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Importance for Absorption of Na^+ from Freshwater of Lysine, Valine and Serine Substitutions in the α1a-Isoform of Na,K-ATPase in the Gills of Rainbow Trout (*Oncorhynchus mykiss*) and Atlantic Salmon (*Salmo salar*)

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Abstract In the gills of rainbow trout and Atlantic salmon, the α1a- and α1b-isoforms of Na,K-ATPase are expressed reciprocally during salt acclimation. The α1a-isoform is important for Na^+ uptake in freshwater, but the molecular basis for the functional differences between the two isoforms is not known. Here, three amino acid substitutions are identified in transmembrane segment 5 (TM5), TM8 and TM9 of the α1a-isoform compared to the α1b-isoform, and the functional consequences are examined by mutagenesis and molecular modeling on the crystal structures of Ca-ATPase or porcine kidney Na,K-ATPase. In TM5 of the α1a-isoform, a lysine substitution, Asn783 → Lys, inserts the ε-amino group in cation site 1 in the E_1 form to reduce the Na^+/ATP ratio. In the E_2 form the ε-amino group approaches cation site 2 to force ejection of Na^+ to the blood phase and to interfere with binding of K^+. In TM8, a Asp933 → Val substitution further reduces K^+ binding, while a Glu961 → Ser substitution in TM9 can prevent interaction of FXYD peptides with TM9 and alter Na^+ or K^+ affinities. Together, the three substitutions in the α1a-isoform of Na,K-ATPase act to promote binding of Na^+ over K^+ from the cytoplasm, to reduce the Na^+/ATP ratio and the work done in one Na,K pump cycle of active Na^+ transport against the steep gradient from freshwater (10–100 μM Na^+) to blood (160 mM Na^+) and to inhibit binding of K^+ to allow Na^+/H^+ rather than Na^+/K^+ exchange.

Keywords Structural/functional Na,K-ATPase · Epithelial transport · Trout gill · α1a-Isoform of Na,K-ATPase · Lysine substitution · Site-directed mutagenesis · Na^+ binding · K^+ binding · Tl^+ binding · Na^+/H^+ exchange

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Abbreviations

E_1	Conformation of Na,K-ATPase with cation sites adapted for Na^+ binding from the cytoplasmic surface
E_2	Conformation of Na,K-ATPase with cation sites adapted for K^+ binding from the extracellular surface
EST	Expressed sequence tags
pdb	Protein data bank
tblastn	Search translated nucleotide database using a protein query
TM	Transmembrane segment
TDS	Tris dodecyl sulphate

Introduction

The migrations of anadromous fish between freshwater and seawater are accompanied by remarkable adaptive changes in the expression of the proteins of a number of ion transport systems in the gills and kidneys (Evans et al. 2005). Acclimation in freshwater to Na^+ uptake across the epithelial cells of the gills can be particularly challenging because Na^+ ion gradients between freshwater and blood (Na^+ ~160 mM) of the fish can become very large in some rivers, e.g., in Canada, where [Na^+] activities in freshwater may drop to the range of 10–50 μM (Kirschner 2004; Bystriansky et al. 2007). In freshwater-adapted fish, Na,K-ATPase pumps Na^+ across the basolateral membrane of the epithelial cells of the gills into the blood to maintain a low intracellular [Na^+] activity and a gradient for apical uptake

of Na^+. The gradient could drive Na^+/H^+ exchange across the apical membrane (Piermarini and Evans 2001). In other models for Na^+ uptake from freshwater, an apical V-type ATPase proton pump secretes H^+ from the cell to generate an electrochemical gradient that drives Na^+ uptake via channels (Kirschner 2004; Bystriansky et al. 2006).

Two α1-subunit isoforms of Na,K-ATPase have been identified in the gills of the rainbow trout (Richards et al. 2003). The α1a- and α1b-subunit isoforms of Na,K-ATPase in the gills of the rainbow trout are expressed reciprocally during acclimation, and it is proposed that the α1a-isoform is responsible for Na^+ uptake in freshwater while the α1b-isoform is involved in ion secretion in fish acclimated to seawater. The α1a-isoform is upregulated in the gills on entry into freshwater and quickly reduced during seawater acclimation in the rainbow trout (*Oncorhynchus mykiss*) (Bystriansky et al. 2006) and in other salmonid fish like the arctic char (*Salvelinus alpinus*) (Bystriansky et al. 2007) and the Atlantic salmon (*Salmo salar*) (Shrimpton et al. 2005; Nilsen et al. 2007).

Another feature of the Na^+ uptake process after freshwater acclimation is that the apparent affinity for Na^+ is increased both for the transcellular transport process in the intact zebrafish (*Danio rerio*) (Boisen et al. 2003) and for the Na,K-ATPase activity in membranes isolated from the lamella of gills. K_M values for [Na^+] activation of Na,K-ATPase activity in freshwater-acclimated rainbow trout (*O. mykiss*) gill are about half of those in seawater trout (Pagliarani et al. 1991), as is also the case in milkfish (*Chanos chanos*) (Lin and Lee 2005). There is also evidence that the Na,K pump transports Na^+ without countertransport of K^+ in freshwater-acclimated trout, and this has been proposed to be due to displacement of the α1a-isoform Na,K-ATPase from rafts with a high content of glycosphingolipids (Lingwood et al. 2005).

The purpose of the present work was to examine if the observed differences between the α1a- and α1b-isoforms are related to substitutions of amino acid residues known to engage in coordination of Na^+ or K^+ ions in the cation sites of Na,K-ATPase (Jorgensen and Pedersen 2001; Morth et al. 2007). Alignment of the α1a- and α1b-isoforms reveals a lysine substitution, Asn783 → Lys, in transmembrane segment 5 (TM5) of the α1a-isoform of the rainbow trout compared to the α1b-isoform. This substitution is located in the same position as a lysine substitution in the α2-subunit of Na,K-ATPase of *Artemia franciscana*. Salt induction of the mRNA of the lysine-substituted α2-isoform of Na,K-ATPase in salt glands plays a key role in the adaptation of *Artemia* to life in highly saline waters at up to 280 g salt per liter (Jorgensen and Pedersen 2001; Jorgensen and Amat 2008).

