J Membrane Biol (2008) 221:39–49
DOI 10.1007/s00232-007-9083-3

Regulation and Function of Lysine-Substituted Na,K Pumps in Salt Adaptation of *Artemia franciscana*

Peter Leth Jorgensen · Francisco Amat

Received: 27 July 2007 / Accepted: 19 October 2007 / Published online: 12 December 2007

Abstract The brine shrimp *Artemia* thrives at extreme conditions of up to 300 g/l salt in hypersaline lakes, but the molecular aspects of this salt adaptation are not clarified. To examine the influence of salt on the expression of two isoforms of Na,K-ATPase, adult *Artemia franciscana* were cultured for 39 days with the microalga *Dunaliella salina* as fodder at increasing salt from 30 to 280 g/l. Quantitative reverse-transcriptase polymerase chain reaction showed that the abundance of mRNA of the lysine-substituted α_2(KK)-subunit was very low at 30 g/l salt but rose steeply in the range of 70–200 g/l to a level at 200–280 g/l salt, similar to the abundance of the mRNA of the α_1(NN)-subunit, which was insignificantly affected by increasing salt. Site-directed mutagenesis showed that Asn324Lys and Asn776Lys in the α_1-subunit of pig kidney Na,K-ATPase reduced the stoichiometry of ^{204}Tl binding from 2 to about 1 $Tl^+(K^+)$ per α-subunit and Na^+-dependent phosphorylation from ATP to 25–30%. In structure models, the ε-amino group of Lys776 is located at cation site 1 in the E_1P form and near cation site 2 in the E_2 conformation, while the side chain of Lys324 points away from the cation sites. Salt-induced expression of the α_2(KK)-subunit Na,K-ATPase in *A. franciscana* may reduce the Na^+/ATP ratio and enable the Na,K pump to extrude Na^+ against steeper gradients and, thus, contribute to salt adaptation.

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Keywords *Artemia franciscana* · Na,K-ATPase · Isoform · Lysine substitution · Salt adaptation · Quantitative polymerase chain reaction · Occlusion of Tl^+ (K^+) · Na^+-dependent phosphorylation · Na^+/ATP ratio · Site-directed mutagenesis

Introduction

The Na,K pump maintains electrochemical gradients across membranes as it couples ATP hydrolysis to the active transport of three Na^+ ions out and two K^+ ions into the cytoplasm of the cell. It operates in a ping-pong sequence while alternating between two conformations, E_1P[3Na] and E_2[2K], with occluded cations (*see* Jorgensen, Håkansson & Karlish, 2003). Mutagenesis screening and direct binding assays showed that each of the four carboxylate residues–Glu327, Glu779, Asp804 and Asp808 in transmembrane segments M4, M5 and M6 of the α_1-subunit–is essential for cation binding and occlusion (Nielsen et al., 1998). In addition to the carboxylates, mutagenesis screening of six oxygen-carrying residues of the specific M5 sequence showed that Asn776, Ser775 and Tyr771 alternately engage in binding of K^+ or Na^+ to stabilize the occluded E_1P[3Na] or E_2[2K] conformation (Pedersen et al., 1998). In homology models based on the high-resolution structures of the Ca-ATPase of the sarcoplasmic reticulum (Toyoshima et al., 2000; Sorensen, Moller & Nissen, 2004), the amphiphilic helices of M4 and the M5–M6 helical loop of the α-subunit of Na,K-ATPase are organized to orient the negatively charged side chains toward a central cavity to contribute to coordinating K^+ or Na^+ ions in cation site 1 and site 2. Homology models for the coordination of the third Na^+ ion have been proposed (Ogawa & Toyoshima, 2002; Håkansson & Jørgensen, 2003).

The sequences in M4 (324-NVPEG), M5 (771-YT-LTSNIPEITP) and M6 (800-ILCIDLGTDM) are unique to the α-subunit of Na,K-ATPase, and they are well conserved among a wide range of animals (P-type database, http://www.patbase.kvl.dk). One important exception is the α_2-KK-subunit (P17326; Baxter-Lowe et al., 1989) of *Artemia franciscana*, where lysines are substituted for Asn324 in M4 and Asn776 in M5, thus introducing two positively charged ε-amino groups of lysine near the center of the binding cavities for cations (Jorgensen & Pedersen, 2000).[1] The ε-amino groups may behave like ammonium ion NH_4^+, which can substitute for K^+ in the activation of ATP hydrolysis by Na,K-ATPase (Skou, 1965).

Artemia is known for its ability to survive as the only crustacean at extreme salt concentrations up to 300 g/l salt in hypersaline lakes inhabited only by a few species of algae, bacteria, ciliates and insects. The shrimps drink seawater; like teleost fish, they excrete salt from glands studded with chloride cells and maintain relatively low salt concentrations (155–185 mM) in the extracellular hemolymph fluid (Conte, 1984; Holliday, Roye & Roer, 1990). The shrimps take up water in coupling with NaCl transport from the gut lumen to the hemolymph. The Na^+ and Cl^- ions are then secreted through the metepipodites at the base of the phyllopodia of adults (Holliday et al., 1990). It is known that high external salt raises the activity of Na,K-ATPase in the salt glands of *Artemia* larvae (Augenfeld, 1969). In the metepipodites the specific activity of Na,K-ATPase is raised 2.2-fold over a time period of 4–7 days when the external salt is increased from 50% (0.49 osmol/kg) to 400% (4.0 osmol/kg) seawater (Holliday et al., 1990). Hybridization experiments with mRNA probes have shown that the α_2-KK-subunit is almost exclusively located in the salt gland of *Artemia* larvae, while the α_1-NN-subunit is ubiquitous (Escalante, Garcia-Saez & Sastre, 1995). The abundance of the α_2-KK-subunit in normal seawater is very low, and it was proposed that the α_1-NN-subunit plays a more important role in the osmoregulatory processes (Sáez, Perona & Sastre, 1997); however, the molecular mechanism behind the adaptation of *Artemia* to high salt has not been clarified.

In an approach to this mechanism, the purpose of the present work was to examine if the lysine-substituted α_2-KK-subunit is involved in the regulatory response to high salinity. We have therefore developed techniques for the growth of adult *A. franciscana* with microalgae in the relevant range of 30–280 g/l salt for 39 days and determined the influence of salt on the relative abundance of mRNA of the α_1-NN and (α2-KK subunits of Na,K-ATPase. Homology models of the α-subunit of Na,K-ATPase were constructed on the basis of the high-resolution structures of Ca-ATPase in the E_1 (1SU4; Toyoshima et al., 2000) and E_2 (1IWO; Toyoshima & Nomura, 2002) forms to examine the localization of the ε-amino groups of the substituted lysines at Asn324 and Asn776 in relation to the putative cation sites 1 and 2. We also applied site-directed mutagenesis in the α_1-subunit of pig kidney Na,K-ATPase expressed in yeast (Pedersen, Rasmussen & Jørgensen, 1996) to determine if the lysine substitutions of Asn324 and Asn776 alter the stoichiometries of K^+ or Na^+ binding. A reduction of the number of Na^+ ions pumped per ATP hydrolyzed would enable the Na,K pump to extrude salt against steeper ion gradients in the salt glands of *Artemia*.

