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Phospholemman (FXYD1) Raises the Affinity of the Human $\alpha_1\beta_1$ Isoform of Na,K-ATPase for Na Ions

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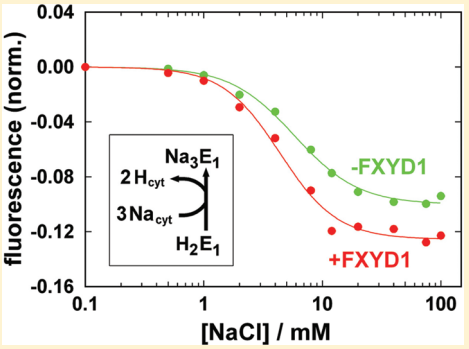
ABSTRACT: The human α_1/His$_{10}$-β_1 isoform of the Na,K-ATPase has been expressed in *Pichia pastoris*, solubilized in *n*-dodecyl-β-maltoside, and purified by metal chelate chromatography. The $\alpha_1\beta_1$ complex spontaneously associates in vitro with the detergent-solubilized purified human FXYD1 (phospholemman) expressed in *Escherichia coli*. It has been confirmed that FXYD1 spontaneously associates in vitro with the α_1/His$_{10}$-β_1 complex and stabilizes it in an active mode. The functional properties of the α_1/His$_{10}$-β_1 and α_1/His$_{10}$-β_1/FXYD1 complexes have been investigated by fluorescence methods. The electrochromic dye RH421 which monitors binding to and release of ions from the binding sites has been applied in equilibrium titration experiments to determine ion binding affinities and revealed that FXYD1 induces an ~30% increase of the Na^+-binding affinity in both the E_1 and P-E_2 conformations. By contrast, it does not affect the affinities for K^+ and Rb^+ ions. Phosphorylation induced partial reactions of the enzyme have been studied as backdoor phosphorylation by inorganic phosphate and in kinetic experiments with caged ATP in order to evaluate the ATP-binding affinity and the time constant of the conformational transition, Na_3E_1-P → P-E_2Na_3. No significant differences with or without FXYD1 could be detected. Rate constants of the conformational transitions Rb_2E_1 → $E_2(Rb_2)$ and $E_2(Rb_2)$ → Na_3E_1, investigated with fluorescein-labeled Na,K-ATPase, showed only minor or no effects of FXYD1, respectively. The conclusion from all these experiments is that FXYD1 raises the binding affinity of $\alpha_1\beta_1$ for Na ions, presumably at the third Na-selective binding site. In whole cell expression studies FXYD1 reduces the apparent affinity for Na ions. Possible reasons for the difference from this study using the purified recombinant Na,K-ATPase are discussed.



The Na,K-ATPase is an integral membrane protein present in virtually all animal cells, where it transports Na^+ and K^+ ions across the plasma membrane and maintains their electrochemical potential gradients, required for electrical excitability and transport of other ions, nutrients, and neurotransmitters, as well as for regulation of cell volume and intracellular pH.

The Na,K-ATPase is a heterodimer composed of the catalytic α subunit that couples ATP hydrolysis and ion transport, and the β subunit which is involved in structural and functional maturation of the enzyme and responsible for trafficking to the cytoplasmic membrane. For about 30 years, a third subunit has been known to be associated with the $\alpha\beta$ complex of the renal Na,K-ATPase.[1] More recently, it was discovered that it belongs to the family of FXYD proteins,[2] a group of seven small transmembrane proteins named after the invariant extracellular motif FXYD. The FXYD proteins are localized in excitable tissues and organs involved in solute and fluid transport, where they are proposed to act as regulators of the ion transport. All members of the family are known to associate with the Na,K-ATPase and modulate its properties in a tissue- and isoform-specific way (for review see refs 3−6).

FXYD1, also known as phospholemman (PLM), was first identified as the major substrate for protein kinases A and C in heart.[7−9] When expressed in *Xenopus oocytes*, it induces chloride-activated currents[10] and transports the zwitterionic amino acid taurine;[11] moreover, it was demonstrated to interact with and regulate the Na^+/Ca^{2+} exchanger[12,13] and the L-type cardiac Ca channel.[14,15]

In 2002, FXYD1 was also discovered to modulate the Na,K-ATPase activity when coexpressed in *X. oocytes* and to interact specifically with the $\alpha_1\beta$ and $\alpha_2\beta$ isozymes in native cardiac and skeletal muscle.[16]

Subsequently, it was localized also in the renal juxtaglomerular apparatus with the β_2 subunit in extraglomerular mesangial cells and with $\alpha_2\beta_2$ in renal cortical blood vessels and the afferent arteriole.[17] FXYD1 was also reported to associate with the $\alpha_1\beta$, $\alpha_2\beta$, and $\alpha_3\beta$ isoforms in cerebellum and choroid plexus.[18] Recent experimental evidence has demonstrated an even wider distribution, suggesting that it may not only be involved in the physiological regulation of contractile tissues, urinary epithelial tissues, and in the nervous system but also in the regulation of the Na,K-ATPase or other ion transporters in

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reproductive, gastrointestinal, hepatic, and pancreatobiliary systems.[19]

To elucidate the physiological significance of Na,K-ATPase modulation by FXYD1, functional interactions have been brought recently into focus. The observations reported differed, however, often from one expression system to another and were sometimes even contradictory. In *X. oocytes* FXYD1 was observed to induce a 2-fold reduction in the apparent affinity for cytoplasmic Na^+ ions, a small decrease in the apparent affinity for extracellular K^+ ions, and no effect on the maximal pump current of Na,K-ATPase.[16]

Functional effects of FXYD1 have been studied extensively in cardiac myocytes, especially the effects of protein kinase A and C activation. In general, FXYD1 has been observed to inhibit active pumping and this inhibition is relieved upon phosphorylation by PKA and PKC. In most studies FXYD1 reduced apparent Na^+ affinity, although effects on the maximal pump rate, v_{max}, have also been detected.[20−24] When FXYD1-knock out mice were compared to wild-type mice no difference in apparent Na^+ affinity was found but a lower v_{max} in sarcolemma membranes from heart.[25]

As described previously, Na,K-ATPase expressed in *Pichia pastoris* has been purified in a detergent-soluble state.[26,27] The Na,K-ATPase can either be coexpressed with FXYD1 in *P. pastoris* and then purified or the purified Na,K-ATPase can be reconstituted in vitro with FXYD1 expressed separately in *P. pastoris*. In vitro reconstitution is the method of choice because it allows experimental control of the molar ratios of $\alpha\beta$ and FXYD1 subunits.[28] FXYD1 was found to raise the apparent affinity of Na,K-ATPase for Na^+ ions in ATPase activity assays, in contrast to the findings in other studies. The availability of the purified recombinant Na,K-ATPase, including the most recent development of in vitro reconstitution of an $\alpha1\beta1$-isoform complex with FXYD1 purified from *Escherichia coli* membranes,[29] now makes it feasible to apply biophysical techniques to study functional modulation by FXYD1. In the current study, the various reaction steps around the Post-Albers cycle were investigated with fluorescence techniques based on the voltage-sensitive dye RH421 that allows the recording of ion movements in the membrane domain of the protein, and with an FITC-labeled enzyme that reports conformation transitions of the protein in the absence of ATP.[30,31] RH421 has been widely and successfully used to characterize the electrogenic partial reactions in the pump cycle of the membrane-bound Na,K-ATPase.[32−37] To obtain significant fluorescence signals, the pump density in the membrane preparations must be high, so that all dye molecules are close to the transmembrane domain of the ion pumps.[32] Recently, we have demonstrated that it is possible to overcome this limitation and to extend the method to detergent-solubilized native and purified recombinant Na,K-ATPase.[38] This is an important development of the RH421 technique that opens up the path to study purified recombinant proteins. In particular, it allows the investigation of Na,K-ATPase reconstituted with FXYD1 in well defined conditions and the characterization of detailed effects of FXYD1 on the various ion-transport properties.

In a first step of the current study, a steady-state fluorescence method was applied to obtain apparent binding affinities for Na^+ and K^+ ions in different substrate-dependent equilibrium states of the pump cycle, especially the two principal conformations, E_1 and E_2-P. In a second step, time-resolved kinetic experiments were performed in which concentration jumps of substrates of the Na,K-ATPase were used to obtain the apparent ATP-binding affinity and the time constants of the conformational transitions E_1-P → E_2-P, E_1 → E_2, and E_2 → E_1 of both the $\alpha_1\beta_1$ and $\alpha_1\beta_1$FXYD1 complexes.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA) was obtained from Bio-Rad and nicotinamide adenine dinucleotide (NADH) was from Roche. *n*-Dodecyl-β-maltoside (DDM) was purchased from Anatrace and BD-Talon metal affinity resin from Clontech. Octaethylene glycol monododecyl ether ($C_{12}E_8$) was obtained from Bachem and 1-stearoyl-2-oleoyl-*sn*-glycero-3-(phospho-l-serine) (SOPS) was from Avanti Polar Lipids. AcTEV Protease was from Invitrogen. ATP (disodium salt, special quality) was obtained from Roche. The electrochromic styryl dye RH421 was ordered from MoBiTec and NPE-caged-ATP (disodium salt) from Molecular Probes. Fluorescein 5′-isothiocyanate (FITC, F7250) was from Sigma. Recombinant cAMP-dependent protein linase (PKA) (2 500 000 units/mL, cat. no. P6000L) and calf intestinal alkaline phosphatase (CIP) (10 000 units/mL, cat. no. M0290S) were obtained from Bio Laboratories. All other reagents were purchased from Merck or Sigma-Aldrich at the highest quality available.

Purification of Na,K-ATPase Expressed in *P. pastoris*. The human α_1/His_{10}-β_1 isoform of Na,K-ATPase was expressed in *P. pastoris* as described previously.[26] The His tag was added to the N′ terminus. The cells were broken with glass beads and urea-treated membranes were prepared.[26] Approximately 1 g of membrane protein was obtained from 100 g of cells. The membranes were solubilized with DDM (DDM/protein 2:1 w/w) and the human α_1/His_{10}-β_1 complex was purified by metal chelate chromatography with BD-Talon beads as described in refs 27 and 39. The protein was eluted from the beads in a solution containing 180 mM imidazole, 20 mM tricine, pH 7.4, 100 mM NaCl, 0.1 mg/mL $C_{12}E_8$, 0.05 mg/ml SOPS, 0.01 mg/mL cholesterol, and 10% glycerol. Approximately 100 μg of purified enzyme was obtained from 100 mg of membrane protein.

To perform the biophysical experiments in the desired conditions, the elution buffer was replaced by a solution of 20 mM tricine, pH 7.4, and 25% glycerol, and concentrated to 0.7−1.1 mg/mL protein with Amicon Ultra Centrifugal Filters 10K (exclusion size 10 kDa). The protein was stored at 0 °C or at −80 °C after freezing with liquid N_2.

The protein concentration was determined either by the Lowry or the BCA assay. The specific ATPase activity was tested by the pyruvate kinase/lactate dehydrogenase assay.[40]

FXYD1 Preparation from *E. coli*. The human FXYD1 was expressed in *E. coli* as described elsewhere.[28,29,39] FXYD1 carried an N-terminal His_6 tag. In short, the cells were broken with two cycles of French Press at 10000 psi in phosphate buffer, pH 7.3, 2 mM $MgSO_4$, 0.5 mM PMSF, and 10 μg/mL DNase. Unbroken cells and heavy membranes were removed by centrifugation at 10000*g* for 15 min and light membranes were collected at 130000*g* for 90 min. The pellet was suspended in NaCl 100 mM, tricine 20 mM, pH 7.4, imidazole 10 mM, glycerol 10%, and protease inhibitors (0.5 mM PMSF, 0.5 mM leupeptin, 0.5 mM pepsatin, 0.5 mM chymostatin), and stored at −80 °C. Approximately 4 g of membrane protein were obtained per 100 g cells. The FXYD1 was purified similarly to the α_1/His_{10}-β_1 complex: the membranes were solubilized with DDM (DDM/protein 2:1 w/w) and the FXYD1 was purified by

metal-chelate chromatography with BD-Talon beads in the presence of 40 μM EDTA at a concentration of 1 mg/mL protein. The protein was eluted as described above and the His-tag was removed by treatment with AcTEV protease as described.[29] Approximately 50 μg of purified protein were obtained from 100 mg of membranes.