With this in mind, BLAST searches of teleost genomes and expressed sequence tag (EST) databases in GenBank were performed to identify lysine substitutions and other substitutions of charged residues in transmembrane segments of potential importance for Na^+/K^+ ion coordination in Na,K-ATPase. Molecular models were constructed to visualize the position of substitutions. In comparison of the E_1 and E_2 conformations (E_1, conformation of Na,K-ATPase with cation sites adapted for Na^+ binding from the cytoplasmic surface; E_2, conformation of Na,K-ATPase with cation sites adapted for K^+ binding from the extracellular surface), the models were based on the high-resolution crystal structures of Ca-ATPase in the E_1 conformation (Protein Data Bank [pdb] 1SU4) (Toyoshima et al. 2000) or the E_2 conformation (pdb 1IWO) (Toyoshima and Nomura 2002). For the E_2[2K] conformation, molecular models were based on the recent crystal structure of porcine kidney Na,K-ATPase (pdb 3B8E) (Morth et al. 2007). The relevant mutations were produced by site-directed mutagenesis of the α1-subunit of Na,K-ATPase from porcine kidney and expressed at high yields in yeast cells (Pedersen et al. 1996), allowing studies of the consequences of the substitutions for binding of Tl^+, K^+ and Na^+ in direct binding assays.

Experimental Procedures

Amino acid sequences were identified by protein blast searches of the teleost genome database in Genbank (www.ncbi.nlm.nih.gov/blast). The tblastn option (search translated nucleotide database using a protein query) was employed in screening of EST databases in GenBank using as protein queries selected sequences of the α1a-isoform (AAQ82790) and α1b-isoform (AAQ82789) of rainbow trout Na,K-ATPase (Richards et al. 2003).

Molecular models using the software Ribbons (http://www.cbse.uab.edu/ribbons/) were kindly constructed by Kjell O. Håkansson on the basis of the coordinates of the Ca-ATPase in the E_1(Ca) form (pdb 1SU4) and in the E_2(Mg-thapsigargin) (pdb 1IWO) conformation (Toyoshima et al. 2000; Toyoshima and Nomura 2002) or the Na,K-ATPase in the E_2[2Rb] conformation (pdb 3B8E) (Morth et al. 2007).

Site-directed mutagenesis and expression of Na,K-ATPase, isolation of yeast membranes, tris dodecyl sulphate (TDS) treatment and [^{3}H]-ouabain binding were performed as described (Pedersen et al. 1996). Assay of Na^+ binding by Na^+-dependent phosphorylation in the presence of oligomycin and assays of occlusion of Tl^+ were done as before (Nielsen et al. 1998; Pedersen et al. 1998). Maximum binding capacities and $K_{0.5}$(Na+) values were estimated and lines were fitted using the equation

$$Na^+\text{dependent } E_1P/\text{ouabain ratio} = a * [NaCl]^n/(c^n + [NaCl]^n)$$

where a is the maximum phosphorylation level, c is $K_{0.5}(Na^+)$ and n is the Hill coefficient.

For K^+ displacement of [^{3}H]-ATP, aliquots of TDS-treated gradient membranes containing 150–200 μg membrane protein were incubated on ice for 30 min in 1 ml of 13 nM [^{3}H]ATP (Amersham, Aylesbury, UK; specific activity 36 Ci/mmol), 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)-Tris (pH 7.2), 10 mM EDTA-Tris and 0.1–10 mM KCl. Choline chloride was added to maintain constant ionic strength. Bound and unbound [^{3}H]ATP were separated by centrifugation at 265,000 × g for 30 min at 4°C. The supernatant was discarded and the remaining buffer removed with a paper towel. The pellet was resuspended for determination of protein and bound [^{3}H]ATP by scintillation counting. Maximum capacities and $K_{0.5}(K^+)$ values were estimated and lines were fitted using the Hill equation in SigmaPlot 9 (Systat, San Jose, CA) (Pedersen et al. 1998).

Results

Identification of Lysine Substitutions in TM5 and Substitutions in TM8 and TM9 of the α1a-Subunit of Na,K-ATPase in Rainbow Trout and Atlantic Salmon

The alignments in Table 1 identify three substitutions, Asn783 → Lys in TM5, Asp933 → Val in TM8 and Glu961 → Ser in TM9, in the Om-α1a-isoform of rainbow trout (*O. mykiss*) compared to the α1b-isoform. The same triad of substitutions was present in the Ss-α1a- and Ss-α1b-isoforms of Na,K-ATPase in the gills of the Atlantic salmon (*Salmo salar*). The lysine substitution in TM5 was found in the Dr-α1a.2- and Dr-α1a.3-isoforms of Na,K-ATPase in zebrafish (*D. rerio*), whereas it was absent from the six other α-subunit isoforms of Na,K-ATPase in zebrafish (Rajarao et al. 2001). The lysine substitutions in zebrafish are not accompanied by the substitutions in TM8 and TM9 as seen in trout and salmon. Lysine substitutions in sequences similar to TM5 of the α1a-isoform were detected in libraries from the carp (*Cyprinus carpio*, CF662950) (Gracey et al. 2004) and a Victoria lake cichlid (*Paralabidochromis chilotes*, BJ680518) (Watanabe et al. 2004) (not shown). The three substitutions observed in the α1a-isoforms in Table 1 were found in TM5, TM8 and TM9 of a complete sequence of the α-subunit of Na,K-ATPase derived from the genome of the planarian flatworm (*Schmidtea mediterranea*, AAWT01016032), renowned for its pluripotent stem cells. The lysine substitution in TM5 was also found in a leach (*Helobdella robusta*, EY372350), suggesting that the substitutions examined in this work are of more general importance for freshwater inhabitants.

Table 1 Sequence alignment of TM5, TM8 and TM9 of the Om-α1a-isoform (AAQ82790) and Om-α1b-isoform (AAQ82789) sequences of rainbow trout (*Oncorhynchus mykiss*) (Richards et al. 2003), the Ss-α1a-isoform (EG355177) and Ss-α1b-isoform (DW007008) sequences of Atlantic salmon (*Salmo salar*) (Adzhubei et al. 2007) and three of the eight isoforms of the α-subunit of Na,K-ATPase in the zebrafish (*Danio rerio*), Dr-Z1(atp1a1a.1) (AF286372), Dr-Z3(atp1a1a.2) (AF286373), and Dr-Z4(atp1a1a.3) (AF308598) (Rajarao et al. 2001) with Ss-α1, the α1-subunit (AAA31002) sequence of porcine (*Sus scrofa*) kidney Na,K-ATPase (Ovchinnikov et al. 1986)