Methods

Cloning and Sequencing of the α_1 and α_2 Subunits of *A. franciscana*

A cDNA clone, pArATNa136 (accession X56650), from the *A. franciscana* Na,K-ATPase α_1-subunit was kindly provided by Leandro Sastre (Madrid, Spain; Macías, Palmero & Sastre, 1991). The *A. franciscana* Na,K-ATPase α_2-subunit was cloned in this laboratory from larvae hatched in 8% NaCl. Total RNA was extracted, and reverse transcription followed by a polymerase chain reaction (RT-PCR) resulted in a cDNA fragment containing the coding sequence of the α_2-subunit plus 22 nucleotides preceding the AUG start codon. The nucleotide sequences of the *A. franciscana* α_1 and α_2 isoforms were determined using the dideoxy chain termination method (Sanger, Nicklen & Coulson, 1977) for sequencing double-stranded DNA (Chen & Seeburg, 1985). Sequencing primers, spaced with an interval of about 400 nucleotides, were designed using the known nucleotide sequences of the *A. franciscana* α_1 (accession P28774) and α_2 (accession P17326) subunits.

Growth and Harvesting of *A. franciscana*

The first step was to develop a mass culture of the green microalga *Dunaliella salina* at high salinities. Then, *A. franciscana* were grown with the microalgae as fodder for 39 days at salinities of 30, 70, 120, 200 and 280 g salt/l in five separate glass cylinders. Aliquots of 4–5 g of *Artemia* were harvested from each cylinder, washed with distilled water, dripped off, weighed, frozen in liquid nitrogen and stored at −80°C.

[1] (In this article, residue numbers Asn324 and Asn776 refer to the α_1-subunit of pig [P05024]); the homologous numbers in the *A. franciscana* α_1-subunit are Asn312 and Asn764 (P28774), and in the α_2-subunit the substituted lysines are Lys308 and Lys758 in P17326.)

Preparation of Total RNA

The procedure is a modification of the method for total RNA isolation by extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture (Chomczynski & Sacchi, 1987). Aliquots containing 250 mg of frozen shrimp were homogenized in 2 ml of Tri Reagent® (Molecular Research Center, Inc., Cincinnati, OH, USA) for 1 min at room temperature in an Ultra Turrax® cell disrupter (Rose Scientific, Alberta, Canada). The homogenate was left at 20°C for 5 min and centrifuged at 10,000 rpm (12,000 × *g*) for 10 min at 4°C to remove insoluble material. The supernatant was removed without disturbing the pellet or the fatty layer at the top and centrifuged again at 10,000 rpm for 10 min at 4°C. After adding 200 µl bromchlorpropane, the mixture was vortexed for 15 s, left at 20°C for 8 min and centrifuged at 10,000 rpm for 15 min at 4°C. The colorless upper aqueous phase was pipetted off and supplemented with 1,000 µl isopropanol, incubated at 20°C for 10 min and centrifuged at 10,000 rpm for 10 min at 4°C to sediment the RNA. The supernatant was removed and the pellet suspended in 2.5 ml 75% ethanol in a vortex. RNA was sedimented by centrifugation at 10,000 rpm for 10 min at 4°C; the pellet was dried in air, dissolved in 100 µl RNAse-free water and stored at −80°C. The yield of RNA was in the range 1.2–1.8 µg RNA/mg wet weight shrimp tissue, and the ratio of wavelengths 260/280 nm was higher than 1.7.

RT-PCR

Generation of first-strand cDNA from total RNA was catalyzed by the ThermoScript™ Reverse Transcriptase (RT; Invitrogen, Carlsbad, CA) using random hexamer primers as follows. Aliquots of 10 µg total RNA were mixed with 100 ng random hexamer primer and 40 nmol dNTP mixture in 24 µl of diethylpyrocarbonate (DEPC)-treated water and incubated for 5 min at 65°C. After cooling on ice, 16 µl DEPC-treated water containing 30 U ThermoScript RT (Invitrogen), 80 U recombinant ribonuclease inbibitor, 200 nmol dithiothreitol (DTT) and cDNA synthesis buffer were added. The mixture was incubated in a PCR instrument for 10 min at 25°C, for 50 min at 50°C and for 5 min at 85°C. A volume of 2 µl containing 4 U of RNase H of *Escherichia coli* was added, and the mixture was incubated for 20 min at 37°C. For precipitation of cDNA, 5 µl 3 M Na-acetate and 100 µl cold 96% ethanol were added and stored at −20°C for 1 h. The tube was centrifuged at 15,000 rpm for 10 min, and the pellet was washed twice with 200 µl 80% ethanol, sedimented for 2 min at 15,000 rpm, resuspended in 10 µl DEPC-treated water and stored at −20°C.

Quantitative PCR

The TaqMan reaction mixture for quantitative PCR (QPCR) is based on the Brilliant® QPCR Master Mix (Stratagene, La Jolla, CA) containing hotstart *Taq* DNA polymerase, standard dNTPs, Core PCR Buffer, glycerol, dimethyl sulfoxide and 5.5 mM $MgCl_2$. ROX (6-carboxy-X-rhodamine) was added as fluorescence reference. The Primer Express 2.0 software (Applied Biosystems, Foster City, CA) was used in the selection of Taqman probes and primers for QPCR of the expression of mRNA of the α_1-NN and α_2-KK subunits as a function of salinity. The concentrations of forward and reverse primers were adjusted to 120 nM and the Taqman probe to 200 nM. The cDNA was added in a series of six 10-fold dilutions in a total volume of 25 µl. In the MX4000 QPCR instrument (Stratagene, La Jolla, CA) the conditions used to amplify the α_1-NN-subunit, α_2-KK-subunit and β-subunit were as follows: one cycle at 95°C for 10 min to activate the *Taq* polymerase, followed by 50 cycles at 95°C for 30 s and 55–60°C for 1 min. The annealing temperature was chosen to be lower than the Tm for the two primers, while the Tm (melting temperature) for the TaqMan probe is higher than that of the forward and reverse primers.