In Vitro Reconstitution of the α_1/His$_{10}$-β_1/FXYD1 Complex. To compare complexes without and with FXYD1, the membranes expressing the α_1/His$_{10}$-β_1 complex were solubilized in DDM and incubated with BD-Talon beads for 4 h at 6 °C, then the loaded BD-Talon beads were split in two parts: one part was incubated in a buffer containing 10 mM imidazole, 20 mM tricine, pH 7.4, 100 mM NaCl, 0.1 mg/mL $C_{12}E_8$, 0.05 mg/ml SOPS, 0.01 mg/mL cholesterol, and 10% glycerol with purified FXYD1 at a molar excess up to 10-fold, the other part was incubated, as control, in the same buffer without FXYD1. After 4 h at 6 °C both preparations were completed in parallel, the excess FXYD1 was removed by washing, and the complexes with and without FXYD1 were eluted as described above. To verify reconstitution, the FXYD1/Na,K-ATPase ratio was checked by quantitative protein determination with gels for the α subunit and with Western Blots for FXYD1.[39]

Two antibodies were used to recognize FXYD1: anti-FXYD1 C-terminus raised against the C-terminal sequence CRSSIRRLSTRRR[16] that recognizes mainly unphosphorylated FXYD1[28] and anti-CP-68, kindly provided by Prof. Donald Bers, which recognizes the phosphorylated Ser 68 of the cytoplasmic domain of FXYD1.[41] PKA phosphorylation and dephosphorylation was done during purification of the FXYD1 while it was bound to the beads.

FITC Labeled-Na,K-ATPase. The membranes expressing $\alpha_1\beta_1$ subunits were suspended at 2 mg/mL in a buffer containing 50 mM NaCl, 1 mM EDTA, 20 mM Tris pH 9.2, with protease inhibitors and incubated for 1 h at 20 °C in the dark with 1 μM FITC. The suspension was then diluted 3-fold with an ice-cold solution of 100 mM MOPS pH 6.45, mixed for 10 min, and centrifuged at 100000*g* for 80 min.[42] The labeled membranes were resuspended at 1 mg/mL in 10 mM MOPS-Tris pH 7.4 and 25% glycerol. Purification of FITC-labeled Na,K-ATPase was performed as described for unlabeled Na,K-ATPase. Reconstitution of FITC-labeled Na,K-ATPase with FXYD1 in solution was done by incubating FXYD1, uncleaved with TEV protease, with the eluted FITC-labeled $\alpha_1\beta_1$ complex at a 10-fold excess of FXYD1 overnight at 0 °C, as described recently.[29]

Steady-State RH421 Fluorescence Measurements. The steady-state fluorescence measurements were carried out in a Perkin-Elmer LS 50B fluorescence spectrophotometer as described before.[43] The excitation wavelength was set to 580 nm and the emission wavelength to 660 nm (slit widths of 15 and 20 nm, respectively). The experiments were performed in a buffer containing 25 mM imidazole and 1 mM EDTA, 5 mM $MgCl_2$, pH 7.2. Subsequently, 200 nM RH421 and 9 μg/mL detergent-solubilized Na,K-ATPase were added to the thermostatted cuvette and equilibrated until a stable fluorescence signal, F_0, was obtained. Titrations were conducted by addition of small aliquots of the indicated substrates from various concentrated stock solutions until signal saturation. To allow comparison between different titration experiments, normalized fluorescence changes with respect to the initial fluorescence level, F_0, were calculated according to $\Delta F/F_0 = (F - F_0)/F_0$. The substrate-dependent fluorescence change, F_{norm}, was fitted by the Hill function,

$$F_{norm}([X^+]) = F_0 + \Delta F_{max}/(1 + ([X^+]/K_{1/2})^{-n}) \quad (1)$$

$[X^+]$ is the substrate ion concentration, ΔF_{max} the maximum fluorescence change, $K_{1/2}$ the half saturating concentration, and n the Hill coefficient. All experiments were performed at 20 ± 0.5 °C.

Measurement of Transient RH421 Fluorescence Signals after Photochemical Release of ATP. The transient fluorescence signals produced by photochemical release of ATP were measured as described in principle previously.[44] The cylindric quartz cuvette was filled with 300 μL of a suspension of 9 μg/mL detergent-solubilized Na,K-ATPase in a medium containing 25 mM imidazole, 1 mM EDTA, 5 mM $MgCl_2$, pH 7.2, 200 nM RH421, 50 mM NaCl, and various amounts of caged ATP. The fluorescence was excited by a HeNe laser set at 580 nm. ATP was released from caged ATP in the cuvette by a light flash (wavelength 308 nm, total energy 150 mJ, duration 10 ns) generated by an EMG 100 excimer laser (Lambda Physics, Göttingen). At pH 7.0 ATP is released from caged ATP with a time constant of 4.6 ms.[45] The concentration of released ATP was determined by the luciferin/luciferase test which was calibrated using solutions of known ATP concentration.[46] About 10% of ATP is released from caged ATP by a single flash. To remove traces of free ATP from the sample of caged ATP, a small amount of apyrase (1.4×10^{-3} U) and 1.4 mM $MgCl_2$ were added to the stock solution of 10 mM caged ATP.

The fluorescence light emitted from the optical cell was collected by an ellipsoidal mirror and focused onto the cathode of the photomultiplier. The photomultiplier current was amplified, digitized by a 12-bit data-acquisition board of a PC with a sampling frequency of 100 kHz and stored for further analysis.

Fluorescein Fluorescence Measurements. Equilibrium fluorescence changes were measured in a Varian fluorimeter at room temperature 20−23 °C. 10−15 μg of FITC-labeled purified α_1/His$_{10}$-β_1 or α_1/His$_{10}$-β_1/FXYD1 complexes were incubated for 30 min at room temperature and then added to a stirred cuvette containing 2 mL of the following solution: 150 mM choline chloride 10 mM Hepes (Tris) pH 7.5 or 7.0. RbCl was added in increments until the fluorescence was constant. The titrations curves were fitted to the Hill function,

$$\Delta F_{norm}([Rb^+]) = \Delta F_{max}/(1 + ([Rb^+]/K_{1/2})^{-n}) \quad (2)$$

where ΔF_{max} is the maximum fluorescence change, $K_{1/2}$ the half saturating concentration, and n the Hill coefficient.

Stopped-flow fluorescence measurements at 23 °C were performed using an Applied Photophysics stopped-flow fluorimeter. The excitation wavelength was set at 495 nm via a monochromator, and the emitted fluorescence was measured at 515 nm using a cutoff filter. Solutions: Total ionic strength of all solutions in both syringe was maintained at 175 mM, consisting of 10 mM Hepes, pH 7.5, and 165 mM choline chloride plus RbCl or NaCl (see ref 42). To monitor the transition $E_2(Rb_2) \rightarrow Na_3E_1$, the applied solutions contained in addition 20−30 μg FITC-labeled α_1/His$_{10}$-β_1 or α_1/His$_{10}$-β_1/FXYD1, and 20 mM RbCl in syringe 1, and 80 mM NaCl in syringe 2. For the transition $E_1 \rightarrow E_2(Rb_2)$ syringe 1 contained additionally 20−50 μg FITC-labeled α_1/His$_{10}$-β_1 or α_1/His$_{10}$-β_1/FXYD1, and syringe 2 20, 40, and 165 mM RbCl. To analyze the data, traces of 4−9 experiments were averaged and fitted by a single or double exponential function using the Kaleidagraph program (Synergy Software).[42]

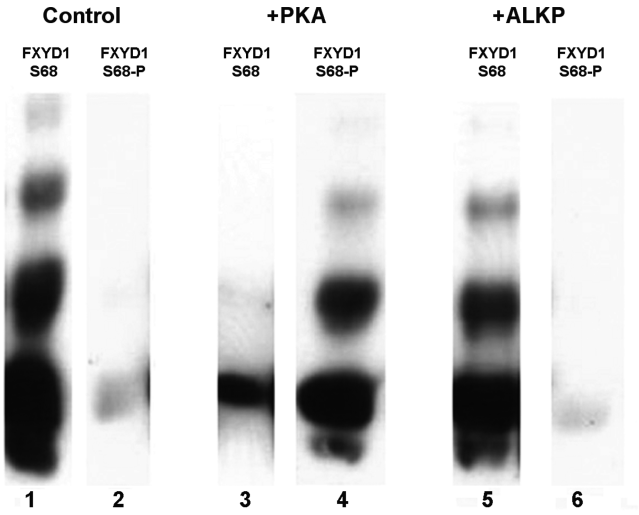

Figure 1. Western blots of phospholemman (FXYD1) isolated from *E. coli*. The antibody anti-FXYD1 was used to label the unphosphorylated form of FXYD1 (S68, lanes 1, 3, 5), while antibody CP68 labels the Ser68-phosphorylated protein (S68-P, lanes 2, 4, 6). Labeling of the isolated FXYD1 by both antibodies shows no significant phosphorylation in the control and after treatment with alkaline phosphatase (+ALKP), in contrast to the state after protein kinase A (+PKA) when phosphorylated FXYD1 is no longer recognized by anti-FXYD1 (lane 3).

RESULTS

In Vitro Reconstitution of the α_1/His$_{10}$-β_1/FXYD1 Complex. As previously demonstrated, incubation of the α_1/His$_{10}$-β_1 complex with a molar excess of FXYD1 allows spontaneous, stoichiometric reconstitution of the α_1/His$_{10}$-β_1/FXYD1 complex.[39]

The specific activity of the enzyme reconstituted with FXYD1 varied between 8.5 and 19.8 μmol of P_i (mg of protein)$^{-1}$ min^{-1} at 37 °C and was always higher than the activity of the complex when prepared without FXYD1. The differences varied between 2.5% and 40%, and are the consequence of a stabilizing effect of FXYD1 that protects the enzyme from partial inactivation during the purification procedure, rather than of a specific effect of FXYD1 on v_{max}. In fact, a strong stabilizing effect of the FXYD1 has been described recently[39] and, in addition, FXYD1 has been shown not to affect the turnover number of the purified recombinant enzyme.[28]

Considering the increased specific activity of the α_1/His$_{10}$-β_1 complex previously detected upon a short incubation at 37 °C, probably due to a thermally induced improvement of the lipid−protein interaction,[27] the enzyme, routinely kept on ice, was incubated for 30 min at RT.