TM5	Om-α1a	ITYTLSS**K**IPEMTPFLFLLLANIP
	Om-α1b	IAYTLTS**N**IPEISPFLLFIIANIP
	Ss-α1a	ITYTLSS**K**IPEMTPFLFLLLANIP
	Ss-α1b	IAYTLTS**N**IPEITPFLFFIIANIP
	Dr-α1a.1	IAYTLTS**N**IPEITPFLLFIIANIP
	Dr-α1a.2	IAYTLTS**K**IPEMSPFLMFVLVGIP
	Dr-α1a.3	IAYTLTS**K**IPEMSPFLMFVVVGIP
	Ss-α1	IAYTLTS**N**IPEITPFLIFIIANIP
TM8	Om-α1a	CHTAYFAAVVIAQWA**V**LIVCK
	Om-α1b	CHTAFFASIVVVQWA**D**LIICK
	Ss-α1a	CHTAYFAAVVIAQWA**V**LIVCK
	Ss-α1b	CHTAFFASIVVVQWA**D**LIICK
	Dr-α1a.1	CHTPFFVTIVVVQWA**D**LVICK
	Dr-α1a.2	CHTAFFTSIVIVQWA**D**LIICK
	Dr-α1a.3	CHTAFFISIVVVQWA**D**LLIVK
	Sc-α1	CHTAFFASIVIVQWT**D**LLICK
TM9	Om-α1a	VLIFGLC**S**ESALALFLSYCP
	Om-α1b	ILIFGLF**E**ETALAVFLSYCP
	Ss-α1a	VLIFGLC**S**ESALALFLSYCP
	Ss-α1b	ILIFGLF**E**ETALAVFLSYCP
	Dr-α1a.1	ILIFGLF**E**ETALAAFLSYCP
	Dr-α1a.2	VLIFAFF**E**EGALAAFLSYCP
	Dr-α1a.3	VLTFGLL**E**ETALAAFLSYCP
	Sc-α1	ILIFGLF**E**ETALAAFLSYCP

Marked in bold text are three substitutions of charged residues in TM5, TM8 and TM9

The amino acid sequences of TM5, TM8 and TM9 in Table 1, as well as those in TM4, TM6 and TM10 (not shown), show a high degree of identity. This is an indication of the conservation from fish to pig of the structural organization of the transmembrane segments and the functional organization of the Na^+/K^+ ion coordinating residues of the cation binding sites. The extensive functional analysis by site-directed mutagenesis of the cation coordinating residues (Jorgensen and Pedersen 2001; Jorgensen et al. 2003) and the crystal structure of porcine kidney α1-Na,K-ATPase (pdb 3B8E; Morth et al. 2007) are therefore relevant models for the arrangement of transmembrane segments and Na^+ and K^+ ion binding sites in the α1a- and α1b-isoform subunits of Na,K-ATPase in the gills of the rainbow trout and Atlantic salmon.

Homology Modeling

Positioning of the Lysine Substitution Asn776 → Lys in the Cation Sites of Na,K-ATPase

To visualize the position of the lysine substitution in the E_1P[3Na] conformation and its movement during transition to the E_2[2K] conformation, molecular models were constructed on the basis of the high-resolution (2.6 Å) crystal structure of Ca-ATPase in the E_1 or E_2 conformation (pdb 1SU4 or 1IWO) (Toyoshima et al. 2000; Toyoshima and Nomura 2002). Figure 1a shows that in the E_1 conformation the ε-amino group of the substituted lysine at position 776 in porcine kidney α1-subunit occupies cation site 1 to interfere with coordination of Na^+ ions to the carboxylates of Glu779 and Asp808. During the conformational change from the E_1 form with preference for binding of Na^+ to the E_2 form with high affinity for binding of K^+, TM5 is tilted with Pro778 as a pivotal point (Toyoshima and Nomura 2002) and the ε-amino group of Lys776 moves from cation site 1 to approach cation site 2 to interfere with K^+ binding, probably due to interactions with the carboxylates of Glu327 and Asp804 (Fig. 1b).

Recently, a three-dimensional structure of porcine kidney α1-Na,K-ATPase was solved at 3.5 Å resolution in the E_2[2Rb] conformation stabilized by MgF_4 (Morth et al. 2007). This allows positioning of the ε-amino group of lysine-776 with more confidence in the E_2[2Rb] conformation. The model in Fig. 2 shows that the substituted lysine inserts into the position of cation site 2, in almost the same position as in Fig. 1b, compatible with interference of the ε-amino group with the carboxylates of Glu327 and Asp804 and the coordination of Rb^+ or K^+ ions.

Positioning the Asp926 → Val Substitution in TM8 and the Glu953 → Ser Substitution in TM9 of the α1-Subunit of Na,K-ATPase

In the molecular model of Fig. 3, the Asp926 → Val substitution is located near the cytoplasmic end of TM8 with the side chain extending toward cation site 2 but outside the coordinating range for Rb^+ or K^+.

The carboxylate group of Glu953 in TM9 is directed toward the transmembrane segment of the incomplete structure of the γ-subunit or FXYD2, as previously observed in a crystallographic analysis of renal Na,K-ATPase (Hebert et al. 2001). The close proximity of TM9 to FXYD2 is confirmed in the 3B8E crystal structure (Morth et al. 2007). Based on this structure, the model in Fig. 3 shows that the Glu953 → Ser substitution may interfere with the association of FXYD2 with TM9 of the α-subunit of Na,K-ATPase. This effect of the mutation was observed before on the basis of a peptide association assay (Li et al. 2004).

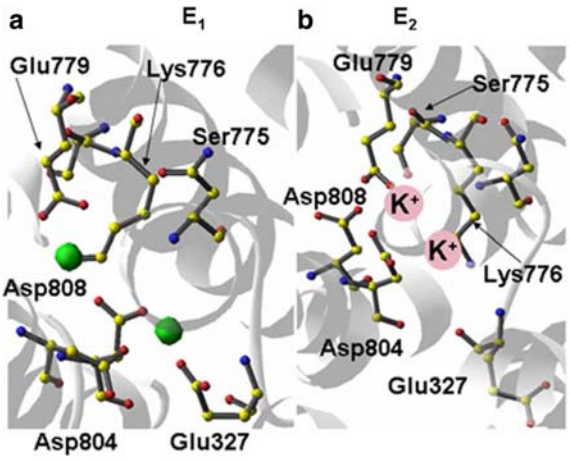

Fig. 1 a, b The position of the Asn776 → Lys substitution in the E_1 or E_2 conformation of Na,K-ATPase modeled on the backbone of Ca-ATPase in the E_1(Ca) form (pdb 1SU4) or on the E_2(Mg-thapsigargin) (pdb 1IWO) conformation. Numbering as in the α1-subunit sequence of pig kidney (P05024). Note that in the E_1 conformation the ε-amino group of the substituted lysine-776 occupies cation site 1 (*green spheres*). During transition to the E_2 conformation, the ε-amino group moves downward and to the right to approach cation site 2 (*pink spheres*)