The cDNA clones in plasmids of the α_1-NN and α_2-KK subunits were used as templates for standard curves in testing the specificity of primers and Taqman probes (Table 1) and in the quantitative estimation of the mRNA expression of the two subunits as a function of salinity. Dilution series of the α_1-NN- and α_2-KK-subunit cDNA were run in parallel to the reverse transcript cDNA preparations to determine the relative changes of the abundance of mRNA of the α_1-subunit, α_2-subunit and β-subunit in Na,K-ATPase of *A. franciscana*.

Table 1 Taqman probes and primers for QPCR of the relative expression of the mRNA of the α_1-NN, α_2-KK and β subunits of *A. franciscana* Na,K-ATPase

	Forward	Taqman	Reverse
α_1	CTCGAAGCCGTCGAAACCCT	TGGCTCCACCTCAACAATTTGCTCA	AAGACTGGCACCCTGACTCAAAA
α_2	GGGTACAAATACGGAAAATGGTT	ACTTCGTCCCAGGCAAAGTCTCA	TTAGAAAATATGGTCCAAATGCA
β	CCTGTTGTTGCTCTCAGTTTGG	TCACGGACAAGCAGTGAACGTGGAA	TGTTGGCCCACGCCTTAC

Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) was used in the selection of TaqMan probes and primers for QPCR

Site-Directed Mutagenesis and Expression of Na,K-ATPase

Site-directed mutagenesis was performed according to Ho et al. (1989), and the PCR fragment containing the mutation was subsequently inserted into the expression plasmid pPAP1666 (Pedersen et al., 1996). Nucleotide sequences of all PCR fragments were confirmed by dideoxy sequencing. Yeast cells were transformed by electroporation according to Becker & Guarente (1991). Growth of transformed yeast cells in an Applicon® fermentor equipped with an ADI 1030 Bio Controller and galactose induction of recombinant Na,K-pump protein synthesis were performed as before (Pedersen et al., 1996). From each fermentation 100–200 g of yeast and 1–2 g of crude membrane protein were produced.

Isolation of Yeast Membranes and Tris Dodecyl Sulfate Treatment

Isolation of crude membranes, fractionation of membranes on sucrose step gradients and assay of protein were performed as before (Pedersen et al., 1996). Both crude and gradient membranes were used for the various assays. Membranes were incubated for 30 min at 20°C with 0.3 mg Tris dodecyl sulfate (TDS)/ml, 5 mg protein/ml, 10 % (w/v) sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethyleneglycoltetraacetic acid (EGTA), 25 mM imidazole-Cl (pH 7.5) supplemented with 1 mM phenylmethylsulfonyl fluoride and 1 μg/ml of chymostatin, pepstatin and leupeptin to prevent proteolysis.

Occlusion of Tl^+

After TDS treatment, the membranes were sedimented at 265,000 × *g* in the Beckman 100A centrifuge for 30 min at 4°C. Membranes were washed twice with ice-cold wash buffer (10% [w/v] sucrose, 0.5 mM EDTA, 25 mM Tris-SO_4 [pH 7.4]) and centrifuged for 10 min as before, followed by resuspension in wash buffer. As in the procedure of Nielsen et al. (1998), the membranes were then divided into two portions and incubated with 1 mM $MgSO_4$, with or without 0.5 mM ouabain, for 20 min at 20°C. The membranes were cooled on ice for at least 20 min before aliquots containing 0.4–0.5 mg protein were incubated with 2–30 μM $^{204}TlNO_3$ (0.5 to 7 · 10^6 cpm/sample) and varying concentrations of unlabeled $TlNO_3$ for 7 min at 0°C. Occluded $^{204}Tl^+$ ions were separated from free $^{204}Tl^+$ ions on Dowex-Tris 50W-X8 columns (50–100 mesh), (Dow Chemical, Midland, MI, USA), and $^{204}Tl^+$ was counted as before (Nielsen et al., 1998).

Ouabain Binding and Na^+-Dependent Phosphorylation

For ouabain binding, aliquots of TDS-treated membranes containing 0.1–0.2 mg protein were incubated at 37°C for 1 h in 3 mM $MgSO_4$, 1 mM $NaTris_2VO_4$, 1 mM EGTA and 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)-Tris (pH 7.2) in the presence of 2–10 nM [^{3}H]-ouabain (18 Ci/mmol) and unlabeled ouabain to final concentrations of 2–300 nM. After standing on ice for 20 min, bound ouabain was isolated by centrifugation in the Beckman 100A centrifuge at 265,000 × *g* for 30 min at 4°C and the pellets were washed and counted as before (Pedersen et al., 1996). Na^+-dependent phosphorylation from [γ-^{32}P]ATP was determined as before (Pedersen et al., 1998), and the ratio of maximum phosphorylation to ouabain binding and $K_{0.5}(Na^+)$ values were estimated using the Hill equation of the Sigmaplot 9.1 program (Jandel Scientific, Corte Madera, CA). Assay of Na,K-ATPase activity was performed as before (Pedersen et al., 1996).

Results

Sequencing of the α_1 and α_2 subunits of *A. franciscana*

As expected, the sequence of the α_1 isoform was identical to the reported sequence of pArATNa136 (Macías et al., 1991). Comparison of the sequence of the *A. franciscana* α_2-subunit from this laboratory with that reported by Baxter-Lowe et al. (1989) (P17326) revealed 195 nucleotide substitutions corresponding to 18 substitutions in the deduced amino acid sequence. Among these amino acid substitutions, 10 are conservative substitutions. Only two are found in the eight conserved segments that are common to all P-type ATPases, whereas 16 are distributed in less conserved segments of the α_2 sequence. This could reflect larger polymorphism than expected for neutral evolution, as observed for the α_1-subunit locus of *A. franciscana* by Sáez, Escalante & Sastre (2000).

Growth of Adult *A. franciscana* in Mass Culture of *Dunaliella salina* at High Salinities

A. franciscana were grown with the microalgae as fodder for 39 days at salinities of 30, 70, 120, 200 and 280 g/l salt corrresponding to osmolarities of 0.56–5.28 Osmol/l. At the time of collection, about 50% of shrimp were females showing reproductive maturity and in each of the five samples 70–79% of the females displayed ovoviviparous offspring inside full ovisacs as an indication of adequate levels of food availability at all salinities. At the time of collection, the female shrimp were on average 14–19%

longer than the males and the average length of both sexes was declining as a function of salinity in the range 30–280 g/l salt, as shown in Figure 1.