Previously, we have shown that FXYD1 isolated from *P. pastoris* is significantly phosphorylated at Ser68 in the PKA-selective sequence RRLS.[28] *E. coli* is known to express a variety of protein Ser/Thr kinases,[47] and it was therefore necessary to determine whether FXYD1 isolated from *E. coli* is phosphorylated at Ser68. Western blots were performed with antibodies against the unphosphorylated FXYD1 (anti-FXYD1) and the Ser68 phosphorylated form of FXYD1 (CP68). The results are shown in Figure 1. The FXYD1 protein isolated from *E. coli* was strongly stained by anti-FXYD1 but not by CP68. After phosphorylation of FXYD1 by protein kinase A, the protein was recognized by CP68 but hardly by anti-FXYD1. As described recently,[29] the purified FXYD1 protein runs on SDS−PAGE gels

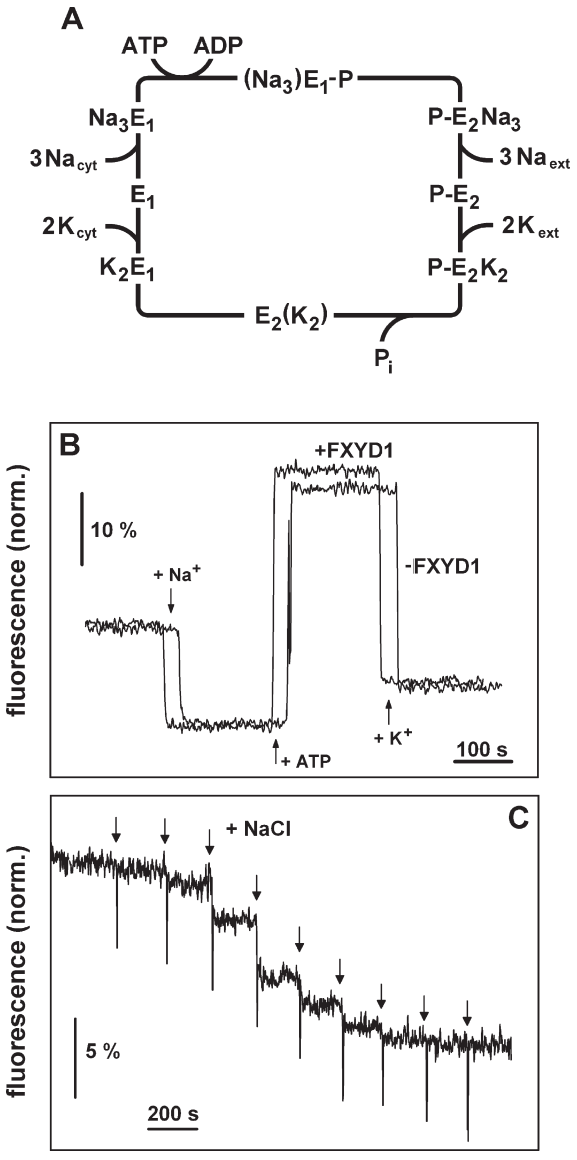

Figure 2. Post-Albers cycle of the Na,K-ATPase and standard experiments performed with the human purified recombinant α_1/His$_{10}$-β_1 and α_1/His$_{10}$-β_1/FXYD1 complexes. (A) The Post-Albers cycle shows the reaction sequence with the E_1 conformation in which the ion binding sites are accessible from the cytoplasm and 2 K^+ are exchanged against 3 Na^+ ions. In the absence of Na^+ and K^+, two binding sites are occupied by protons (not shown). In the P-E_2 conformation, the binding sites are open to the extracellular side and allow ion exchange of 3 Na^+ against 2 K^+. In between both conformations the so-called occluded states, $(Na_3)E_1$-P and $E_2(K_2)$, confine the bound ions and prevent a short circuit between both aqueous phase during the conformation transitions. (B) In the initial state, the Na,K-ATPase is accumulated in the state H_vE_1 ($v \sim 1.6$). Addition of 50 mM NaCl (+ Na^+) induces the transition to state Na_3E_1, reflected by a fluorescence decrease of ~14%. Addition of 1 mM ATP (+ ATP) starts the reaction sequence $Na_3E_1 \rightarrow \ldots \rightarrow$ P-E_2 and the release of the 3 Na^+ induces the fluorescence increase of ~35%. When subsequently 20 mM KCl ($+K^+$) are added all substrates are present, the pumps are transferred to the turnover mode, and the proteins are preferentially in both occluded states which precede the corresponding rate-limiting reaction steps of each half cycle. (C) When instead of 50 mM NaCl the substrate is added in small aliquots, titration of the binding sites can be monitored by the successive fluorescence decrease.

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with an apparent mass of 11−12 kDa after treatment with TEV protease. This corresponds to the monomer. While the monomer is the principal species, the purified FXYD1 protein has a tendency to oligomerize to dimers and higher oligomers. These are seen easily in Western blots such as in Figure 1 due to the sensitivity of the blots, but they represent only a small fraction of the total FXYD1 (as shown in ref 29). Subsequent dephosphorylation by alkaline phosphatase restored the condition of the initial control. This result clearly proves that the isolated FXYD1 used in the following experiments was not significantly phosphorylated at Ser68.

RH421 Standard Experiments. Standard experiments are used to characterize functional properties of the isolated ion pumps. In these experiments, the fluorescence changes of the RH421 allow detection of different states and degrees of occupancy of the ion binding sites, upon addition of the various substrates, and reveal the electrogenicity of the pump cycle.[33] In buffer with no Na^+ and K^+ present (as described above), the Na,K-ATPase is mainly in the state H_2E_1,[37] and the stable fluorescence level is used as reference, F_0, for normalization. After addition of 50 mM NaCl the pump undergoes the transition to the state Na_3E_1 (Figure 2A). Electrogenic binding of the third Na^+ causes an observable fluorescence decrease. Subsequent addition of saturating ATP induces the transition to the P-E_2 conformation and the release of all three Na^+, producing a fluorescence increase about three times larger than the previous fluorescence change. When finally 20 mM KCl are added, the protein works under turnover conditions, and the resulting fluorescence level is produced mainly by the occluded states $(Na_3)E_1$-P and $E_2(K_2)$, since in both states the subsequent conformational transitions are rate limiting steps in each half cycle.

As we have reported recently,[38] the recombinant α_1/His_{10}-β_1 complex follows the same scheme as the native protein in membrane fragments although the fluorescence changes are smaller, probably due to a reduced response of the RH421 in the modified detergent−lipid−protein environment.

Standard experiments performed in the presence and absence of FXYD1 are compared in Figure 2B. Two specific properties can be deduced from the substrate-addition induced amplitude changes. At first, the ATP-induced fluorescence changes are larger in the presence of FXYD1. The difference of ~8% is similar to the difference in specific activity between the two enzyme preparations. In the numerous enzyme preparations purified in the framework of this study, the difference in enzyme activity of the complexes with and without FXYD1 reflected the difference in the ATP-induced fluorescence change and the two measures of active pumps are linearly correlated (not shown). It is known that the (normalized) substrate-induced fluorescence changes are proportional to the concentration of active ion pumps.[32] Therefore, there have to be more active ion pumps in the presence of FXYD1. The second observation is that for both recombinant enzymes the ATP-induced fluorescence increase was smaller than 3-fold the fluorescence change after Na^+ addition. Since in the presence of 50 mM NaCl the pumps are saturated with 3 Na^+ ions in the E_1 conformation and after addition of ATP virtually all (active) pumps have performed the transition into the P-E_2 state and released all Na^+ ions, the fluorescence increase represents the release of three elementary charges from the binding sites. When this fluorescence change is compared to the initial Na^+-induced jump, it can be estimated that, before Na^+ addition, on average only 1.7 protons were bound in the presence of FXYD1 and 1.6 in its absence.

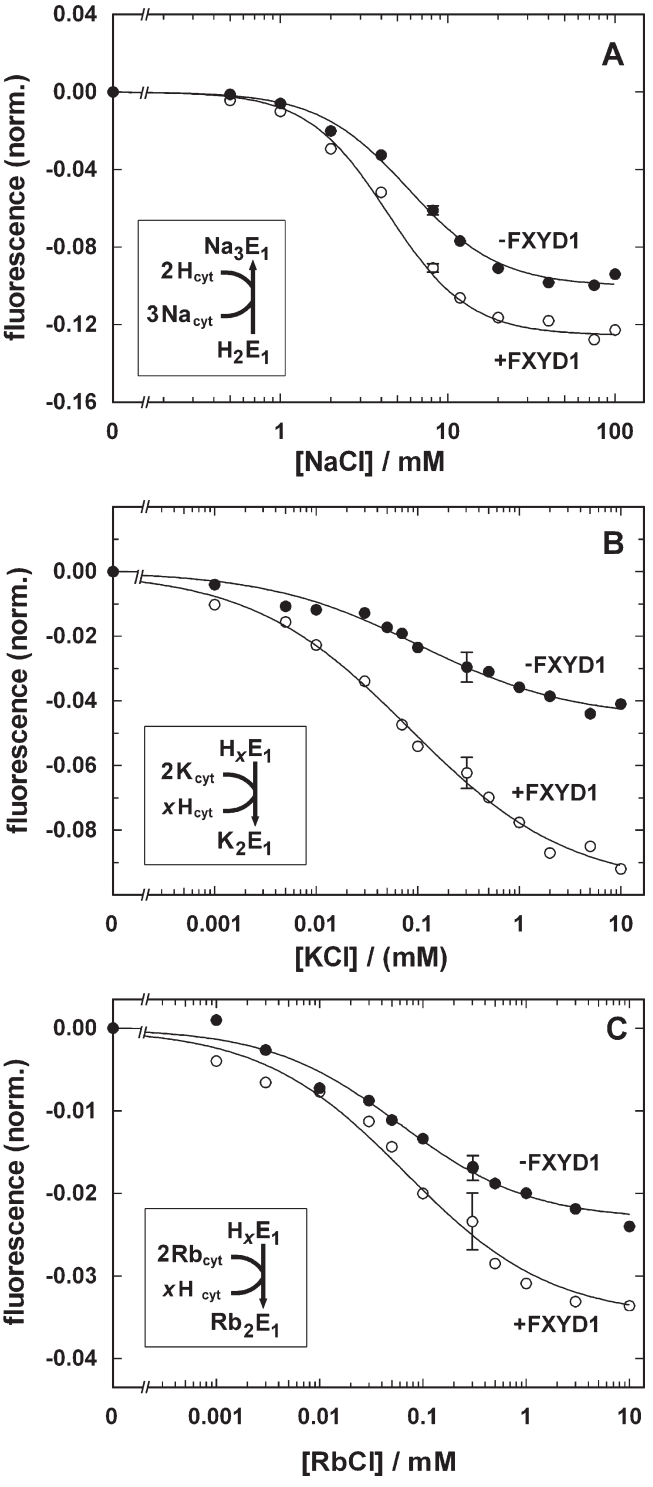

Figure 3. Titration of the ion-binding sites in the E_1 conformation. Evaluation of experiments corresponding to that in Figure 2C provide the concentration dependence of the fluorescence signal that reflects binding of (A) Na^+, (B) K^+, and (C) Rb^+ to the binding sites of α_1/His_{10}-β_1 (solid circles) and α_1/His_{10}-β_1/FXYD1 (open circles) complexes. Binding of the cations was determined from titration experiments as shown in Figure 2C. Data points are the average of three experiments and were fitted by the Hill function (solid lines) to determine the half-saturation ion concentrations. For the sake of clarity, typical error bars were shown only at one concentration.

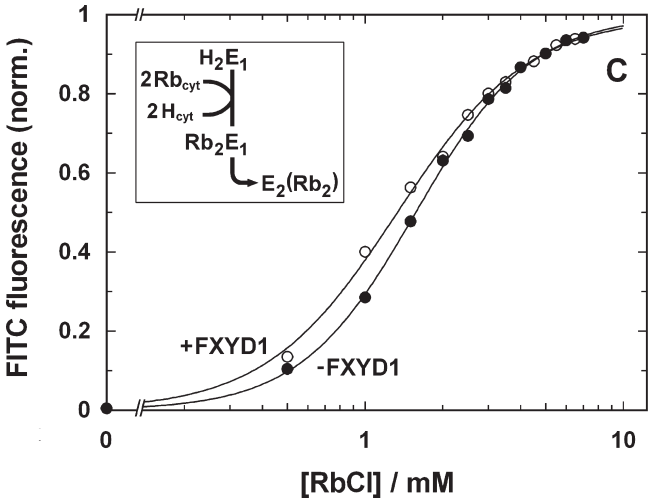

Figure 4. Rb^+-binding induced conformation transition detected by FITC-labeled enzyme. This fluorescent label does not report the Rb^+ binding step but the subsequent conformation transition, $Rb_2E_1 \rightarrow E_2(Rb_2)$.[31] The E_2 conformation exhibits a higher fluorescence level. Data were fitted by the Hill function (solid lines) to determine the apparent half-saturation Rb^+ concentrations for both α_1/His_{10}-β_1 (solid circles) and α_1/His_{10}-β_1/FXYD1 (open circles) complexes.