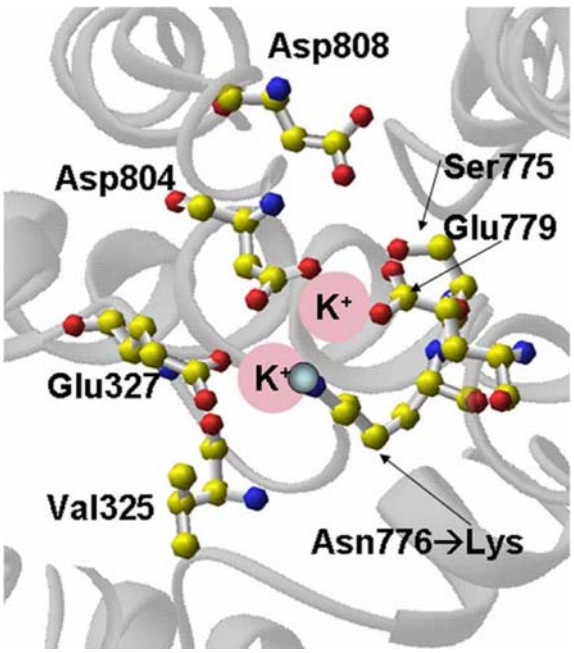

Fig. 2 Model of the position of the lysine substitution Asn776 → Lys in TM5 in the α1-subunit, based on the coordinates (pdb 3B8E) of the crystal structure of porcine kidney Na,K-ATPase (Morth et al. 2007). The ε-amino group of the lysine is seen to overlap with K^+ in cation site 2 to interfere with K^+ ion binding. Numbering of residues as in the α1-subunit sequence of pig kidney (P05024)

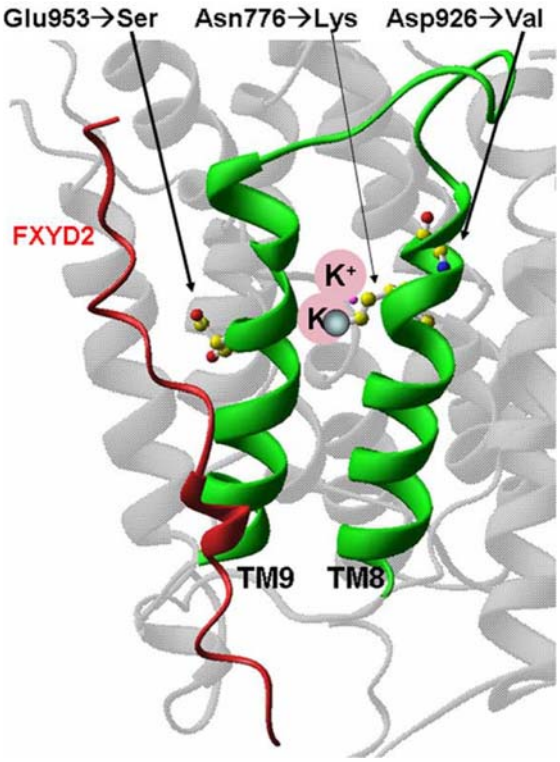

Fig. 3 Model of the position of substitutions Asp926 → Val in TM8 and Glu953 → Ser in TM9 of the α1a-isoform relative to the position of Asn776 → Lys in TM5. The model, in Ribbons, is based on the coordinates (pdb 3B8E) of the crystal structure of porcine kidney Na,K-ATPase (Morth et al. 2007). The Asp926 → Val substitution is seen to be remote from cation site 1. The Glu953 → Ser substitution in TM9 removes a carboxylate group of potential importance for interaction with FXYD2 or γ-subunit (*red color*) of Na,K-ATPase. Numbering of residues as in the α1-subunit sequence of pig kidney (P05024)

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

The ouabain binding data in Table 2 show that the level of expression of the three substitutions in yeast was equal to or higher than that of the wild-type. The Asn776–>Lys mutation increased the dissociation constant for ouabain (K_d) by 17-fold, as an indication that the ε-amino group of lysine acts as an NH_4^+ substitute for K^+ to stabilize a conformation with reduced affinity for ouabain. It is seen that the lysine substitution reduced the Na,K-ATPase activity to 43% of wild-type activity.

Table 2 Consequences for Na,K-ATPase activity and ouabain binding of lysine substitution in TM5, valine in TM8 and serine in TM9 of α1-subunit Na,K-ATPase expressed in yeast

	Na,K-ATPase activity (%)	Ouabain binding	
		(pmol/mg protein)	K_d (nM)
Asn776 → Lys	43 ± 6	19.3 ± 0.3	82 ± 7
Asp926 → Ala	81 ± 5	13.2 ± 0.5	7.8 ± 0.9
Glu953 → Ala	76 ± 6	21.4 ± 1	10.6 ± 1.7
Wild-type	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 for three or more consecutive preparations. Ouabain binding data are estimated by nonlinear regression analysis of binding at a series of [^{3}H]-ouabain concentrations. Specific activities of Na,K-ATPase were estimated and expressed in percent of wild-type activity: 100% = 71 ± 6 nmol/(min x mg protein)

Consequences of the Lysine Substitution Asp776 → Lys in TM5 of the α1-Subunit of Na,K-ATPase for Binding of Na^+ or K^+ (Tl^+)

Binding of Na^+

The apparent affinity for Na^+ was estimated from curves of Na^+ dependence of phosphorylation from ATP in the presence of oligomycin to prevent transition from E_1P to E_2P and allow monitoring of the formation of the $MgE_1P[3Na]$ complex as a dead-end reaction (Pedersen et al. 1998). It is seen from Fig. 4a that the Asn776 → Lys mutation caused a significant decrease of the apparent affinity for Na^+ binding, without reducing the capacity for phosphorylation. The $K_{0.5}$(Na+) was increased from 0.5 to 4.9 mM, corresponding to a reduction of the Gibbs free energy of Na^+ binding ($\Delta\Delta G_{b(Na+)}$) of +5.4 kJ/mol (Table 3).