QPCR Analysis of the Influence of Salinity on Expression of α_1-NN and α_2-KK Subunits of *A. franciscana*

Separation of the RT reaction from the QPCRs was preferred since a two-step procedure is more sensitive for detection of mRNAs occurring at low abundance. Comparison of the amplification plots in Figures 2 and 3 shows that the increase in salinity from 30 to 280 g/l caused only minor changes in the course of the amplification of the α_1-NN-subunit template, while the amplification curves for the α_2-KK-subunit were distributed over a wide range of cycles. Accordingly, the extrapolated threshold cycle numbers for the α_1-NN-subunit in Figure 4 revealed an insignificant decrease, from Ct = 21.4 at 30 g salt/l to Ct = 19.4 at 280 g/l salt, indicating that the high salinity had only a minor influence on the abundance of mRNA of the α_1-NN-subunit in *Artemia*. In contrast, the Taqman probes for the α_2-KK-subunit barely amplified the product from shrimp grown at 30 g/l salt, while the curves for amplification of the template for the α_2-KK-subunit from *Artemia* grown at 70, 120 and 200 g/l salt were shifted considerably to the left (Fig. 3). The estimated threshold cycle numbers in Figure 4 were reduced significantly along a steep slope from Ct = 30.5 at 70 g/l salt to a plateau at

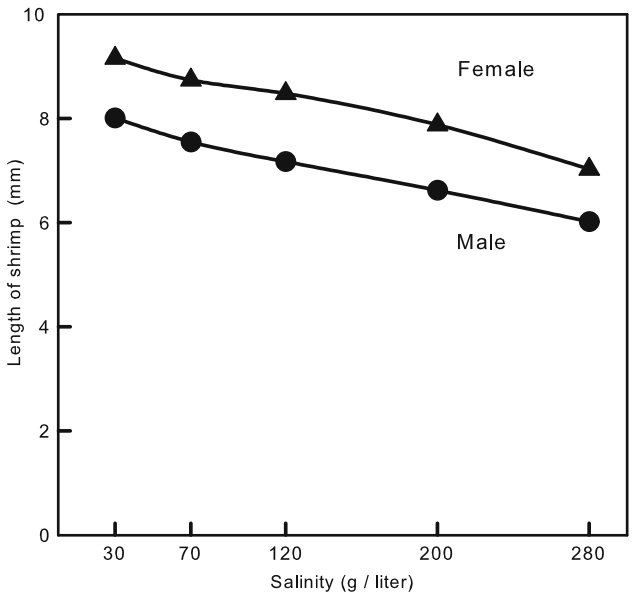

Fig. 1 Average length of male and female *A. franciscana* after growth with the microalga *Dunaliella salina* as fodder for 39 days at salinities of 30, 70, 120, 200 and 280 g/l salt. The length was measured for 30 specimens of each sex

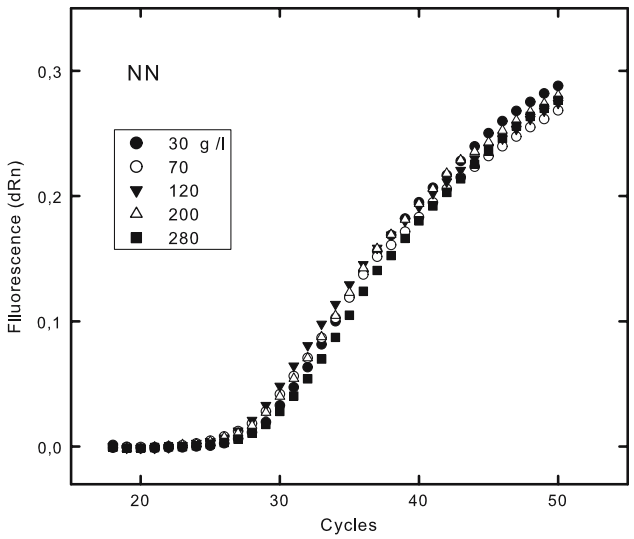

Fig. 2 Amplification in the MX4000 QPCR instrument of the cDNA of the α1-NN-subunit from *A. franciscana* grown for 39 days at salinities increasing from 30 to 280 g/l salt. The TaqMan reaction mixtures with Taq DNA polymerase were run for 50 cycles at 95°C for 30 s and 55–60°C for 1 min. The cDNA was added in aliquots of 1 µl from a series of six 10-fold dilutions of cDNA in a total reaction volume of 25 µl. *Curves* show amplification after adding 1 µl of a 100-fold dilution of the cDNA preparation to the reaction mixture

Ct = 20.0 and 19.4 at 200 and 280 g/l salt, respectively. This plateau is at the same level as the threshold cycle numbers for the α_1-NN-subunit. As the standard plots of Ct vs. the concentration of template cDNA of the α_1-NN and

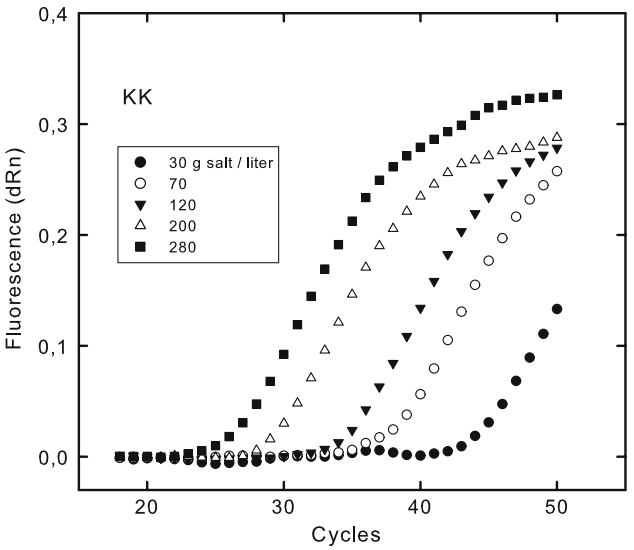

Fig. 3 Amplification in the MX4000 QPCR instrument of the cDNA of the α_2-KK-subunit from *A. franciscana* grown for 39 days at salinities ranging from 30 to 280 g/l salt. The TaqMan reaction mixture with Taq DNA polymerase was run for 50 cycles at 95°C for 30 s and 55–60°C for 1 min. The cDNA was added in aliquots of 1 µl from a series of six 10-fold dilutions of cDNA in a total reaction volume of 25 µl. *Curves* show amplification after adding 1 µl of a 100-fold dilution of the cDNA preparation to the reaction mixture

α_2-KK subunits are semilogarithmic with slopes close to -3.2, the drop of the Ct number from 30.5 to about 20 represents an increase of the abundance of α_2-KK-subunit mRNA and cDNA of more than 1,000-fold. Using cDNA clones in plasmids of the α_2-KK-subunit as standard, the abundance of α_2-KK-subunit mRNA was estimated to be raised from 0.96 pg cDNA/mg *Artemia* at 30 g/l salt to 1.1–1.6 ng cDNA/mg *Artemia* at 200–280 g/l salt.