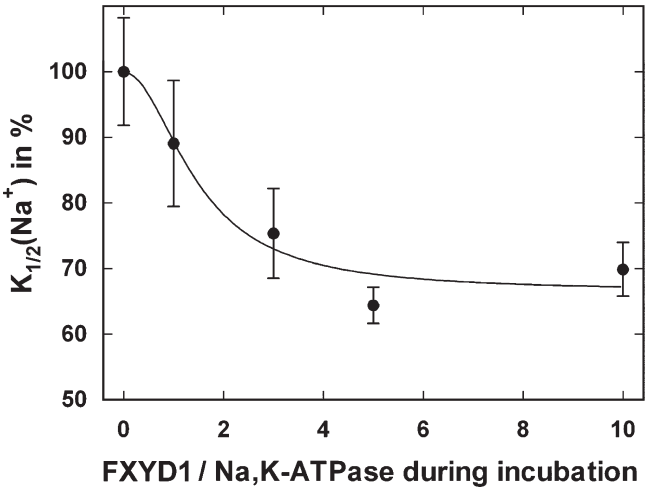

Figure 5. Effect of the FXYD1 molar excess during reconstitution on the Na^+ binding affinity. A 5-fold or higher excess of FXYD1 over the Na,K-ATPase concentration led to a saturation of α_1/His_{10}-β_1/FXYD1 complex formation. The half-saturating Na^+ concentration, $K_{1/2}$, was reduced by ~30% due to the interaction of FXYD1 with α_1/His_{10}-β_1.

Both the α_1/His_{10}-β_1 and the α_1/His_{10}-β_1/FXYD1 complexes exhibit a strong fluorescence decrease upon addition of 20 mM KCl, corresponding to 2.5−2.6 positive charges bound in the average to the ion sites in this preparation. Since under turnover conditions the fluorescence level is primarily a mixture of both occluded states, $(Na_3)E_1$-P and $E_2(K_2)$, the results indicate a comparable occupation of both states, not significantly different from what was found in the α_1 isoform of the Na,K-ATPase from rabbit kidney.[38] Obviously, the presence of FXYD1 does not notably affect the rate-limiting steps of the pump cycle.

Ion Binding Affinities in the E_1 Conformation. The initial fluorescence drop induced by addition of 50 mM NaCl can be titrated in small steps by addition of aliquots of NaCl, and thus the concentration-dependent fluorescence decrease, as shown in Figure 2C, allows the determination of the Na^+-binding kinetics presented in Figure 3A.

When starting with the initial $H_\nu E_1$ ($\nu \sim 1.6$) state, it is possible to determine the binding affinities for the Na^+ and K^+ in the E_1 conformation (Figure 3A,B). Small aliquots of NaCl or KCl were added up to 100 mM and 10 mM, respectively. Because of the small fluorescence changes, at least three identical experiments were performed and averaged to improve the signal-to-noise ratio. The concentration dependence was fitted with the Hill function (eq 1).

In the Na^+-binding experiments, a 30% higher affinity was detected in the presence of FXYD1 (Figure 3A). The half-saturating Na^+ concentration, $K_{1/2}$, was 5.8 ± 0.4 mM for α_1/His_{10}-β_1 and 4.6 ± 0.2 mM for α_1/His_{10}-β_1/FXYD1. The Hill coefficient, n, was comparable for both preparations, 1.6 ± 0.1, respectively. The larger fluorescence amplitude indicates a higher fraction of active pump molecules in the presence of FXYD1, which was, averaged over the three sets of experiments, 25% larger, and reflects again the enhanced number of active pumps in the presence of FXYD1. When the enzyme preparation was repeated with a doubled amount of SOPS (0.1 mg/mL SOPS), despite the increased number of negative surface charges no significant difference in the ratio of the Na^+-binding affinities of the enzymes with and without FXYD1 has been observed.

The K^+ binding kinetics in the E_1 conformation can be studied since at pH 7.2 about 1.6 H^+ are present in the binding sites, and therefore, the exchange with 2 K^+ is accompanied by a small electrogenic component.[37] The K^+ titration data could be fitted also by the Hill function (Figure 3B) and resulted again in a slightly higher binding affinity in the presence of FXYD1. $K_{1/2}$ was 0.10 ± 0.02 mM for α_1/His_{10}-β_1 and 0.08 ± 0.01 mM for α_1/His_{10}-β_1/FXYD1. Because of the low fluorescence change, $\Delta F_{max} < 5\%$, in the case of the α_1/His_{10}-β_1 the error of the fitted result is large, and therefore, the difference has to be accounted as not significant. The difference in ΔF_{max} on the order of a factor of 2 indicates, however, that there is a significant difference in the fraction of ion pumps to which K^+ is able to bind in the E_1 conformation. The Hill coefficients, n, were comparable 0.57 ± 0.06 (-FXYD1) and 0.55 ± 0.04 (+FXYD1), respectively. In a corresponding way binding kinetics of Rb^+ has been studied. Rb^+ is known to be a congener of K^+ with a slightly higher binding affinity than K^+.[36] The concentration dependence of the titration curves (Figure 3C) has been fitted by the Hill function, and for both preparations, α_1/His_{10}-β_1 and α_1/His_{10}-β_1/FXYD1, $K_{1/2}$ was 0.07 ± 0.02 mM with a Hill coefficient of 0.65 ± 0.06.

Binding of Rb^+ ions was studied also with FITC-labeled pumps at pH 7.5 (Figure 4) and 7.0 (not shown). In contrast to the experiments with RH421, FITC does not monitor the electrogenic binding of the ions in the binding sites, $E_1 + 2Rb \rightarrow Rb_2E_1$, but the subsequent conformation transition, $Rb_2E_1 \rightarrow E_2(Rb_2)$. At both pHs the half-saturating Rb^+ concentration was slightly higher in the absence of FXYD1. At pH 7.0 the apparent $K_{1/2}$ was 1.03 ± 0.03 mM for α_1/His_{10}-β_1 and 0.86 ± 0.01 mM for α_1/His_{10}-β_1/FXYD1. At pH 7.5 the respective values were 1.56 ± 0.03 mM and 1.33 ± 0.05 mM. The presence of FXYD1 caused a reduction of $K_{1/2}$ of about 15%, independent of pH. The Hill coefficient of the fits was 2 and indicates cooperative binding. When the apparent $K_{1/2}$ values from the FITC experiments are compared to the $K_{1/2}$ values from the RH421 experiments the

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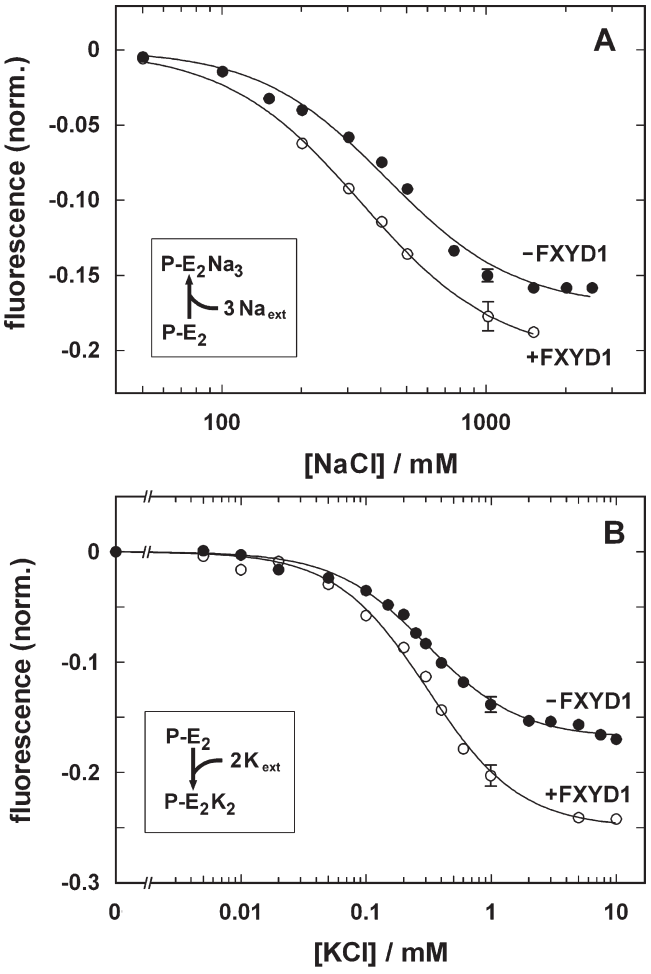

Figure 6. Titration of the ion-binding sites in the P-E_2 conformation. Binding of (A) Na^+ and (B) K^+, to the ion sites of α_1/His_{10}-β_1 (solid circles) and α_1/His_{10}-β_1/FXYD1 (open circles) complexes were performed and fitted by the Hill function (solid lines) to determine the half-saturation ion concentrations. Data points are the average of three experiments. For the sake clarity, typical error bars were shown only at one concentration.

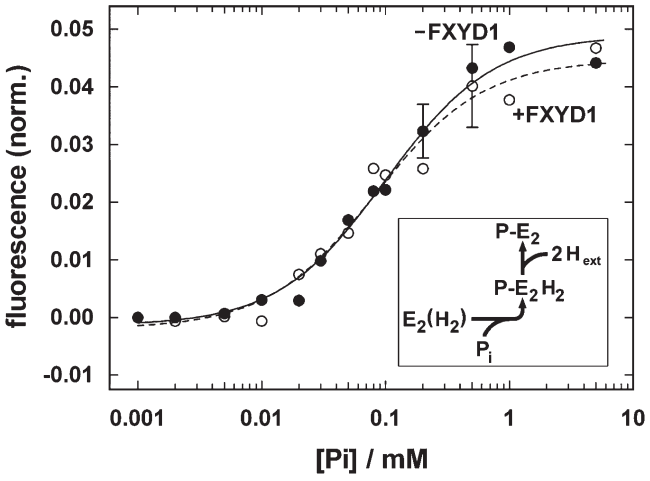

Figure 7. Effect of FXYD1 on backdoor phosphorylation in the absence of Na^+ and K^+ ions. Experiments were performed with α_1/His_{10}-β_1 (solid circles) and α_1/His_{10}-β_1/FXYD1 (open circles) complexes. The concentration dependence of the RH421 fluorescence signals could be fitted by a single binding isotherm (solid and dashed lines). No significant differences in the binding kinetics could be found. Data points are the average of three experiments. For the sake clarity, typical error bars were shown only at one concentration.

discrepancy in the order of a factor of 10 has to be assigned to the second reaction step, the conformation transition, $Rb_2E_1 \rightarrow E_2(Rb_2)$, which is detected by FITC. The FITC experiments were performed in the presence of 150 mM choline chloride, and it is known that the increased ionic strength stabilizes the E_1 conformation. Therefore, this effect has to be taken into account to explain the large discrepancy in the detected $K_{1/2}$ values between the RH421 and FITC experiments.

The effect of the excess of FXYD1 during the incubation with the α_1/His_{10}-β_1 complex prebound to BD-Talon beads in the reconstitution procedure was investigated to ensure that the experiments in the presence of FXYD1 were performed under saturating FXYD1 binding. The Na^+ binding affinity was studied as a crucial parameter indicating the complex formation obtained during incubation with molar excesses of FXYD1 up to 10-fold, which was used routinely. A maximum effect was found already at a 5-fold molar excess of FXYD1. The results are shown in Figure 5. When the FXYD1-dependent half-maximum Na^+ concentration was fitted by the Hill function (eq 1) it was found that about 50% of the Na,K-ATPase molecules were reconstituted together with a FXYD1 when a molar excess of 1.5 ± 0.3 was present during incubation.