Binding of Tl^+

In wild-type, two Tl^+ ions are bound per ouabain binding site with high affinity, $K_{0.5(Tl+)} = 9$ μM (Fig. 4b, Table 3), as described (Nielsen et al. 1998). The Asn776 → Lys mutation completely eliminated Tl^+ occlusion in the range 0–100 μM Tl^+. This corresponds to a large reduction (>12 kJ/mol) of Gibbs free energy ($\Delta\Delta G_{b(Tl+)}$) of Tl^+ binding. The magnitude of this change is similar to that observed after removal by mutagenesis of the carboxylate side chains of Glu327, Asp804 or Asp808 (Pedersen et al. 1998).

Binding of K^+

In wild-type, K^+ ions displaced binding of [^{3}H]-ATP at 13 nM from the E_1ATP conformation and stabilized the alternative conformation, E_2[2K], with $K_{0.5} = 0.040$ mM (Pedersen et al. 1998). In the Asn776 → Lys mutation, K^+ ions displaced [^{3}H]-ATP with a $K_{(0.5,K+)}$ value of 0.65 mM, 16-fold higher than the value for wild-type, corresponding to a reduction of the free energy of Tl^+ binding ($\Delta\Delta G_{b(K+)}$) of +8.5 kJ/mol (Table 3). The ε-amino group of Lys776 therefore interferes with binding of K^+ ions and prevents stabilization of the E_2[2K] conformation.

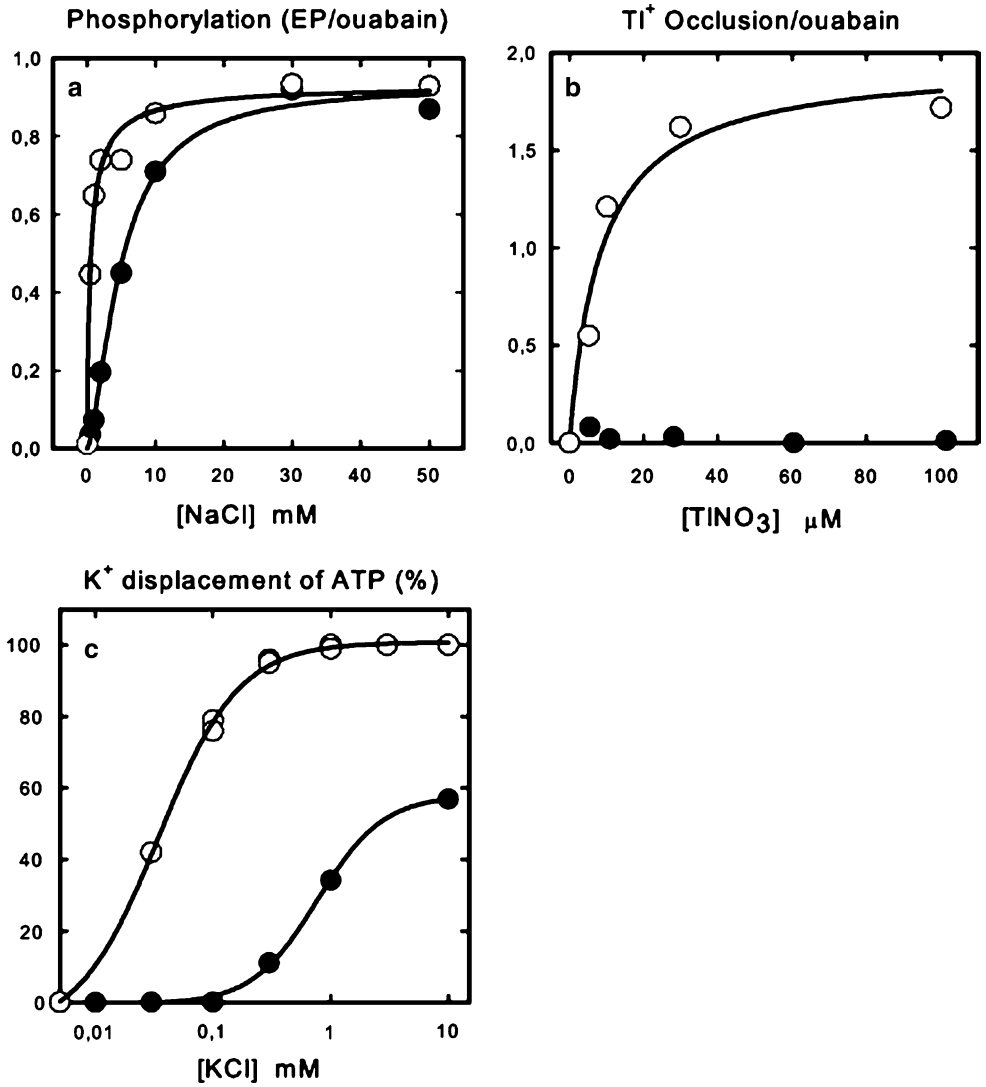

Fig. 4 (**a**) Na^+-dependent phosphorylation from [γ-^{32}P]ATP of wild-type (○) or Asn776 → Lys (●) substitution in the α1-subunit of pig kidney Na,K-ATPase expressed in yeast. Data points are average values of two separate experiments. Lines were fitted and maximum phosphorylation levels and $K_{0.5}(Na^+)$ values were estimated by nonlinear least-squares regression analysis using the Hill equation and are shown in Table 3. (**b**) Concentration dependence of $^{204}Tl^+$ occlusion in wild-type (○) or Asn776 → Lys (●) substituted α1-subunit of pig kidney Na,K-ATPase expressed in yeast. Data points are average values of two separate experiments with double determinations at each cation concentration. Maximum $^{204}Tl^+$ occlusion/ouabain binding ratio and $K_{0.5}(Tl^+)$ values were estimated by nonlinear least-squares regression analysis using the Hill equation and are shown in Table 3. (**c**) K^+ ion displacement of [^{3}H]ATP binding of wild-type (○) or Asn776 → Lys (●) substitution in the α1-subunit of pig kidney Na,K-ATPase expressed in *Saccharomyces cerevisiae*. Capacities and $K_{0.5}(K^+)$ values were estimated using the Hill equation and are shown in Table 3

Consequences of the Substitution of Asp926 → Val in TM8 of Na,K-ATPase for Binding of Na^+ or K^+ (Tl^+)

It is seen from Table 3 that the substitution Asp926 → Ala had a moderate effect on Na^+ binding and a more pronounced effect on binding of K^+. The mutation caused a threefold increase of the $K_{0.5Na+}$ and $K_{0.5Tl+}$ values and an eightfold increase of $K_{0.5K+}$ for displacement of [^{3}H]-ATP. This is in line with the 4.2-fold decrease of the $K_{0.5K+}$ for K^+ previously observed in electrophysiological assays of Na,K pump current of the Asp926 → Leu mutation (Yamamoto et al. 1996). Alanine, leucine and valine are hydrophobic side chains; and these results predict that the Asp926 → Val substitution will cause a similar reduction of the affinity for binding of K^+.