The decrease of the average values of Ct for the α_1-NN-subunit from 21.4 at 30 g/l salt to 19.4 at 280 g/l salt is not statistically significant (Fig. 4). As estimated from a standard curve of plasmids containing the α_1-NN-subunit clone, this change does correspond to an increase of mRNA abundance from 0.30 ng cDNA/mg *Artemia* at 30 g/l salt to 1.5 ng cDNA/mg *Artemia* at 280 g/l salt.

The extrapolated threshold cycle numbers for the β-subunit were reduced from an average value of 22.5 at 30 g/l salt to 20.3 at 280 g/l salt (Fig. 4). Although the data are not significantly different, they are compatible with an increase in expression of the β-subunit corresponding to an increased expression of the α-subunit isoforms.

Expression in Yeast of Single and Double Mutations of Asn324 and Asn776 in the α_1-subunit of *Sus scrofa*

Ouabain binding capacities and affinities were determined to monitor the expression of mutations in yeast as described before (Pedersen et al., 1996). Data in Table 2 show that the mutations were expressed at yields similar to those of the wild type, in agreement with previous studies of mutations to the cation sites of Na,K-ATPase (Nielsen et al., 1998; Pedersen et al., 1998). The lysine substitutions caused a decrease of the affinity for ouabain binding as the dissociation constant for ouabain (K_D) was 17-fold higher than wild type for the single Asn776Lys mutation and 26-fold higher than wild type for the double mutation Asn776Lys/Asn324Lys. The potassium-bound E_2[2K] conformation of Na,K-ATPase is known to have a reduced affinity for ouabain (Jorgensen & Andersen, 1988), and we therefore compared the influence of K^+ and NH_4^+ ions on ouabain binding and found that NH_4^+ ions displaced [^{3}H]-ouabain with almost the same affinity as K^+ ions (*data not shown*). The ε-amino group of lysine may therefore act like an NH_4^+ cation substitute for K^+ and stabilize an occluded E_2 conformation of Na,K-ATPase with lower affinity for ouabain than the Mg-E_2-vanadate conformation.

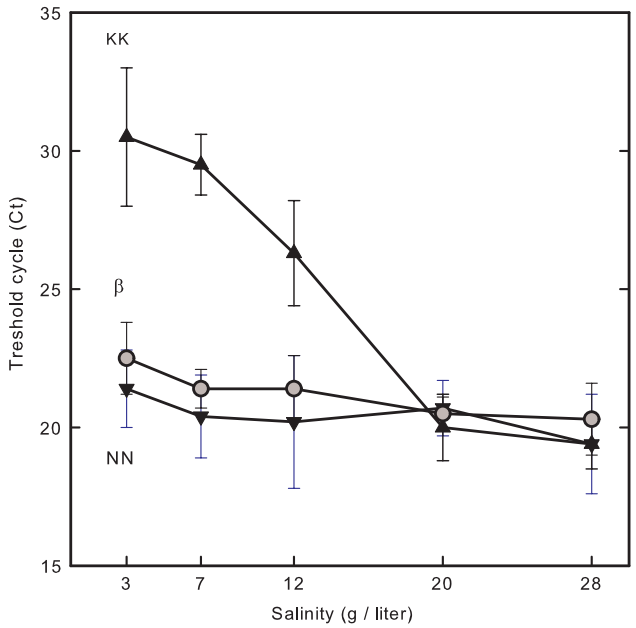

Fig. 4 Extrapolated threshold cycle values estimated from data like those in Figures 2 and 3 from a series of six 10-fold dilutions of the cDNA using the specific TaqMan probes for the α_1-NN, α_2-KK or β subunits. The data are mean values ± SD of a series of five consecutive experiments on different preparations of cDNA from *A. franciscana* grown for 39 days at salinities from 30 to 280 g/l salt

Consequences for Binding of Na^+ of Single and Double Lysine Substitutions of Asn324 and Asn776 in the α_1-Subunit of Na,K-ATPase

The presence of oligomycin in the phosphorylation assay prevents E_1P–E_2P transition and allows monitoring of the formation of the MgE_1P[3Na] complex as a dead-end reaction. The apparent affinity of the complex for Na^+ can therefore be estimated from curves of Na^+ dependence of phosphorylation from ATP (Pedersen et al., 1998). It is seen from Figure 5 and Table 3 that single mutations of Asn776Lys or Asn324Lys caused 10-fold or 15-fold increases of the $K_{0.5(Na+)}$. Introduction of a second lysine further reduced Na^+ binding. In the double mutation Asn776Lys/Asn324Lys, the phosphorylation level was reduced to 30% of wild type. The Na^+ binding data in Table 6 show that the single mutation Asn776Lys caused

Table 2 Consequences for ouabain binding of single and double lysine substitutions of Asn324 and Asn776 of α_1-subunit Na,K-ATPase expressed in yeast

	Na,K-ATPase activity (%)	Ouabain binding (pmol/mg pr)	K_D (nM)
Lys324/Asn776	32 ± 4	13.2 ± 0.5	25 ± 3
Asn324/Lys776	43 ± 6	19.3 ± 0.3	82 ± 7
Lys324/Lys776	14 ± 5	9 ± 1	123 ± 7
Asn324/Asn776 WT	100 ± 8	9.6 ± 0.4	4.6 ± 0.5

The Na,K-ATPase activities and the capacities and affinities of ouabain binding are average values of three or more consecutive preparations. Ouabain binding data are estimated by nonlinear regression analysis of binding at a series of [^{3}H]-ouabain concentrations

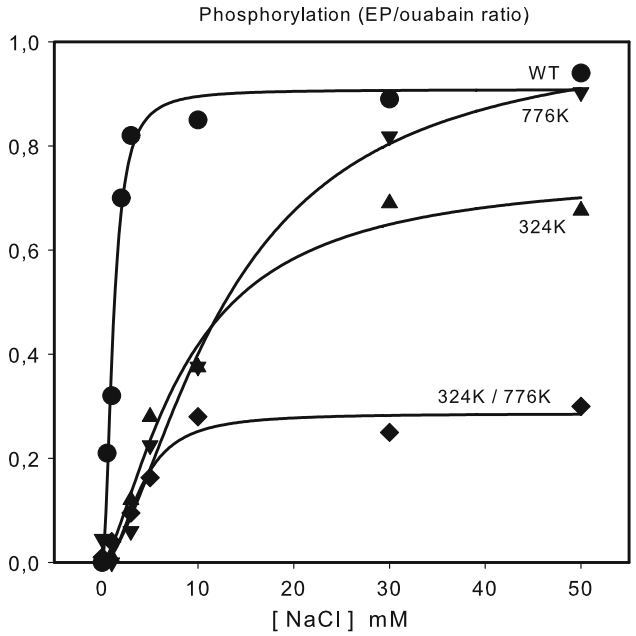

Fig. 5 Consequences for Na^+-dependent phosphorylation from [γ-^{32}P]ATP of single or double lysine substitution of Asn324 and Asn776 in the α_1-subunit of pig kidney Na,K-ATPase expressed in *Saccharomyces cerevisiae*. Data points are average values of two separate experiments. Lines were fitted and maximum phosphorylation levels and $K_{0.5}(Na^+)$ values were estimated by nonlinear least-squares regression analysis using the equation Phosphorylation/ouabain ratio = a *$[NaCl]^n/(c^n + [NaCl]^n)$, where a is the maximum phosphorylation level, c is $K_{0.5}(Na^+)$ and n is the Hill coefficient. The estimated maximum capacities and $K_{0.5}(Na^+)$ values are shown in Table 3