Ion Binding Affinities in the P-E_2 Conformation. When Na,K-ATPase is equilibrated in standard buffer with 5 mM $MgCl_2$, 50 mM NaCl, and 1 mM ATP (pH 7.2), the P-E_2 state is maintained with virtually empty binding sites since the back reaction to the E_1 conformation in the absence of K^+ is extremely slow in comparison to the forward reaction. Thus, extracellular Na^+ and K^+ binding can be studied by titration experiments (Figure 6). Aliquots of NaCl up to 2.5 M or KCl up to 10 mM were added. Because NaCl concentrations of 2 M and above produced unspecific artifacts on the RH421 fluorescence in the case of the α_1/His_{10}-β_1/FXYD1 complex, the data points above 1.5 M were ignored. The half-saturating Na^+ concentration could be determined although saturation was not completely obtained. Again, a 30% higher apparent binding affinity for Na^+ was detected in the presence of FXYD1 (Figure 6A). By fitting of the data with the Hill function (eq 1) $K_{1/2}$ values of 428 ± 33 mM (−FXYD1) and 333 ± 12 mM (+FXYD1) were found with n = 1.7 ± 0.1 and 1.6 ± 0.2, respectively.

When K^+ titration experiments were performed, binding of two K^+ ions enabled dephosphorylation and as a consequence pump turnover occurred. At low concentrations (<1 mM) K^+ binding was still rate limiting. At higher K^+ concentrations, the pump turnover was controlled by both conformation transition steps of the cycle, and the occupation of the respective preceding enzyme states, $(Na_3)E_1$-P and $E_2(K_2)$, defined the observed fluorescence levels. The results are shown in Figure 6B and reveal, in contrast to the Na^+ titration, identical $K_{1/2}$ values for the K^+ affinity, $K_{1/2}$ = 0.31 ± 0.02 mM (n = 1.17 ± 0.07) for α_1/His_{10}-β_1, and $K_{1/2}$ = 0.31 ± 0.02 mM (n = 1.18 ± 0.08) for α_1/His_{10}-β_1/FXYD1. The bigger fluorescence decrease in the presence of FXYD1 reflects, again, a higher fraction of active Na,K-ATPase molecules.

Backdoor Phosphorylation. Addition of inorganic phosphate (Pi) in the absence of Na^+ induces the so-called backdoor phosphorylation reaction, in which the lower half cycle of the

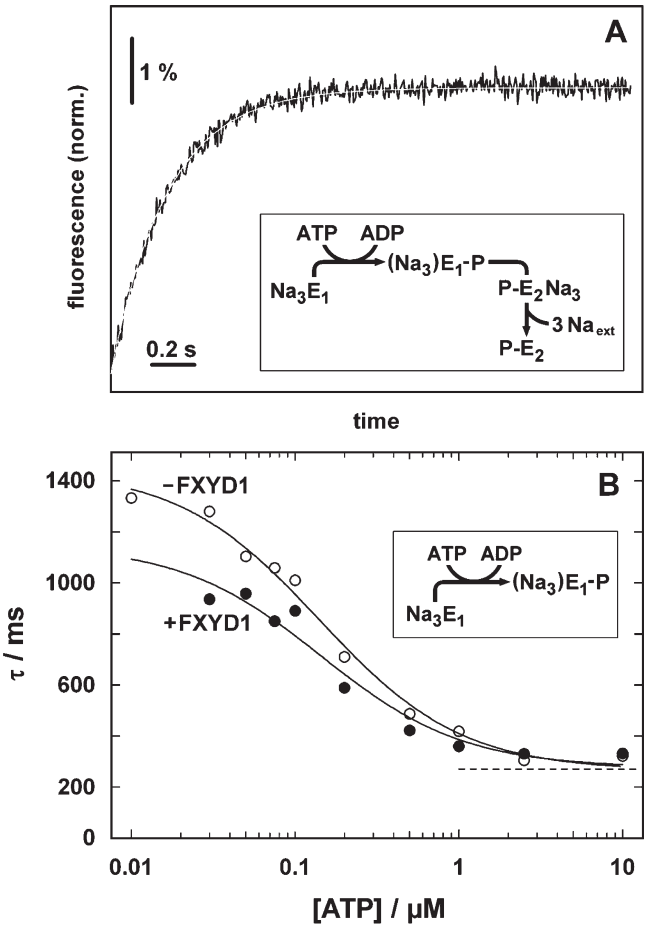

Figure 8. Analysis of time-resolved RH421 fluorescence signals of ATP-induced Na^+ transport by the Na,K-ATPase. (A) Time course of the fluorescence relaxation upon an ATP-concentration jump from 0 to 2.5 μM performed with α_1/His_{10}-β_1/FXYD1 complexes. The experimental data could be fitted with the function, $F(t) = \Delta F_{max}(1 - \exp(-t/\tau))$, as shown by the gray line. At 2.5 μM the reaction sequence was mostly controlled by the conformation transition, $(Na_3)E_1$-P → P-E_2Na_3. (B) Dependence of the time constant, τ, on the ATP concentration released. At low concentration the reaction sequence was controlled by enzyme phosphorylation. Experiments were performed with α_1/His_{10}-β_1 (solid circles) and α_1/His_{10}-β_1/FXYD1 (open circles) complexes. The concentration dependence of τ could be fitted by a single binding isotherm (solid lines). The dashed line indicates value of τ at the saturating ATP concentrations which was the same in the presence and absence of FXYD1.

Post-Albers scheme is reversed, E_1 → P-E_2. The addition of Tris phosphate triggers in the absence of K^+ a H^+-transferring partial reaction, $(H_\nu E_1 \rightarrow)$ $H_2E_1 \rightarrow E_2(H_2) \rightarrow$ P-$E_2H_2 \rightarrow$ P-E_2. Because of the low H^+-binding affinity in the P-E_2 conformation, at pH 7.2 the two bound protons are electrogenically released on the extracellular side, and a fluorescence increase is detected (Figure 7). Because of the rather small fluorescence changes (<5%), the signal-to-noise ratio is quite high, even after averaging several identical experiments. However, similar Pi half-saturating concentrations have been detected when the concentration dependence was fitted by a single binding isotherm: $K_M = 100 \pm 13$ μM for α_1/His_{10}-β_1 and $K_M = 87 \pm 13$ μM for α_1/His_{10}-β_1/FXYD1.

Time-Resolved RH421 Fluorescence Signals after ATP-Concentration Jump. The kinetics of ATP binding and the conformation transition E_1-P → P-E_2 was studied in time-resolved experiments. Enzyme phosphorylation was triggered by an ATP-concentration jump when ATP was released from its inactive precursor, caged ATP. At a saturating NaCl concentration (50 mM), the enzyme is trapped completely in the Na_3E_1 state. The release of ATP triggers the reaction sequence $Na_3E_1 \rightarrow Na_3E_1 \cdot ATP \rightarrow (Na_3)E_1$-P → P-$E_2Na_3$ → P-E_2. The last reaction step is detected by RH421 as fluorescence increase due to the electrogenic Na^+ release to the extracellular side (Figure 8A). The shown fluorescence trace represents an experiment in which 2.5 μM ATP were released. The time course can be fitted by a single exponential function with a time constant, τ, of 270 ms.

At high ATP concentrations, ATP binding and enzyme phosphorylation are fast compared to the conformational transition E_1-P → P-E_2. As previously established by time-resolved experiments with the 5-IAF dye in the presence of the cardiotonic steroid strophanthidin, the rate constant of the fluorescence increase reflects the rate of the conformational transition $(Na_3)E_1$-P → P-$E_2(Na_3)$, which is followed by the fast release of the first Na^+ ion.[32] The remaining two Na^+ ions are released subsequently with even higher rate constants.[48] At saturating ATP concentrations, a time constant of 276 ± 48 ms was detected in the absence and 264 ± 30 ms in the presence of FXYD1 (Figure 8B). Since the transition $(Na_3)E_1$-P → P-E_2-(Na_3) is supposed to be the rate-determining process of the pump cycle (see above), these rate constants represent a characteristic value of the turnover rate of the pump cycle. They are the same in the presence and absence of FXYD1.

At low ATP concentrations, ATP binding is rate limiting, and therefore, controls the rate of the fluorescence change. Since only part of the enzyme in the cuvette is phosphorylated and undergoes the conformational transition, the amplitude of the signal is lower than at saturating ATP concentrations. By fitting the ATP dependence of the time constant and of the amplitude of the fluorescence increase, the apparent ATP affinity can be evaluated. However, because of large scattering of fluorescence amplitudes, it was impossible to extract sufficiently accurate values from the fitted data, in contrast to the analysis of the time constants. In Figure 8B, the time constant, τ, is plotted against the concentration of the released ATP concentration. From a Michaelis−Menten fit the half-saturating ATP, $K_{1/2}$, concentrations were obtained to be 0.14 ± 0.06 μM for the α_1/His_{10}-β_1 and 0.14 ± 0.02 μM for the α_1/His_{10}-β_1/FXYD1 complexes. In conclusion, ATP binding and phosphorylation are also not affected by FXYD1. At saturating ATP concentrations, the time constants for the α_1/His_{10}-β_1 and α_1/His_{10}-β_1/FXYD1 complexes were both 270 ms. They are about 50% larger than the respective time constant obtained for the rabbit kidney enzyme, $\alpha_1\beta_1\gamma$, in membrane preparations under comparable conditions.[49]

Time-Resolved FITC-Fluorescence Signals in Stopped-Flow Experiments. The conformation transition of the dephosphorylated Na,K-ATPase as shown in the lower half of the pump cycle (Figure 2A) can be monitored in stopped-flow experiments by Na^+-concentration jump experiments when Rb^+ ions as congener of K^+ are present initially, $E_2(Rb_2) \rightarrow Rb_2E_1 \rightarrow Na_3E_1$, and in the opposite direction when the Na,K-ATPase is kept in the absence of Na^+ and K^+ and mixed with RbCl, $H_\nu E_1 \rightarrow Rb_2E_1 \rightarrow E_2(Rb_2)$.

The rate of the $E_2(Rb_2) \rightarrow Na_3E_1$ (Figure 9A) reaction sequence is identical with and without FXYD1 (see Table 2). The reverse transition was studied at three concentrations of

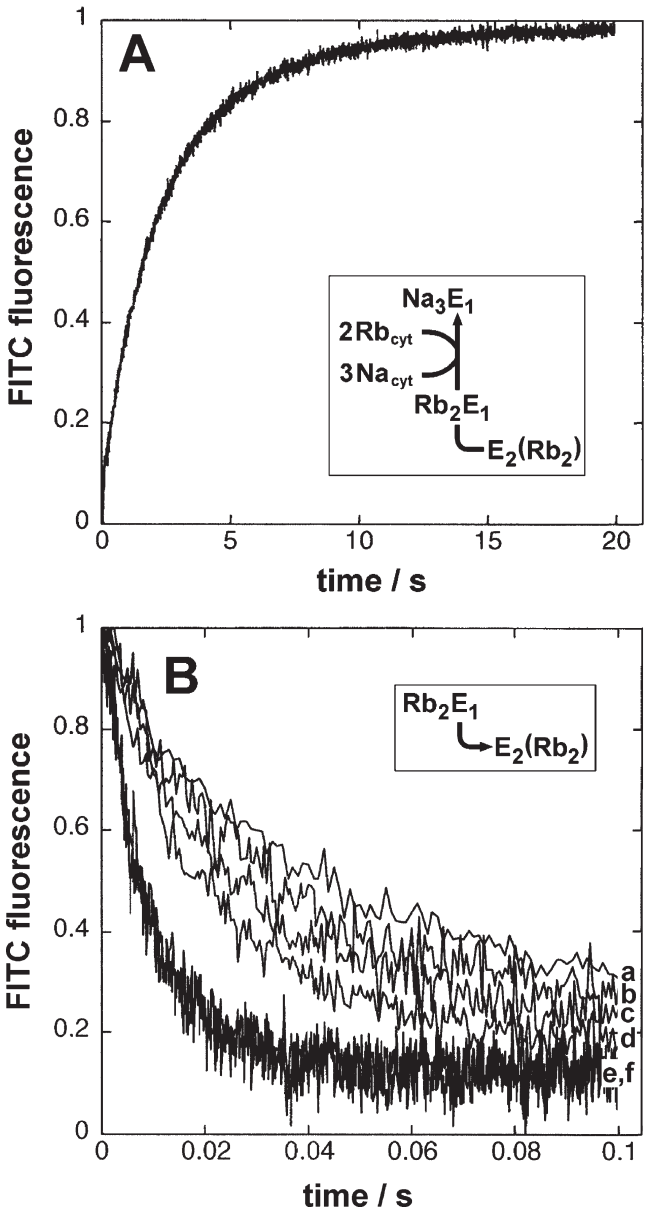

Figure 9. Stopped-flow experiments performed with FITC-labeled α_1/His_{10}-β_1 and α_1/His_{10}-β_1/FXYD1 complexes to study Na^+ and K^+ induced conformation transitions. (A) The conformation transition $E_2 \rightarrow E_1$ was induced by mixing the protein complexes in the $E_2(Rb_2)$ state with 40 mM NaCl. The fluorescence traces for the α_1/His_{10}-β_1 and α_1/His_{10}-β_1/FXYD1 complexes superimpose indistinguishably. The time-course was fitted by the function $F(t)/F_\infty = 1 - \exp(-k_1 t)$. k_1 is the rate constant of the limiting reaction step represented by the exponential. (B) The transition $E_1 \rightarrow E_2$ was studied by mixing the protein complexes without and with FXYD1 with RbCl solutions of various concentrations (a, b: 10 mM; c, d: 20 mM; e, f: 83 mM). Traces with α_1/His_{10}-β_1 are a, c, e and with α_1/His_{10}-β_1/FXYD1 are b, d, f. Time-courses were fitted by a double exponential function: $F(t) = F_\infty + \Delta F_1 \exp(-k_1 t) + \Delta F_2 \exp(-k_2 t)$. k_1, k_2 are the rate constants of first and second exponentials. ΔF_1 and ΔF_2 are the respective amplitudes and F_∞ is fluorescence level of the stationary state.