Table 3 Consequences of substitutions Asn776 → Lys, Asp926 → Ala and Glu953 → Ala of the α1-subunit of pig kidney Na,K-ATPase for Na^+, Tl^+ or K^+ ion as estimated by regression analysis from data like those in Fig. 4

	EP/ouabain ratio	$K_{0.5Na+}$ (mM)	$\Delta G_{b(Na+)}$ (kJ/mol)
Na^+-dependent phosphorylation EP/ouabain ratio and $K_{0.5Na+}$			
Wild-type	0.93 ± 0,03	0.49 ± 0.4	−17.3
			$\Delta\Delta G_{b(Na+)}$ (kJ/mol)
766K	0.94 ± 0.05	5.4 ± 0.4	+5.4
926A	0.91 ± 0.05	1.5 ± 0.3	+2.8
953A	0.82 ± 0.02	0.81 ± 0.05	+1.4
	Tl^+ occlusion/ouabain ratio	$K_{0.5Tl+}$ (μM)	$\Delta G_{b(Tl+)}$ (kJ/mol)
Tl^+ occlusion/ouabain ratio and $K_{0.5Tl+}$			
Wild-type	1.70 ± 0.02	7.0 ± 0.2	−28.3
			$\Delta\Delta G_{b(Tl+)}$ (kJ/mol)
766K	0.02	Nd	>12
926A	1.32 ± 0.8	22.6 ± 0.51	+3.3
953A	1.6 ± 0.3	17 ± 9	+2.2
	Capacity (%)	$K_{0.5K+}$ (μM)	$\Delta G_{b(K+)}$ (kJ/mol)
K^+ displacement of [^{3}H]ATP (%) and $K_{0.5K+}$			
Wild-type	108 ± 0.03	35 ± 2	−23.6
			$\Delta\Delta G_{b(K+)}$ (kJ/mol)
766K	59 ± 1	790 ± 38	+8.5
926A	96 ± 2	300 ± 13	+5.2
953A	111 ± 6	93 ± 0.01	+2.2

Gibbs free energy of Na^+, Tl^+ or K^+ ion binding was estimated from the data using the equation $\Delta G_b(X^+) = -RT \ln(EP/ouab/K_{0.5(X+)})WT$, where X can be Na^+, Tl^+ or K^+. The changes of free energy, $\Delta\Delta G_b$, were estimated using the equation of Fersht (1999): $\Delta\Delta G_b = -RT \ln[(\text{maximum binding}/K_{0.5})\text{mut}/(\text{maximum binding}/K_{0.5})\text{WT}]$

Consequences of the Glu953 → Ala Substitution in TM9 of Na,K-ATPase for Association with FXYD and Binding of Na^+

As seen from Table 3, the Glu953 → Ala substitution had only minor effects on binding of Na^+, Tl^+ or K^+. This was expected as the side chain of Glu953 in TM9 points away from the cation sites and toward the transmembrane segment of the γ-subunit or FXYD2 regulatory peptide of Na,K-ATPase. Interference with binding of FXYD peptides after substitution of the carboxylate of Glu953 by alanine or serine cannot be monitored in this system since the yeast cells are not transformed to express FXYD peptides.

GenBank tblastn searches using rat Na,K-ATPase FXYD2 or FXYD4 as probe revealed nucleotide sequences of multiple FXYD peptides in rainbow trout, Atlantic salmon, zebrafish and carp with derived amino acid sequence identities in the range of 30–47%. FXYD peptides with similarity to FXYD2 (γ-subunit of Na,K-ATPase) were identified in rainbow trout (CX255728), carp (CA969834) and zebrafish (XM_001334460). Using FXYD4 as probe, a series of derived peptides with FXYD signature sequences were found in rainbow trout (CA352723) and zebrafish (BC054135, FXYD6), but FXYD4 was not identified with certainty among these sequences. In Atlantic salmon the two probes detected several sequences: FXYD2 (BK006252), FXYD5 (BK006253), FXYD6 (BK006241), FXYD7 (NM_001123727), FXYD8 (NM_001123731), FXYD9 (NM_001123725), FXYD11 (NM_001123728) and FXYD12 (BK006249). Among these, FXYD9 and FXYD11 show the highest identities (%) of amino acid sequences with tetrapod FXYD4 or CHIF. Expression of mRNA of several of these FXYD genes in gill and kidney of Atlantic salmon was recently shown to differ between freshwater and seawater (Tipsmark 2008).

Discussion

The data point at functional differences between the α1a- and α1b-isoforms of Na,K-ATPase in the gills of rainbow trout and Atlantic salmon. The structural and functional analyses of the three substitutions in TM5, TM8 and TM9 of the α1a-isoform compared to the α1b-isoform raise important questions concerning the significance of the reduction of the 3 Na^+/ATP ratio and the pronounced inhibition of K^+ ion binding due to the lysine substitution Asn783 → Lys in TM5 as well as the additional inhibition of K^+ ion binding by the Asp933 → Val substitution in TM8. The third substitution, Glu961 → Ser in TM9, raises the possibility that the ion transport regulators of the FXYD family have important effects on Na,K-ATPase in the gills of anadromous fish comparable to their role in the tubular cells of mammalian kidneys (Garty and Karlish

2006) and in the salt glands of the shark, *Squalus acanthias* (Mahmmoud et al. 2003).

Detection of a lysine substitution in TM5 of the α-subunit of Na,K-ATPase in rainbow trout, salmon, zebrafish, carp and a cichlid suggests that this is a widespread evolutionary modification among fish migrating into freshwater. At present, the number of identified lysine substitutions in this group appears to be limited by the dearth of full-length amino acid sequences of teleost α-subunits of Na,K-ATPase.