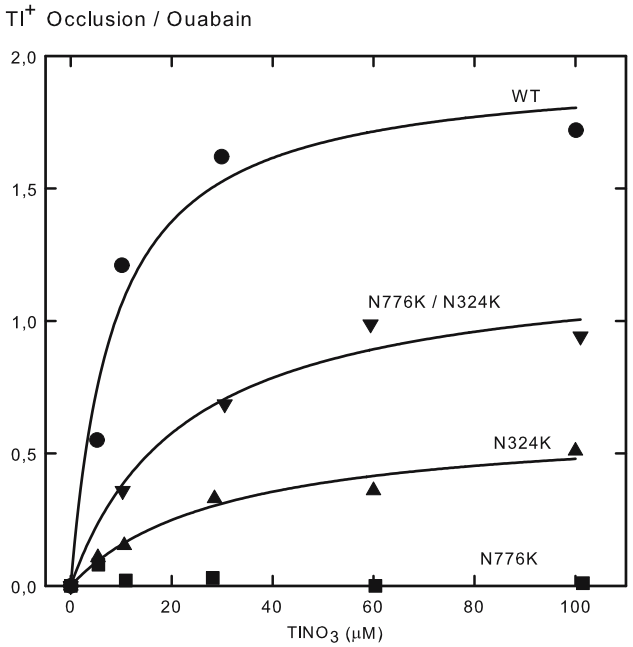

Fig. 6 Concentration dependence of $^{204}Tl^+$ occlusion after single or double lysine substitution of Asn324 and Asn776 in the α_1-subunit of pig kidney Na,K-ATPase expressed in *Saccharomyces cerevisiae*. Data points are average values of two separate experiments with double determinations at each cation concentration. Lines were fitted by nonlinear least-squares regression analysis to the Hill/Michaelis-Menten equations: occluded Tl^+/ouabain binding ratio = a *$[TlNO_3]^n/(c^n + [TlNO_3]^n)$, where a is the maximum Tl^+ occlusion/ouabain binding ratio, c is $K_{0.5}(Tl^+)$ and n is the Hill coefficient. For the Michaelis-Menten equation, n = 1. The estimated maximum capacities and $K_{0.5}(Tl^+)$ values are shown in Table 4

Table 3 The consequences of single or double lysine substitutions of Lys324 and Lys776 in the α_1-subunit of pig kidney Na,K-ATPase for phosphorylation and binding of Na^+ as estimated by regression analysis from the data in Figure 5

	EP/ouabain binding ratio	$K_{0.5}(Na^+)$ (mM)
Asn324/Asn776	0.93 ± 0.1	0.5 ± 0.1
Lys324/Asn776	0.74 ± 0.1	7.3 ± 2.7
Asn324/Lys776	0.9 ± 0.03	4.9 ± 0.4
Lys324/Lys776	0.29 ± 0.02	4.0 ± 0.6

Table 4 The consequences of single or double lysine substitutions of Lys324 and Lys776 in the α_1-subunit of pig kidney Na,K-ATPase for the occluded Tl^+/ouabain binding ratio and $K_{0.5}(Tl^+)$ as estimated by regression analysis from the data in Figure 6

	Tl^+ occlusion/ouabain binding ratio	$K_{0.5}(Tl^+)$ (μM)
Asn324/Asn776	2.0 ± 0.2	9 ± 3
Lys324/Asn776	0.62 ± 0.08	30 ± 9
Asn324/Lys776	0.02 ± 0.004	20
Lys324/Lys776	1.3 ± 0.1	27 ± 7

almost the same change in Na^+ binding energy as the double mutation Asn776Lys/Asn324Lys. This indicates that interactions between the two lysines have little influence on binding of Na^+ in the E_1P conformation.

Concequences of Single or Double Lysine Substitutions of Asn324 or Asn776 for Binding of Tl^+ or K^+

In wild type, two Tl^+ ions are bound per ouabain binding site with high affinity, $K_{0.5(Tl+)}$ = 9 μM (Fig. 6, Table 4), in agreement with the data of Nielsen et al. (1998). The single Asn324Lys mutation caused a large depression of the affinity for Tl^+ occlusion with an 11-fold reduction of the ratio of maximum binding to $K_{0.5(Tl+)}$ relative to wild type. In the Asn766Lys mutation, Tl^+ occlusion was almost completely eliminated.

Compared to the single mutations, the double mutations Asn324Lys/Asn766Lys caused an increase of Tl^+ occlusion to approach one Tl^+ ion occluded per ouabain binding site with threefold higher $K_{0.5(Tl+)}$ than in wild type (Fig. 6, Table 4). A possible interaction between the two

substituted lysines therefore appears to facilitate Tl^+ occlusion. Double mutant cycle energy analysis (Fersht, 1999) allows detection of interaction between two side chains in a protein if they are mutated singly and then in a double mutation. Analysis of the binding data (Tables 5 and 6) shows that the single substitution of Asn324 or Asn776 to lysine reduces the free energy of Tl^+ binding with 5.5 kJ/mol or 13.5 kJ/mol, while the interaction between the two lysine residues after double lysine substitution favors Tl^+ binding by −15.0 kJ/mol. It is probable that electrostatic repulsion between the two lysines rearranges the structure to favor the binding of a single Tl^+ ion in the E_2 conformation.