Rb^+ ions (Figure 9B). The time course of these experiments is best fitted by a double exponential function. The first and faster process amounts to about 80% of the signal, it increases with Rb^+ concentration and provides a more reliable measurement than the second process which tends to be somewhat variable. Table 2 includes only the major rate constant.

At a high Rb^+ concentration (83 mM), the rate constant is unaffected by the presence of FXYD1. At lower, nonsaturating Rb^+ concentrations the presence of FXYD1 slightly increased the rate constant. For the reverse reaction, the presence of FXYD1 had no effect on the rate constant.

DISCUSSION

So far, the effects of FXYD1 on the Na,K-ATPase have been mainly investigated in intact cells, in *X. oocytes*,[16,28] and native cardiac tissue.[25] These systems allow a characterization of the physiological effects but have limited possibilities for studying the mechanistic and structural interactions between both proteins. By contrast, the detergent-solubilized purified recombinant preparations provide a system that should be highly suited for carrying out detailed functional analyses of FXYD1 effects under well-defined conditions. Unlike in native cells, the effects of FXYD1 on the different isoforms of the Na,K-ATPase may be investigated separately, and modifying substrate conditions will affect functions only of the Na,K-ATPase and Na,K-ATPase-FXYD1 complexes. When solubilized ion pumps are prepared in the absence and in the presence of added purified FXYD1, it is possible to compare the modulation of Na,K-ATPase properties directly, with and without FXYD1. It has been pointed out that in vitro reconstitution allows experimental control of the optimal ratio of FXYD1:$\alpha\beta$ subunits, and shown that the $\alpha\beta$/FXYD1 complexes purified after coexpression in *P. pastoris* or obtained by in vitro reconstitution have the same properties.[28] The stoichiometry of FXYD1 bound to $\alpha\beta$ is not determined directly in these experiments, but it can be assumed to be $\alpha\beta$:FXYD1 1:1, as inferred previously for $\alpha\beta$/FXYD complexes in coimmunoprecipitation experiments[50] and shown directly in the crystal structures of both the renal and shark rectal gland Na,K-ATPase.[51,52] Since in the purified preparations the phosphorylation state of FXYD1 is easily controllable, the functional role of the protein kinases mediated phosphorylation of FXYD1 should be detectable without interference by other cellular components. Moreover, in principle, the characterization of the structural interactions could be facilitated by experiments with truncated forms of FXYD1, point mutants, and synthetic peptides representing either the transmembrane or cytoplasmic segment.

Both fluorescence methods, utilizing the electrochromic styryl dye RH421 and the conformation-sensitive FITC label, have been applied in steady-state and time-resolved kinetic experiments to obtain a detailed characterization of the FXYD1 effects on different partial reactions of the pump cycle. A general constraint of the RH421 method applied to solubilized recombinant enzymes is a reduced fluorescence response upon substrate addition in comparison to measurements with native protein in membrane fragments. In view of this limitation, obtaining reliable kinetic parameters requires averaging several identical experiments to improve the signal-to-noise ratio and provide sufficiently accurate data for a quantitative, or at least qualitative, comparison between the enzymes in the presence and in the absence of FXYD1.

The reconstitution method provides complexes of Na,K-ATPase, lipids, detergent molecules, and (when indicated) FXYD1, which display enzymatic activity and substrate-dependent partial reactions according to the Post-Albers cycle in agreement with results

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Table 1. Equilibrium Dissociation Constants for the Substrates of the α_1/His$_{10}$-β_1 Isoform of Na,K-ATPase in the Absence and Presence of FXYD1[a]

		$K_{1/2}$ ($\alpha_1\beta_1$) (human)	$K_{1/2}$ ($\alpha_1\beta_1$FXYD1) (human)	$K_{1/2}$ ($\alpha_1\beta_1\gamma$) (ref) (rabbit kidney)	notes
$Na^+_{cytoplasmic}$	mM	5.8 ± 0.4	4.6 ± 0. 2	4.2 ± 0.1[43]	RH421
$K^+_{cytoplasmic}$	mM	0.10 ± 0.02	0.08 ± 0.01	0.10 ± 0.003[58]	RH421
$Rb^+_{cytoplasmic}$	mM	0.07 ± 0.02	0.07 ± 0.02	0.08 ± 0.03[36]	RH421
$Rb^+_{cytoplasmic}$	mM	1.56 ± 0.03	1.33 ± 0.05		FITC, pH 7.5
	mM	1.03 ± 0.03	0.86 ± 0.01		FITC, pH 7.0
$Na^+_{extracellular}$	mM	428 ± 33	333 ± 12	387 ± 21.8[59]	RH421
$K^+_{extracellular}$	mM	0.31 ± 0.02	0.31 ± 0.02	0.19 ± 0.009[60]	RH421
ATP	μM	0.14 ± 0.06	0.14 ± 0.02	0.05−0.2 [34]	RH421
Pi	μM	87 ± 13	100 ± 13	24 ± 1 [35]	RH421

[a] The experiments were performed in the presence of 5 mM $MgCl_2$, pH 7.2, and at 20 °C, if not mentioned otherwise. For comparison, respective data obtained from rabbit $\alpha_1\beta_1\gamma$ are supplemented from the literature.

obtained with $\alpha_1\beta_1$ Na,K-ATPase in native membrane preparations.[38] Therefore, it is possible to derive from the results presented above details on the FXYD1-induced modifications of the functional properties of the α_1/His$_{10}$-β_1 isoform of the sodium pump.

FXYD1 Stabilizes the Enzyme during the Purification Procedure. In the presence of FXYD1, the enzyme showed higher specific activity. The reason for the broad range of this increase (2.5−40%) encountered in the numerous preparations used is unclear and could be due to minor differences of each preparation (e.g., in slightly varying lipid/detergent composition). Nevertheless, in all cases the FXYD1 effect on the Na^+ binding affinity was found to be reproducible. In principle, this effect could result from either a specific effect of FXYD1 on the turnover rate of the pump, v_{max}, or enzyme stabilization or both. Previously, it has been shown that FXYD1 stabilizes the protein, without affecting the turnover rate,[28] and more recently FXYD1, FXYD2, and FXYD4 have all been found to stabilize against both thermal and detergent-induced inactivation by a mechanism involving amplification of specific protein−phosphatidylserine interactions.[29]

The time-resolved experiments monitoring the rate-limiting reaction steps in both half cycles, which are $(Na_3)E_1$-P → P-E_2Na_3 and $E_2(K_2)$ → K_2E_1, reveal that the rate constants are the same under saturating substrate concentration in the presence and absence of FXYD1 (Figures 8B and 9A). This finding demonstrates clearly that v_{max} is not affected by the presence of FXYD1. The larger fluorescence changes upon substrate additions which were detected in the presence of FXYD1 throughout all RH421 experiments, and the linear correlation between enzyme activity and fluorescence change upon addition of ATP in the standard experiments support the stabilizing effect of FXYD1, and can be explained by the following arguments. Because of the purification of the complexes by metal chelate chromatography, the preparation is free of lipid/detergent micelles without protein. After addition and equilibration of RH421 with the protein complexes, the charge-monitoring dye is evenly distributed and all complexes contribute to the total fluorescence signal, independently of their activity. When, for example, ATP is added only active enzyme molecules will contribute to the observed fluorescence increase. Thus, a linear relation exists between fraction of active enzymes and fluorescence change.

The purified recombinant $\alpha_1\beta_1$ complex has a lower Na,K-ATPase activity (≈10 μmol/min/mg) compared to the purified renal Na,K-ATPase (30−40 μmol/min/mg, $\alpha_1\beta_1$FXYD2). A part of this difference is due to inactivation during the preparation in the absence of the FXYD protein, but the activity of the $\alpha_1\beta_1$FXYD1 complex (≈15 μmol/min/mg) is also much lower than that of the membrane bound Na,K-ATPase. Since the FXYD1 has a strong stabilizing effect, but no effect on the turnover rate or conformational transition E_1 to E_2 at saturating substrate concentrations (see also refs 28, 29, and 39), it is likely that the difference between renal and recombinant enzyme is due mainly to the different environment in intact membranes versus lipid/detergent complexes, respectively.

Effect of FXYD1 on the Substrate-Dependent Reaction Steps. The experimentally determined binding affinities of the substrates of the Na,K-ATPase are compiled in Table 1 and compared with respective values of Na,K-ATPase from rabbit kidney ($\alpha_1\beta_1$FXYD2). The results indicate that ATP and Pi binding in both conformations of α_1/His-β_1 are not significantly affected by FXYD1 binding. Significant differences were observed for Na^+ binding in both conformations, and possibly for Rb^+ as congener of K^+ in the E_1 conformation. These results will be discussed in the following. When the equilibrium dissociation constants of the reconstituted α_1/His$_{10}$-β_1 with or without FXYD1 are compared to the respective results obtained from rabbit kidney enzyme, it is obvious that there is a broad agreement between the data of the α_1/His$_{10}$-β_1/FXYD1 complexes and the rabbit enzyme which is known to be formed as heterotrimer by $\alpha_1\beta_1\gamma$.[53] γ (or FXYD2) is a regulatory subunit closely related to FXYD1, although γ is reported to decrease Na^+ binding affinity.[5,54]

When the Rb^+-induced conformation transition E_1 to E_2 was studied (Figure 4), the presence of FXYD1 caused a minor reduction of the apparent $K_{1/2}$ which has to be assigned to slightly facilitated conformation transition Rb_2E_1 → $E_2(Rb_2)$. This interpretation is in agreement with the finding that at low Rb^+ concentrations the rate constant of this reaction step is increased in the presence of the FXYD1 subunit (Table 2).

FXYD1 Increases the Binding Affinities of Na^+ Ions to the Binding Sites in E_1 and P-E_2. When the equilibrium dissociation constants for the ions in the E_1 conformation are scrutinized in Table 1, it can be seen that the α_1/His$_{10}$-β_1/FXYD1 complexes have a higher Na^+-binding affinity. There is virtually no effect of FXYD1 on K^+ and Rb^+ binding. All monovalent cations (here, K^+, Na^+, and Rb^+) are able to bind to the two central cation binding sites within transmembrane segments M4, M5, M6, and M8. However, only Na^+ is able to occupy the third exclusively Na^+-selective site after the two other sites are occupied by Na^+.