It is also intriguing that extreme salinity challenges at either end of the spectrum elicit the same modification, expression of a lysine-substituted α-isoform of Na,K-ATPase, as part of the solution by natural evolution to survival of fish in salt-depleted freshwater and to survival of a crustacean, *Artemia franciscana*, in salt lakes at up to 5 M salt. Lysine substitutions have not been identified in Na,K-ATPase sequences other than those shown here in fish, flatworm and leach or earlier in *Artemia* (Jorgensen and Amat 2008). In the closely related gastric H,K-ATPase, a lysine substitution is inserted in a position similar to Ser775 in Figs. 1a and 2 (Maeda et al. 1990) to adjust the ratio to one H^+ per ATP hydrolyzed. In the α-subunit of Na,K-ATPase, the mutation Ser775 → Lys (adjacent to Asn776 studied in this work) strongly inhibits extracellular K^+ binding and Rb^+ uptake in oocytes (Burnay et al. 2003).

Positively charged arginine or histidine residues are inserted in the cation sites of P-type proton pumps in plants and yeast to keep the H^+/ATP ratio close to a value of 1 and to allow the buildup of steep electrochemical H^+ gradients with membrane potentials up to 250 m*V* (Pedersen et al. 2007).

The cartoon model in Fig. 5 illustrates the effects of the substituted lysine in TM5 on the Na^+ stoichiometry and the movement of the positively charged ε-amino group during the E_1–E_2 transition. In the E_1 conformation the ε-amino group occupies cation site 1 and acts as a fixed internal cation to prevent binding of at least one Na^+ ion, thus reducing the ratio of the pump from $3Na^+$/ATP to $2Na^+$/ATP. In the E_1–E_2 conformational change, twisting or tilting of the transmembrane helices adapts distances between cation coordinating oxygen atoms to the dimensions suitable for coordination of K^+ ions (Jorgensen and Pedersen 2001). In the E_2 conformation the ε-amino group of the substituted lysine has moved to cation site 2, where the protonated group can interact with Glu327 of TM4 and Asp804 of TM6 and displace the transported Na^+ ions. The movements of the transmembrane helices also open a pathway to the extracellular space (Olesen et al. 2007), allowing for exchange of Na^+ for K^+. The electrostatic repulsion of the positively charged ε-amino group of the lysine contributes to the ejection of the transported Na^+ ions from the water-filled pathway to the extracellular medium and to the inhibition of the binding of K^+ ions.

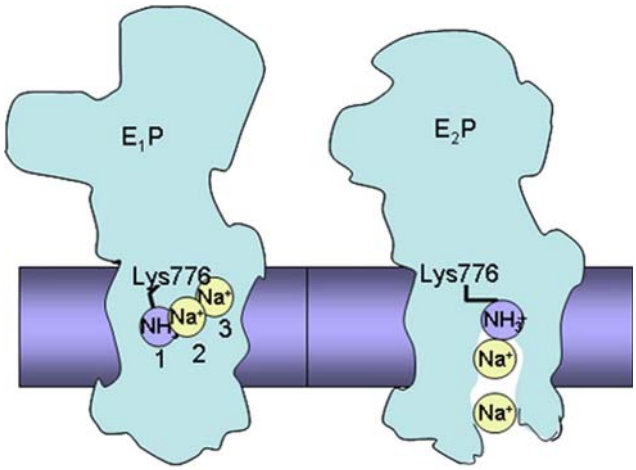

Fig. 5 Cartoon model of the position and functions of the lysine substitution in the α1-subunit of Na,K-ATPase. In the E_1 conformation, the ε-amino group of Lys776 (Lys783 in trout α1a-isoform) occupies cation site 1 and acts as a fixed internal cation to prevent binding of at least one Na^+ ion, thus reducing the stoichiometry of the pump to 2Na/ATP. During transition to the E_2 conformation, the ε-amino group of Lys776 approaches cation site 2, where the protonated group can displace the transported Na^+ ions and interfere with binding of K^+

The present data are compatible with a reduction of the Na^+/ATP ratio in the α1a-isoform of Na,K-ATPase, but the precise stoichiometry cannot be determined because the relatively low affinity for Na^+ prevents direct assay of radiolabeled $^{22}Na^+$ binding at equilibrium in recombinant Na,K-ATPase preparations. A reduction of the Na^+/ATP ratio is important as the work done will be reduced if only two Na^+ ions are transported per Na,K pump cycle, as shown in Fig. 6 in calculations based on the assumptions of Kirschner (2004). At freshwater [Na^+] activities of 10–100 μM, the cost of one Na,K pump cycle with $3Na^+$/ATP will exceed the free energy of ATP hydrolysis: $-\Delta G =$ 50–60 kJ/mol at 25°C (Lauger 1991). Reduction by a lysine substitution of the ratio to two Na^+ ions transferred per ATP hydrolyzed would probably allow Na,K-ATPase to handle absorption against this steep gradient. In the arctic char (*Salvelinus alpinus*) the increase in expression of the α1a-isoform of Na,K-ATPase in the gills is particularly pronounced during its migration into rivers in northern Canada, where the fish can thrive at [Na^+] activities in the range of 10–50 μM (Bystriansky et al. 2007); but amino acid sequences are not available to identify putative lysine substitutions in the α1a-isoform of the arctic char.

The rate-limiting step for Na,K pump turnover in epithelial cells during Na^+ absorption from freshwater is the loading of cytoplasmic Na^+ sites from the intracellular medium with a relatively low [Na^+] and a much higher