Molecular Models of the Position of Single and Double Lysine Substitutions of Asn324 and Asn776 in α_1-Subunit of Na,K-ATPase

The homology models in Figure 7 illustrate the positions of lysine substitutions Lys324 and Lys776 relative to the side chains of selected cation coordinating residues of TM4, TM5 and TM6 in the α_1-subunit of pig kidney Na,K-ATPase (Jorgensen & Pedersen, 2000). In the E_1 conformation, the protonated ε-amino group of Lys776 occupies cation site 1, thus interfering with coordination of Na^+ ions to the carboxylates of Glu779 and Asp808. The side chain of Asp324 points away from cation sites 1 and 2. During the conformational change from the E_1 form with preference for

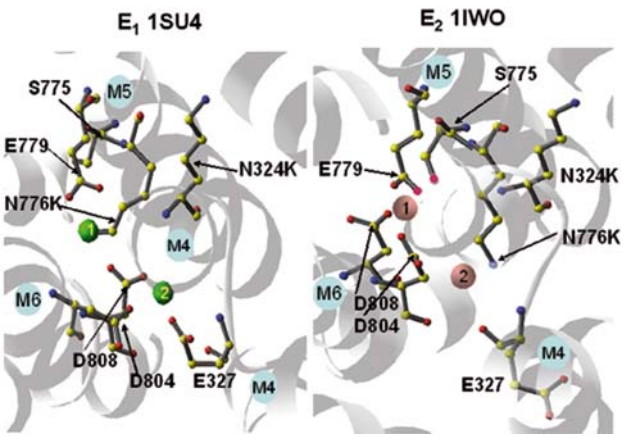

Fig. 7 Model of the position of residues involved in binding of Na^+ (*green circles*) or K^+ (*red circles*) in the E_1 or E_2 conformation of Na,K-ATPase. Numbering as in the α_1-subunit sequence of pig kidney (P05024). The side chains are modeled on the backbone of Ca-ATPase in the E_1 (Ca) form or in the E_2(Mg-thapsigargin conformation. Note that in the E_1 conformation cation site 1 is occupied by the ε-amino group of the substituted lysine at position 766. During transition to the E_2 conformation, this ε-amino group moves downward and to the right to approach cation site 2

binding of Na^+ to the E_2 form with high affinity for binding of $K^+(Tl^+)$, transmembrane segment M5 is tilted with Pro778 as a pivotal point (Toyoshima & Nomura, 2002) and the ε-amino group of Lys776 movies from cation site 1 to approach cation site 2, with possible interactions with the carboxylates of Glu327 and Asp808. This movement of the Lys776 side chain also suggests that the positively charged end group can contribute to ejecting a transported Na^+ ion from cation site 2 to the extracellular medium.

Table 5 Change in Gibbs free energy of Tl^+ and Na^+ binding to wild-type recombinant Na,K-ATPase

Allele	$\Delta Gb_{(Tl+)}$ (kJ/mol)	$\Delta Gb_{(Na+)}$ kJ/mol
Wild-type Asn324/Asn776	−28.0	−14.8

Gibbs free energy of Na^+ ion binding was estimated from the data in Figure 6 and Table 4 using the equation $\Delta Gb_{(Na+)} = -RT \ln (EP/ouab/K_{0.5(Na+)})_{WT}$.

Gibbs free energy of Tl^+ ion binding was estimated from the data in Figure 5 and Table 3 using the equation $\Delta Gb_{(Tl+)} = -RT \ln (Tl^+ occl/ouab/K_{0.5(Tl+)})_{WT}$

Table 6 Alterations in Gibbs free energy (ΔΔGb) of Tl^+ or Na^+ ion binding at 4°C of single and double lysine substitutions of Asn324 and Asn776 in M5 of the α-subunit of Na,K-ATPase

Allele	$\Delta\Delta Gb_{(Tl+)}$ (kJ/mol)	$\Delta\Delta Gb_{(Na+)}$ (kJ/mol)
Lys324/Asn776	+5.5	+4.7
Asn324/Lys776	+13.5	+4.9
Lys324/Lys776	+3.5	+5.3

ΔΔGb values were calculated from data in Figures 5 and 6 using the equation of Fersht (1999): $\Delta\Delta Gb = -RT \ln [(\text{maximum binding}/K_{0.5})_{mut} / (\text{maximum binding}/K_{0.5})_{WT}]$

Discussion

The selective salt induction of the expression of α_2-KK-subunit mRNA and the fixed internal positive charges of the substituted lysines in the cation sites of the α_2-KK-subunit of Na,K-ATPase appear to be of primary importance for understanding the molecular aspects of the adaptation of *A. franciscana* to salinity challenges.

This is the first study of the relative expression of the α_2-KK and α_1-NN subunits as a function of salinity to demonstrate that salinity challenge from 30 to 280 g/l salt causes large increases of the mRNA abundance of the α_2-KK-subunit by more than three orders of magnitude (from 0.96 pg to 1.1–1.6 ng cDNA/mg *Artemia*) to levels which are similar to those of the mRNA of the α_1-NN-subunit (0.6–1.4 ng cDNA/mg *Artemia*). Only insignificant changes are observed in mRNA abundance of the α_1-NN-subunit and the β-subunit. The low abundance at 30 g/l of the α_2-KK-subunit relative to that of the α_1-NN-subunit is in agreement with previous observations in normal

seawater (Sáez et al., 1997), but the influence of salinity on the expression of the α_2-KK and α_1-NN subunits has not been examined before. The specific activity of Na,K-ATPase in the metepipodites is raised 2.2-fold over a time period of several days when the external salt is increased from 15 to 120 g/l salt (Holliday et al., 1990), but it is difficult to estimate the relative contributions of the α_1- and α_2-subunit Na,K-ATPase to these changes since the molecular activities of the two isoforms in *Artemia* are not known at present.

The other important property of the α_2-KK-subunit is that the double lysine substitutions interfere with binding of Na^+ and K^+ (Tl^+) in the cation sites of Na,K-ATPase. The mutagenesis data together with molecular modeling show that the lysine substitutions reduce the number of K^+ (Tl^+) ions bound from two to about one per α-subunit. Still, the number of Na^+ ions bound per phosphorylated residue and the important Na^+/ATP stoichiometry ratio of the α_2-KK-subunit Na,K-ATPase in *Artemia* remain unknown since the low affinity for Na^+ of Na,K-ATPase does not allow a direct assay of Na^+ binding at equilibrium after heterologous expression.

In the homology models at high resolution (Fig. 7), the positions of the ε-amino group of Lys776 are in agreement with the interference of the lysine substitutions with both Na^+-dependent phosphorylation and direct binding of Tl^+(K^+) in the mutagenesis studies.

Cytoplasmic Na^+ ions activate phosphorylation from ATP, and the Na^+ ions to be transported out of the cell bind at the cation sites of the E_1P conformation with preference for binding of Na^+ over K^+ (Jorgensen & Pedersen, 2000). Both the first and the second lysine substitutions reduce Na^+-dependent phosphorylation from ATP, but 30% of Na^+-dependent phosphorylation capacity remains after the double lysine substitution. It is probable that the protonated ε-amino group of Lys776 near cation site 1 interacts with the carboxyl groups of Glu779 of M5 or Asp808 in M6 and, thus, interferes with binding of Na^+ at this site.