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Table 2. Rate Constants, k, of Rb_2E_1 to $E_2(Rb_2)$ and $E_2(Rb_2)$ to Na_3E_1 Transitions for Recombinant $\alpha_1\beta_1$ Na,K-ATPase without or with FXYD1, as Detected in the Experiment with FITC-Labeled Enzyme[a]

		k ($\alpha_1\beta_1$) (human)	k ($\alpha_1\beta_1$FXYD1) (human)	k ($\alpha_1\beta_1$) (pig)	k ($\alpha_1\beta_1\gamma$) (pig kidney)
			$Rb_2E_1 \rightarrow E_2(Rb_2)$		
10 mM RbCl	k/s^{-1}	21.1 ± 0.48	29.8 ± 0.93	20.6 ± 3.96	19.4 ± 0.65
20 mM RbCl	k/s^{-1}	43.3 ± 1.04	47.9 ± 0.96	122.8 ± 30.3	30.1 ± 1.52
83 mM RbCl	k/s^{-1}	117.7 ± 2.53	119.3 ± 2.7	162.7 ± 10.9	168.4 ± 45.6
			$E_2(Rb_2) \rightarrow Na_3E_1$		
80 mM NaCl	k/s^{-1}	0.38 ± 0.001	0.38 ± 0.001	0.48 ± 0.002	0.10 ± 0.001

[a] For comparison respective results are shown obtained from pig kidney Na,K-ATPase.

Since the binding affinity for K^+ and Rb^+ are independent of the presence of FXYD1, and the increase of the Na^+ binding affinity is, however, greater than 30% when FXYD1 is present, it stands to reason to assign this difference to an additional effect of FXYD1 on the third Na^+ site.

In the P-E_2 conformation the K^+ binding affinity is insensitive to the presence of FXYD1 (Table 1). Only for Na^+ a similarly enhanced affinity (~30%) is observed that may reflect again an effect of FXYD1 on the specific third binding site. Because the C-terminal sequence of the α subunit is proposed to be involved in the binding of the third Na^+ ion,[51] our result fits with the proximity of the FXYD proteins to helix M9 of the α subunit.[51,55]

Since the presented experiments cover all reaction steps around the Post-Albers cycle (Figure 2A), it can be stated that FXYD1 affects significantly only the third Na^+-specific site, independent of the conformation, with an affinity increase of 30−40%. By contrast to the effect on cation binding, no effects on enzyme phosphorylation and dephosphorylation, as well as on both conformation transitions E_1-E_2 and E_2-E_1, have been detected in the purified α_1/His_{10}-β_1/FXYD1 complexes.

Comparison with Effect of FXYD1 in Whole Cells. The effect of FXYD1 on Na^+ occlusion reported here is consistent with a higher apparent Na^+ affinity that was observed in the Na,K-ATPase activity assay for the purified α_1/His_{10}-β_1/FXYD1 complex obtained from the yeast system, compared to the control.[28] However, this effect of FXYD1 on the Na^+-binding affinity is different from published data obtained from intact cells, where the predominant effect of FXYD1 was a decrease in the apparent Na^+-binding affinity in cardiac myocytes,[20−24] HeLa cells,[28] or *X. oocytes*,[16] or no effect at all.[25] Phosphorylation of the FXYD1, at PKA or PKC sites Ser 63 and Ser 68, has been shown to alleviate the lower Na^+ affinity in cardiac myocytes. In a previous paper, it was found that FXYD1 expressed in *P. pastoris* is partially phosphorylated at Ser68, and it was proposed that the discrepancy between results in intact cell systems and the purified recombinant protein might be attributable to that fact.[28] However, the FXYD1 purified from *E. coli* is not phosphorylated at Ser68 (Figure 1). Therefore, a different explanation is required. One possibility that can be excluded is that the FXYD1/$\alpha\beta$ interaction in vitro is in some way nonspecific. On the contrary, it is clear that FXYD1 binds specifically to the $\alpha\beta$ complex, as shown by the saturating curve at low molar excess of FXYD1/$\alpha\beta$ (Figure 5), similar to the stabilizing effect of FXYD1 and other FXYD proteins described recently[29] and, in particular, the selectivity of the functional effect for Na ions compared to all other ligands of the pump.

A working hypothesis to reconcile the contradictory experimental findings in whole cells and the purified protein could be based on an assumption of multiple (at least two) functional and structural interactions of FXYD1 with the pump. On the one hand, the interaction of the transmembrane segment of FXYD1 with the membrane domain of the α subunit is assumed to raise the Na^+-binding affinity as suggested by the current findings, as well as the stabilizing effects of FXYD1 described recently.[29] On the other hand, interaction of the C-terminal sequence with the cytoplasmic domain of the pump in the cellular environment may be assumed to affect the enzymatic activities of the Na,K-ATPase, such as enzyme phosphorylation or E_1-E_2 conformation transitions, and thus modulate v_{max}. A decrease of v_{max} leads to a redistribution between the various states of the Post-Albers cycle with the result that the state preceding the rate-limiting step is highly populated. A secondary consequence of such an effect would be to reduce the apparent Na^+ binding affinity since the E_1 states are less populated. In order to explain the discrepancy between cellular and isolated Na,K-ATPase/FXYD1 complexes, one would have to assume that the interaction of the C-terminal segment with the cytoplasmic domain is absent in the purified enzyme, leaving intact only the interaction between the transmembrane domains of FXYD1 and α.

Excluding FXYD1 phosphorylation as the reason for an impaired interaction between the cytoplasmic domains, the results in the detergent-solubilized complexes might be explained in the following way. Recently, the structure of the free FXYD1 monomer in SDS micelles has been determined by NMR spectroscopy.[56] It is organized in four α-helices, H1 at the extracellular side, H2 as transmembrane domain, and H3 and H4 on the cytoplasmic side. As a monomer, FXYD1 adopts a L-shaped conformation and H4 is associated to the membrane.

Isothermal titration calorimetry experiments and solid-state NMR measurements show that a 35-amino acid peptide representing the FXYD1 cytoplasmic domain ($FXYD1_{38-72}$) strongly associates with highly anionic phospholipid headgroups of the membrane. This interaction is, however, significantly weakened when the fraction of negatively charged phospholipids is reduced to represent a more physiological environment.[57]

The presence of the membrane and its lipid composition, in particular the surface charge density, might be important for an appropriate interaction of FXYD1 with the pump. In the reconstituted complexes the proteins are surrounded by an annulus of lipid and detergent molecules that forms an hydrophobic environment and allows functional integrity of the Na,K-ATPase. However, it does not form a planar membrane surface as present in a cell, and therefore, may potentially affect the cytoplasmic interaction of FXYD1 and pump. A determination of the complete phosphorylation state of the FXYD1 and the introduction of a technique that allows an investigation of the

transport properties of the purified detergent-solubilized recombinant enzymes reconstituted in lipid vesicles could help to clarify the discrepancies. If such projected experiments do not provide a satisfactory explanation of the observed differences, other explanations will have to be considered. For example, it is conceivable that in the whole cell environment, interactions of the C-terminal helix depend on additional interactions with cellular proteins or other ligands, which are absent in the purified α_1/His$_{10}$-β_1/FXYD1 complexes. Moreover, it cannot be excluded that in the cell the association of the pump with FXYD1 is assisted by other proteins, and therefore, the complexes obtained by in vitro reconstitution or coexpressed in non-native systems do not represent the correct association. The physiological relevance of the results still has to be confirmed.

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ABBREVIATIONS USED

RH421, *N*-(4-sulfobutyl)-4-(4-(*p*-(dipentylamino)phenyl)butadienyl)-pyridinium inner salt; FITC, fluorescein 5′ isothiocyanate; NADH, nicotinamide adenine dinucleotide; DDM, *n*-dodecyl beta-D-maltoside; EDTA, diaminoethane-tetraacetic acid; $C_{12}E_8$, octaethylene glycerol mondodecyl ether; SOPS, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine; PMSF, phenylmethylsulfonyl fluoride; MOPS, 3-morpholinopropane-1-sulfonic acid; caged ATP, P3-(l-(2-nitrophenyl)ethyl)-adenosine5′-triphosphate

REFERENCES

(1) Forbush, B., III, Kaplan, J. H., and Hoffman, J. F. (1978) Characterization of a new photoaffinity derivative of ouabain: Labeling of the large polypeptide and of a proteolipid component of the Na,K-ATPase. *Biochemistry 17*, 3667–3676.

(2) Sweadner, K. J., and Rael, E. (2000) The FXYD gene family of small ion transport regulators or channels: cDNA sequence, protein signature sequence, and expression. *Genomics 68*, 41–56.

(3) Cornelius, F., and Mahmmoud, Y. A. (2003) Functional modulation of the sodium pump: the regulatory proteins "Fixit". *News Physiol Sci. 18*, 119–124.

(4) Garty, H., and Karlish, S. J. (2005) FXYD proteins: tissue-specific regulators of the Na,K-ATPase. *Semin. Nephrol. 25*, 304–311.

(5) Geering, K. (2005) Function of FXYD proteins, regulators of Na, K-ATPase. *J. Bioenerg. Biomembr. 37*, 387–392.

(6) Geering, K. (2006) FXYD proteins: new regulators of Na-K-ATPase. *Am. J. Physiol. Renal Physiol. 290*, F241–F250.

(7) Presti, C. F., Scott, B. T., and Jones, L. R. (1985) Identification of an endogenous protein kinase C activity and its intrinsic 15-kilodalton substrate in purified canine cardiac sarcolemmal vesicles. *J. Biol. Chem. 260*, 13879–13889.

(8) Pretorius, P. J., Pohl, W. G., Smithen, C. S., and Inesi, G. (1969) Structural and functional characterization of dog heart microsomes. *Circ. Res. 25*, 487–499.

(9) Palmer, C. J., Scott, B. T., and Jones, L. R. (1991) Purification and complete sequence determination of the major plasma membrane substrate for cAMP-dependent protein kinase and protein kinase C in myocardium. *J. Biol. Chem. 266*, 11126–11130.

(10) Moorman, J. R., Palmer, C. J., John, J. E., III, Durieux, M. E., and Jones, L. R. (1992) Phospholemman expression induces a hyperpolarization-activated chloride current in *Xenopus oocytes*. *J. Biol. Chem. 267*, 14551–14554.

(11) Moorman, J. R., Ackerman, S. J., Kowdley, G. C., Griffin, M. P., Mounsey, J. P., Chen, Z., Cala, S. E., O'Brian, J. J., Szabo, G., and Jones, L. R. (1995) Unitary anion currents through phospholemman channel molecules. *Nature 377*, 737–740.

(12) Zhang, X. Q., Qureshi, A., Song, J., Carl, L. L., Tian, Q., Stahl, R. C., Carey, D. J., Rothblum, L. I., and Cheung, J. Y. (2003) Phospholemman modulates Na^+/Ca^{2+} exchange in adult rat cardiac myocytes. *Am. J. Physiol. Heart Circ. Physiol. 284*, H225–H233.

(13) Mirza, M. A., Zhang, X. Q., Ahlers, B. A., Qureshi, A., Carl, L. L., Song, J., Tucker, A. L., Mounsey, J. P., Moorman, J. R., Rothblum, L. I., Zhang, T. S., and Cheung, J. Y. (2004) Effects of phospholemman downregulation on contractility and $[Ca^{2+}]$i transients in adult rat cardiac myocytes. *Am. J. Physiol. Heart Circ. Physiol. 286*, H1322–H1330.

(14) Guo, K., Wang, X., Gao, G., Huang, C., Elmslie, K. S., and Peterson, B. Z. (2010) Amino acid substitutions in the FXYD motif enhance phospholemman-induced modulation of cardiac L-type calcium channels. *Am. J. Physiol. Cell Physiol. 299*, C1203–C1211.

(15) Wang, X., Gao, G., Guo, K., Yarotskyy, V., Huang, C., Elmslie, K. S., and Peterson, B. Z. (2010) Phospholemman modulates the gating of cardiac L-type calcium channels. *Biophys. J. 98*, 1149–1159.