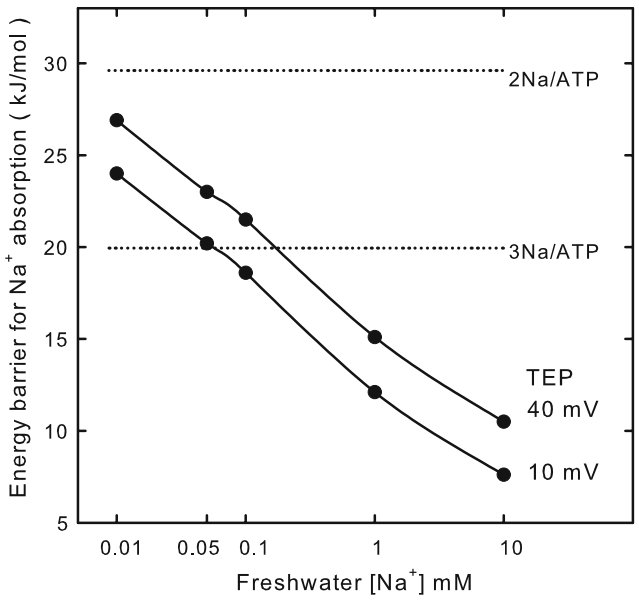

Fig. 6 Estimation of the energy barrier for absorption of Na^+ (kJ/mol Na^+ transported) across gill epithelial cells as a function of freshwater [Na^+] activity from 10 μM to 10 mM at transepithelial potentials (TEP) of 10 or 40 m*V* and blood Na^+ at 170 mM. Calculations based on the equation by Kirschner (2004): $\Delta\mu Na = RT \log[Na^+]s/[Na^+]a + zF\Delta\Psi$, where *R*, the gas constant, is 8.31 J $(°K)^{-1}$ $mole^{-1}$; *T* is temperature (~287°K); *F* is the Faraday constant (96.5 kJ $mole^{-1}$); *z* is valence (here +1); $\Delta\Psi$ is the transepithelial potential (V); s is serosal or blood side; and a is apical. *Dotted lines* represent the estimated energy barrier for Na^+ absorption (kJ/mol Na^+) at $3Na^+$ or $2Na^+$ per Na,K-ATPase cycle, assuming 100% utilization of the free energy of ATP hydrolysis ($-\Delta G = 60$ kJ/mol) (Lauger 1991)

[K^+] activity (Jorgensen 1980; Jørgensen et al. 2003). In an unmodified α1-Na,K pump, the ratio of the apparent affinities for Na^+ and K^+ at the cytoplasmic surface corresponds to a 16-fold preference for Na^+ over K^+ (Jorgensen and Pedersen 2001). As the combined effect of substitutions Asn783 → Lys and Asp933 → Val is to reduce the apparent affinity for K^+ much more than that for Na^+, they will increase the preference for binding of Na^+ over binding of K^+ at the cytoplasmic pump surface. This can contribute to the increase of the apparent affinity for Na^+ observed in titrations of the α1a-isoform Na,K-ATPase activity in vitro (Pagliariani et al. 1991; Lin and Lee 2005) and to the increased apparent affinity for Na^+ in the uptake process in vivo in freshwater-adapted zebrafish (Boisen et al. 2003).

For the estimates in Fig. 6 it is assumed that the active transcellular transport of Na^+ is catalyzed by Na,K-ATPase, and the calculations must be modified if other primary active transporters contribute to Na^+ uptake across the apical membrane. One proposal is that an apical V-type ATPase secretes H^+ from the cell to generate an electrochemical gradient that drives Na^+ uptake via channels (Kirschner 2004; Evans et al. 2005). Another possibility is that the severe reduction of the affinity for K^+ can alter the transport mode of Na,K-ATPase. In the E_2 conformation, extracellular K^+ ions bind at sites with a large preference for K^+ over Na^+ ions but protons compete with K^+ for binding to the cation sites of Na,K-ATPase (Apell 2003). When the apparent affinity for K^+ is strongly reduced by the fixed internal positively charged ε-amino group of lysine, H^+ can substitute for K^+ ions and the α1a-isoform Na,K-ATPase can mediate Na^+/H^+ exchange and contribute to the gradient required for Na^+/H^+ exchange across the apical membrane of the gill cells. Na^+/H^+ exchange has earlier been demonstrated as a transport mode in Na,K-ATPase (Polvani and Blostein 1988), and the P-type Ca-ATPase catalyzes $Ca^{2+}/2H^+$ exchange (Olesen et al. 2007).

With respect to K^+ binding, the α1a-isoform Na,K-ATPase in fish is different from the α2-NaK-ATPase in *Artemia franciscana*. In the salt glands of *Artemia*, increasing salinity strongly induces the mRNA of the α2-subunit of Na,K-ATPase with double lysine substitutions to drive secretion of Na^+ from blood (170 mM Na^+) to salt brine at 280 g/l salt (~5 M Na^+). In the α2-subunit of *Artemia*, the Asn776 → Lys substitution in TM5 is supplemented by a second Asn324 → Lys substitution in TM4. The side chain of Lys324 points away from the cation sites but interacts with Lys776 to restore binding to about one Tl^+ or K^+ ion per α2-subunit and to allow Na^+/K^+ exchange (Jorgensen and Amat 2008). In contrast, K^+ and Tl^+ binding remain severely reduced in the α1a-isoform Na,K-ATPase, suggesting that it can catalyze Na^+/H^+ exchange in the gills of trout and salmon. An important aspect of this transport mode of the α1a-isoform Na,K-ATPase is that it may represent a solution to the problem of taking up Na^+ from freshwater with high affinity without losing K^+ to the environment.

The contribution of the Glu953 → Ser substitutions in TM9 of the α1a-isoform in the gills of trout and salmon is more uncertain. The structural data (Hebert et al. 2001) and previous mutagenesis experiments (Li et al. 2004) suggest that this substitution can interfere with the association of FXYD peptides with TM9 of the α1a-subunit and thus alter the affinities for Na^+ or K^+ (Garty and Karlish 2006). Recent data reveal a complex pattern of expression of mRNA of several FXYD peptides in gill and kidney of the Atlantic salmon depending on salinity. A substantial number of regulators—FXYD5, FXYD6, FXYD7, FXYD8, FXYD9 and FXYD11—appear to be expressed in the gills of freshwater-adapted salmon (Tipsmark 2008).

In seawater-adapted gills, Na,K-ATPase appears to be embedded in glycosphingolipid-enriched rafts, but the α1a-isoform Na,K-ATPase in freshwater trout is insensitive to arylsulfatase and probably not associated with rafts (Lingwood et al. 2005). These authors suggest that the freshwater α1a-isoform Na,K-ATPase catalyzes uncoupled Na^+ efflux

and that Na^+/K^+ exchange depends on interactions with glycosphingolipid. The present experiments rather show that the ε-amino group of lysine interferes with K^+ binding to alter the transport mode of the α1a-isoform Na,K-ATPase from Na^+/K^+ to Na^+/H^+ exchange. The dissociation of the α1a-isoform Na,K-ATPase from rafts could be due to substitutions other than those in the segments of transmembrane helices of importance for cation binding.

The predictions in the present work are based on bioinformatics, molecular modeling and site-directed mutagenesis experiments on the α1-subunit of porcine Na,K-ATPase. Better understanding of the physiological consequences of the substitutions requires further progress in localization of the α1a- and α1b-isoforms of Na,K-ATPase in the epithelial cells of the gills and identification of the ion transport regulators of the FXYD family in these cells. Equally important are direct measurements of the transport modes, Na^+/K^+ or Na^+/H^+ exchange, and identification of the channels and cotransporters involved in the transcellular Na^+ absorption from freshwater.

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