The molecular models do not explain the functional significance of Lys324 as its charged group points away from cation sites 1 and 2 (as shown in Fig. 7). The possibility that Lys324 could interfere with binding at a putative third Na^+ site should also be considered. A model by Ogawa & Toyoshima (2002) proposes that Tyr771 of M5, Gly806 and Thr807 of M6 and Glu954 of M9 are engaged in coordination of a third Na^+ ion; however, the ε-amino group of Lys324 also points away from these residues. Similarly, the side chain of Lys324 is far away from Ser768 and Thr772, where a partly dehydrated Na^+ ion is proposed to bind at a putative third Na^+ site on the cytoplasmic side of cation sites 1 and 2 (Håkansson & Jorgensen, 2003).

In the E_1–E_2 conformational change, the ion-binding sites change from being accessible from the cytoplasmic face of the enzyme to being accessible from the extracellular side of the enzyme, where K^+ ions to be transported into the cytoplasm bind at sites with a large preference for K^+ over Na^+ ions. In the molecular model of the E_2 form in Figure 7, the ε-amino group of Lys776 has moved near site 2, where the protonated group can interact with Asp808 of M6 and Glu327 of M4 and, thus, displace a transported Na^+ ion and interfere with binding of extracellular K^+.

The cation binding data show that the single lysine substitution of Asn324 or Asn776 reduces the Gibbs free energy of Tl^+ binding, while the interaction between the two lysine residues after double lysine substitution favors Tl^+ binding by 15.0 kJ/mol. Electrostatic repulsion between the two substituted lysines may therefore rearrange the structure to favor the binding of a single Tl^+ ion. This is supported by the ouabain binding data since both the single Asn776Lys mutation and the double mutation Asn776Lys/Asn324Lys cause large reductions of the affinity for ouabain. The ε-amino group of Lys776 can therefore act as fixed internal NH_4^+ ions to stabilize a cation-bound conformation of Na,K-ATPase with reduced affinity for ouabain. Both the cation binding and the ouabain binding data therefore support the notion that the ε-amino group of Lys776 acts as a fixed internal cation to displace binding of K^+(Tl^+) in the E_2 conformation and binding of Na^+ in the E_1P conformation.

The energy required for transport of Na^+ from cytoplasm to hemolymph in *A. franciscana* is from + 15.3 to + 18.1 kJ/mol at 25°C if it is assumed that the cytoplasmic activities in the epithelial cells are 5–15 mM Na^+, that the paracellular activity of Na^+ is 150 mM like in the hemolymph (115–185 mM) and that the basolateral potential is 100 m*V*, positive in hemolymph (Conte, 1984; Holliday et al., 1990). At external salt rising toward 280 g/l, the electrochemical gradient for Na^+ across the basolateral membrane of the epithelial cells of the salt glands increases significantly and the energy required for Na^+ transport will exceed + 20 kJ/mol Na^+ ion. At a stoichiometry of 3 Na^+/ATP the energy requirement then exceeds the energy available from hydrolysis of ATP: $-\Delta G = 50$–60 kJ/mol at 25°C as estimated by Lauger (1991). A reduction of the Na,K-pump stoichiometry from 3Na^+/ATP to 1 or 2Na^+/ATP hydrolyzed is therefore required to translocate Na^+ against the steep electrochemical gradients under salt lake conditions.

Analogues to the fixed internal positive charge in the cation sites of the α_2-KK-subunit of Na,K-ATPase (Jorgensen & Pedersen, 2000) are seen in proton pumps of plants and yeast and in the mammalian stomach. It is known that P-type plasma membrane proton pumps are capable of transporting protons or hydroxonium ions against steep gradients of [H^+] activity, e.g., from close to

pH = 7 in the cytoplasm of the parietal cells of the stomach mucosa to pH = 1–2 in gastric juice (Hersey & Sachs, 1995). Accordingly, fixed internal positive charges in cation site 1 of the E_1 conformation can be demonstrated in molecular models based on the structure of Ca-ATPase of plant and yeast plasma membrane proton pumps (Bukrinsky et al., 2001). In the model of AHA2 of *Arabidopsis thaliana*, the guanidinium group of Arg655 occupies a position equivalent to that occupied by Ca^{2+} in site 1 of SERCA1a, i.e., the same position as the ε-amino group of the substituted Lys776 in the α_1-subunit of Na,K-ATPase (Fig. 7). In a similar position, the side chain of His701 protrudes into cation site 1 of the PMA1 proton pump of *Saccharomyces cerevisiae*. In the α-subunit of H,K-ATPase, the proton pump in human gastric mucosa (Maeda et al., 1990), a serine in a position similar to Ser775 in the E_1 form of Figure 7 is substituted for a lysine with the ε-amino group near cation site 1. These charged groups can interfere with ion binding at cation site 1, and the stoichiometry in transport models for these proton pumps is assumed to be $1H^+$ or $1H_3O^+$/ATP hydrolyzed (Buch-Pedersen & Palmgren, 2003).

More distant analogues are the pyruvate kinases of *Corynebacterium glutamicum* and *E. coli*, which do not require external K^+ ions for maximum activity, while the activity of rabbit muscle pyruvate kinase depends on external K^+ for activation. Alignment reveals a lysine at position 117 in the two bacterial pyruvate kinases, whereas the rabbit muscle pyruvate kinase has a Glu at this position and substitution of Lys for Glu in the rabbit muscle pyruvate kinase eliminates the requirement for external K^+ ions (Laughlin & Reed, 1997).

The gastric H,K-ATPase is a close relative of Na,K-ATPase, and the proton pumps in the plasma membrane of plants and yeast also belong to the family of P-type ATPases. The analogous lysine substitutions in bacteria show that the ε-amino group of a lysine can substitute for K^+ ions as activator of other enzymatic reactions. With this in mind, our observations fit the notion that the lysine substitutions of α_2-KK-Na,K-ATPase act as fixed internal cations to reduce the number of ions transported per ATP hydrolyzed and, thus, enable the Na,K pump to extrude Na^+ ions against steeper gradients. The induction by salt of the expression of the α_2-KK-subunit of Na,K-ATPase in *A. franciscana* therefore plays a key role in the adaptation of brine shrimp to life in highly saline waters.

The authors are grateful to Anne M. Thomsen and David Sorensen for excellent technical assistance. This work was supported by the Danish Natural Science Research Council, the Novo-Nordic and Carlsberg Foundations and the Spanish government MCyT project (AGL2001-4582-E).

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