(16) Crambert, G., Fuzesi, M., Garty, H., Karlish, S., and Geering, K. (2002) Phospholemman (FXYD1) associates with Na,K-ATPase and regulates its transport properties. *Proc. Natl. Acad. Sci. U. S. A. 99*, 11476–11481.

(17) Wetzel, R. K., and Sweadner, K. J. (2003) Phospholemman expression in extraglomerular mesangium and afferent arteriole of the juxtaglomerular apparatus. *Am. J. Physiol. Renal Physiol. 285*, F121–F129.

(18) Feschenko, M. S., Donnet, C., Wetzel, R. K., Asinovski, N. K., Jones, L. R., and Sweadner, K. J. (2003) Phospholemman, a single-span membrane protein, is an accessory protein of Na,K-ATPase in cerebellum and choroid plexus. *J. Neurosci. 23*, 2161–2169.

(19) Floyd, R. V., Wray, S., Martin-Vasallo, P., and Mobasheri, A. (2010) Differential cellular expression of FXYD1 (phospholemman) and FXYD2 (gamma subunit of Na, K-ATPase) in normal human tissues: a study using high density human tissue microarrays. *Ann. Anat. 192*, 7–16.

(20) Despa, S., Bossuyt, J., Han, F., Ginsburg, K. S., Jia, L. G., Kutchai, H., Tucker, A. L., and Bers, D. M. (2005) Phospholemman-phosphorylation mediates the beta-adrenergic effects on Na/K pump function in cardiac myocytes. *Circ. Res. 97*, 252–259.

(21) Despa, S., Tucker, A. L., and Bers, D. M. (2008) Phospholemman-mediated activation of Na/K-ATPase limits $[Na]_i$ and inotropic state during β-adrenergic stimulation in mouse ventricular myocytes. *Circulation 117*, 1849–1855.

(22) Han, F., Bossuyt, J., Despa, S., Tucker, A. L., and Bers, D. M. (2006) Phospholemman phosphorylation mediates the protein kinase C-dependent effects on Na^+/K^+ pump function in cardiac myocytes. *Circ. Res. 99*, 1376–1383.

(23) Silverman, B. Z., Fuller, W., Eaton, P., Deng, J., Moorman, J. R., Cheung, J. Y., James, A. F., and Shattock, M. J. (2005) Serine 68 phosphorylation of phospholemman: acute isoform-specific activation of cardiac Na/K ATPase. *Cardiovasc. Res. 65*, 93–103.

(24) Bers, D. M., and Despa, S. (2009) Na/K-ATPase--an integral player in the adrenergic fight-or-flight response. *Trends Cardiovasc. Med. 19*, 111–118.

(25) Jia, L. G., Donnet, C., Bogaev, R. C., Blatt, R. J., McKinney, C. E., Day, K. H., Berr, S. S., Jones, L. R., Moorman, J. R., Sweadner, K. J., and Tucker, A. L. (2005) Hypertrophy, increased ejection fraction, and reduced

Na-K-ATPase activity in phospholemman-deficient mice. *Am. J. Physiol. Heart Circ. Physiol 288*, H1982–H1988.

(26) Cohen, E., Goldshleger, R., Shainskaya, A., Tal, D. M., Ebel, C., leMaire, M., and Karlish, S. J. (2005) Purification of Na^+,K^+-ATPase expressed in *Pichia pastoris* reveals an essential role of phospholipid-protein interactions. *J. Biol. Chem. 280*, 16610–16618.

(27) Haviv, H., Cohen, E., Lifshitz, Y., Tal, D. M., Goldshleger, R., and Karlish, S. J. (2007) Stabilization of Na^+,K^+-ATPase purified from *Pichia pastoris* membranes by specific interactions with lipids. *Biochemistry 46*, 12855–12867.

(28) Lifshitz, Y., Lindzen, M., Garty, H., and Karlish, S. J. (2006) Functional interactions of phospholemman (PLM) (FXYD1) with Na^+, K^+-ATPase. Purification of $\alpha1/\beta1$/PLM complexes expressed in Pichia pastoris. *J. Biol. Chem. 281*, 15790–15799.

(29) Mishra, N. K., Peleg, Y., Cirri, E., Belogus, T., Lifshitz, Y., Voelker, D. R., Apell, H. J., Garty, H., and Karlish, S. J. (2011) FXYD proteins stabilize Na,K-ATPase: Amplification of specific phosphatidylserine-protein interactions. *J. Biol. Chem. 286*, 9699–9712.

(30) Karlish, S. J. D. (1980) Characterization of conformational changes in (Na,K) ATPase labeled with fluorescein at the active site. *J. Bioenerg. Biomembr. 12*, 111–136.

(31) Rephaeli, A., Richards, D., and Karlish, S. J. (1986) Conformational transitions in fluorescein-labeled (Na,K)ATPase reconstituted into phospholipid vesicles. *J. Biol. Chem. 261*, 6248–6254.

(32) Bühler, R., Stürmer, W., Apell, H.-J., and Läuger, P. (1991) Charge translocation by the Na,K-pump: I. Kinetics of local field changes studied by time-resolved fluorescence measurements. *J. Membr. Biol. 121*, 141–161.

(33) Stürmer, W., Bühler, R., Apell, H.-J., and Läuger, P. (1991) Charge translocation by the Na,K-pump: II. Ion binding and release at the extracellular face. *J. Membr. Biol. 121*, 163–176.

(34) Heyse, S., Wuddel, I., Apell, H.-J., and Stürmer, W. (1994) Partial reactions of the Na,K-ATPase: determination of rate constants. *J. Gen. Physiol. 104*, 197–240.

(35) Apell, H.-J., Roudna, M., Corrie, J. E., and Trentham, D. R. (1996) Kinetics of the phosphorylation of Na,K-ATPase by inorganic phosphate detected by a fluorescence method. *Biochemistry 35*, 10922–10930.

(36) Schneeberger, A., and Apell, H.-J. (2001) Ion selectivity of the cytoplasmic binding sites of the Na,K-ATPase: II. Competition of various cations. *J. Membr. Biol. 179*, 263–273.

(37) Apell, H.-J., and Diller, A. (2002) Do H^+ ions obscure electrogenic Na^+ and K^+ binding in the E_1 state of the Na,K-ATPase? *FEBS Lett. 532*, 198–202.

(38) Habeck, M., Cirri, E., Katz, A., Karlish, S. J., and Apell, H. J. (2009) Investigation of electrogenic partial reactions in detergent-solubilized Na,K-ATPase. *Biochemistry 48*, 9147–9155.

(39) Lifshitz, Y., Petrovich, E., Haviv, H., Goldshleger, R., Tal, D. M., Garty, H., and Karlish, S. J. (2007) Purification of the human $\alpha2$ isoform of Na,K-ATPase expressed in *Pichia pastoris*. Stabilization by lipids and FXYD1. *Biochemistry 46*, 14937–14950.

(40) Schwartz, A. K., Nagano, M., Nakao, M., Lindenmayer, G. E., and Allen, J. C. (1971) The sodium- and potassium-activated adenosine triphosphatase system. *Meth. Pharmacol. 1*, 361–388.

(41) Rembold, C. M., Ripley, M. L., Meeks, M. K., Geddis, L. M., Kutchai, H. C., Marassi, F. M., Cheung, J. Y., and Moorman, J. R. (2005) Serine 68 phospholemman phosphorylation during forskolin-induced swine carotid artery relaxation. *J. Vasc. Res. 42*, 483–491.

(42) Belogus, T., Haviv, H., and Karlish, S. J. (2009) Neutralization of the charge on Asp 369 of Na^+,K^+-ATPase triggers $E_1 \leftrightarrow E_2$ conformational changes. *J. Biol. Chem. 284*, 31038–31051.

(43) Schneeberger, A., and Apell, H.-J. (1999) Ion selectivity of the cytoplasmic binding sites of the Na,K-ATPase: I. Sodium binding is associated with a conformational rearrangement. *J. Membr. Biol. 168*, 221–228.

(44) Stürmer, W., Apell, H.-J., Wuddel, I., and Läuger, P. (1989) Conformational transitions and change translocation by the Na,K pump: comparison of optical and electrical transients elicited by ATP-concentration jumps. *J. Membr. Biol. 110*, 67–86.

(45) McCray, J. A., Herbette, L., Kihara, T., and Trentham, D. R. (1980) A new approach to time-resolved studies of ATP-requiring biological systems; laser flash photolysis of caged ATP. *Proc. Natl. Acad. Sci. U. S. A 77*, 7237–7241.

(46) Deluca, M., and McElroy, M. D. (1978) Purification and properties of firefly luciferase. *Methods Enzymol. 57*, 3–15.

(47) Alber, T. (2009) Signaling mechanisms of the *Mycobacterium tuberculosis* receptor Ser/Thr protein kinases. *Curr. Opin. Struct. Biol. 19*, 650–657.

(48) Holmgren, M., Wagg, J., Bezanilla, F., Rakowski, R. F., de Weer, P., and Gadsby, D. C. (2000) Three distinct and sequential steps in the release of sodium ions by the Na^+/K^+-ATPase. *Nature 403*, 898–901.

(49) Apell, H. J., Benz, G., and Sauerbrunn, D. (2011) Proton diet for the sodium pump. *Biochemistry 50*, 409–418.

(50) Lindzen, M., Gottschalk, K. E., Fuzesi, M., Garty, H., and Karlish, S. J. (2006) Structural interactions between FXYD proteins and Na^+,K^+-ATPase: α/β/FXYD subunit stoichiometry and cross-linking. *J. Biol. Chem. 281*, 5947–5955.

(51) Morth, J. P., Pedersen, B. P., Toustrup-Jensen, M. S., Sorensen, T. L., Petersen, J., Andersen, J. P., Vilsen, B., and Nissen, P. (2007) Crystal structure of the sodium-potassium pump. *Nature 450*, 1043–1049.

(52) Shinoda, T., Ogawa, H., Cornelius, F., and Toyoshima, C. (2009) Crystal structure of the sodium-potassium pump at 2.4 Å resolution. *Nature 459*, 446–450.

(53) Therien, A. G., Goldshleger, R., Karlish, S. J., and Blostein, R. (1997) Tissue-specific distribution and modulatory role of the gamma subunit of the Na,K-ATPase. *J. Biol. Chem. 272*, 32628–32634.

(54) Beguin, P., Crambert, G., Guennoun, S., Garty, H., Horisberger, J. D., and Geering, K. (2001) CHIF, a member of the FXYD protein family, is a regulator of Na,K-ATPase distinct from the gamma-subunit. *EMBO J. 20*, 3993–4002.

(55) Li, C., Grosdidier, A., Crambert, G., Horisberger, J. D., Michielin, O., and Geering, K. (2004) Structural and functional interaction sites between Na,K-ATPase and FXYD proteins. *J. Biol. Chem. 279*, 38895–38902.

(56) Teriete, P., Franzin, C. M., Choi, J., and Marassi, F. M. (2007) Structure of the Na,K-ATPase regulatory protein FXYD1 in micelles. *Biochemistry 46*, 6774–6783.

(57) Hughes, E., Whittaker, C. A., Barsukov, I. L., Esmann, M., and Middleton, D. A. (2010) A study of the membrane association and regulatory effect of the phospholemman cytoplasmic domain. *Biochim. Biophys. Acta 145*, 165–173.

(58) Schulz, S., and Apell, H.-J. (1995) Investigation of ion binding to the cytoplasmic binding sites of the Na,K-pump. *Eur. Biophys. J. 23*, 413–421.

(59) Wuddel, I., and Apell, H.-J. (1995) Electrogenicity of the sodium transport pathway in the Na,K-ATPase probed by charge-pulse experiments. *Biophys. J. 69*, 909–921.

(60) Bühler, R., and Apell, H.-J. (1995) Sequential potassium binding at the extracellular side of the Na,K-pump. *J. Membr. Biol. 145*, 165–173.