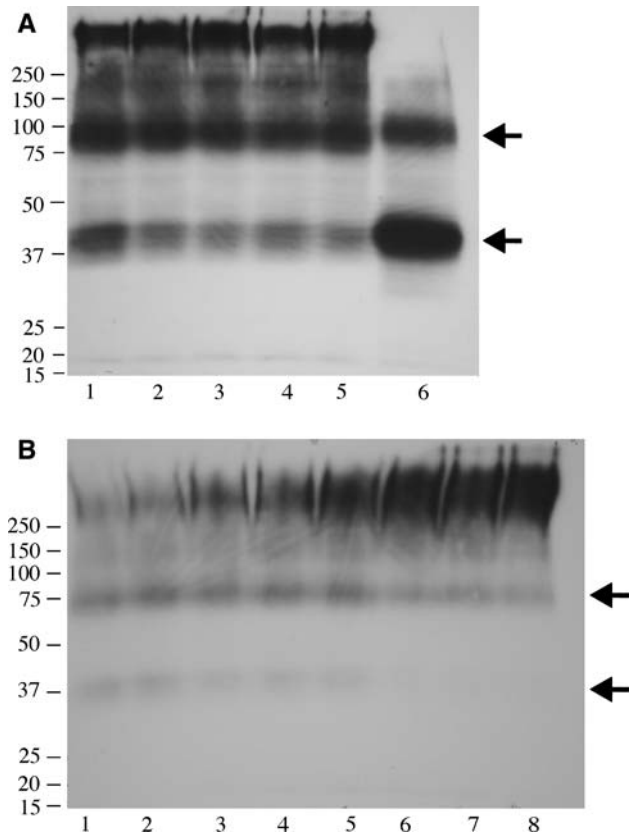

Fig. 5 Cross linking of sod2 protein: (**a**) Cross linking via DSS. Crude membranes (Lanes 1–5) from yeast expressing V5-tagged sod2 were incubated for 30 min at room temperature with the cross linker DSS at concentrations of 0, 0.005, 0.01, 0.02, 0.04, and 0 mM. Lanes 6 was partially purified protein. The products were subjected to SDS-PAGE and Western blotting analysis using anti-V5 monoclonal antibody. Arrows denote the monomeric and dimeric forms of sod2. (**b**). Cross linking via *o*-PDM. Lanes 1–8 contained were treated with 0, 0.005, 0.01, 0.02, 0.04, 0.1, 0.5, 2 mM *o*-PDM for 30 min at room and analyzed as described in Fig. 5a

oligomers. Increasing concentrations of *o*-PDM caused decreasing amounts of the monomeric protein. Interestingly, the amount of 80 kDa form of the protein decreased at high *o*-PDM concentrations also and the amount of high molecular weight oligomers increased greatly.

Discussion

Sod2 is the principal Na^+/H^+ exchanger of the yeast *S. pombe*. Under normal physiological conditions it is responsible for salt tolerance in this species, and removes excess intracellular sodium [4, 17]. Disruption of the sod2 gene results in sensitivity to LiCl or NaCl [3] and sod2 is homologous to plant Na^+/H^+ exchangers making it an important model system for study [18]. Research on membrane proteins, such as sod2 is hampered because membrane proteins are much more difficult to express than soluble ones. In this study, we examined the use of *S. cerevisiae* as an expression system for this membrane protein. *S. cerevisiae* as an expression system has proven successful with transport proteins of similar overall function and origin [12, 19, 20]. We successfully expressed sod2 in *S. cerevisiae* in amounts large enough for partial purification and reconstitution. The reconstituted protein was capable of Na^+-dependent transport.

In the process of development of an expression system for this protein, several important parameters came to light. First, we found that there was colony to colony variation in expression levels of the protein. The cause of this variability was not known, but did result in noticeable differences in expression of the protein. Further, we found that with longer times of induction, the level of expressed protein declined. This was likely due to degradation of the protein. Solubilization of the protein was achieved successfully by using the detergent DDM. A detergent to protein ratio of 3:1 gave a balance between optimal solubilization and detergent use. Only partial purification of the sod2 protein was achieved. Surprisingly other proteins co-purified with the sod2 protein. It is not known whether

these proteins bound directly to the sod2 protein forming a complex (note below), or whether they were contaminants. Further purification of the sod2 protein to homogeneity proved elusive. The fact that the protein demonstrated functional activity after reconstitution confirmed that it was not misfolded.

We noted that the protein appeared to dimerize, even in SDS containing gels. Human Na^+/H^+ exchanger type 1 has been reported to dimerize in several studies [21–23]. In order to confirm that sod2 exists as a dimer, we cross-linked the protein using DSS. DSS is a homobifunctional reagent that principally reacts with amines and has been used earlier in studies to demonstrate close association of membrane proteins [24, 25]. DSS has a spacer arm length of 11.4 A. The cross-linked products were resolved by SDS-PAGE followed by immunoblotting. We found that a large amount of sod2 was present as a dimeric species. There was also several very high molecular weight species present which did not enter the gel very far, possibly indicating the presence of a multimer of proteins. DSS reduced the amount of sod2 monomer slightly. Similar experiments with *o*-PDM were more effective. Increasing concentrations of *o*-PDM caused the disappearance of the 40 kDa sod2 monomer. There was also an increased amount of sod2 oligomer present of varying sizes and at high *o*-PDM concentrations, the amount of 80 kDa sod2 decreased and the amount of oligomer increased. The spacer arm length of *o*-PDM is 7.7–10.5 Å and several different conformations of this cross linker are available so that its increased efficacy of cross linking might be due to use of a configuration of significantly smaller size [26]. The larger amount of sod2 oligomers suggests that sod2 may be present as a complex with a number of other proteins present in the crude membranes.

In summary, our results demonstrate that the sod2 protein can be expressed in *S. cerevisiae* in a functional form. We show that DDM solubilized the protein effectively and demonstrate functional reconstitution of the protein. Sod2 monomer is present as a 40 kDa protein, but could be cross linked to larger species, suggesting that it exists in membranes linked to itself or other proteins. Future studies may identify sod2-linked proteins.

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