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KdpFABC Reconstituted in *Escherichia coli* Lipid Vesicles: Substrate Dependence of the Transport Rate

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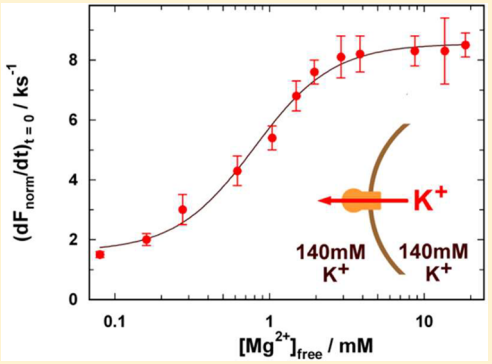
ABSTRACT: KdpFABC complexes were reconstituted in *Escherichia coli* lipid vesicles, and ion pumping was activated by addition of ATP to the external medium which corresponds to the cytoplasm under physiological conditions. ATP-driven potassium extrusion was studied in the presence of various substrates potentially influencing transport rate. The pump current was detected as a decrease of the membrane potential by the voltage-sensitive dye $DiSC_3(5)$. The results indicate that high cytoplasmic K^+ concentrations have an inhibitory effect on the KdpFABC complex. The pump current decreased to ~25% of the maximal value at 140 mM K^+ and minimal Mg^{2+}concentrations. This effect could be counteracted with increased Mg^{2+} concentrations on the cytoplasmic side. This observation may be explained by the Gouy−Chapman effect of two Mg^{2+} ions probably bound with a $K_{1/2}$ of 0.8 mM close to the entrance of the access channel to the binding sites. This factor ensures that under physiological conditions the rate-limiting effect of K^+ release is significantly reduced. Also both ADP and inorganic phosphate are able to reduce the turnover rate of the pump by reversing the phosphorylation step (K_i of 151 μM) and the dephosphorylation step (K_i of 268 μM), respectively. In the case of the DDM-solubilized KdpFABC complex, activation energy under turnover conditions was previously found to be 55 kJ/mol, and the *o*-vanadate inhibition constant is shown here to be ~1 μM, which is in agreement with values reported for other P-type ATPases. In the case of the reconstituted enzyme, however, significant differences were observed that have to be assigned to effects of the lipid bilayer environment. The activation energy was increased by a factor of 2, whereas the inhibition by *o*-vanadate became reduced in a way that only ~66% of the enzyme could be inhibited and the inhibition constant was increased to a value of ~60 μM.



KdpFABC complex of *Escherichia coli*, a member of the P-type ATPase family, is an emergency and high affinity potassium uptake system, only expressed under K^+-limiting conditions. In contrast to other members of the P-type ATPase family that consist of a catalytic subunit performing both ion transport and ATP hydrolysis, the KdpFABC complex has a unique structure−function relationship of four different subunits. The largest subunit, KdpB, represents the ATP-hydrolysis catalytic subunit[1] and is classified as type 1a P-type ATPase.[2] The KdpA subunit, structurally similar to the KcsA-like K^+ channels, is proposed to mediate ion binding and transport.[3] KdpC acts as a molecular chaperone and is involved in the ATP binding process,[4] whereas the smallest subunit, KdpF, has the role of a functional lipid that is responsible for the structural integrity of the complex and the stability of the dimeric, functional form.[5]

According to the recently proposed Post-Albers cycle for the KdpFABC complex, binding and transport of $2K^+$ occur in the dephosphorylation half cycle in correspondence with the "classical" schemes accepted for the Na,K-ATPase and H,K-ATPase.[6] The presence of $3H^+$ bound inside the membrane domain of the KdpFABC is a prerequisite for the ATP-driven half cycle, although they are not translocated but remain bound throughout the whole pump cycle. Apart from electrogenic binding to these "functional" sites, protons can act in the transport sites as weak congener of K^+ in the absence of K^+, or as noncompetitive inhibitor affecting the enzyme activity and/or the coupling between KdpA and KdpB in an unspecific but pH-dependent manner.

It has been reported previously that enzyme activity and K^+ transport are dependent on K^+ concentration, and it has been shown that the KdpFABC is capable of transporting other monovalent cations, although to a lesser extent.[7,8] Furthermore, influence of pH on K^+ transport and possible proton transport in the absence of K^+ were investigated with reconstituted KdpFABC complex in *E. coli* lipid vesicles.[9] The rather small pump current was found to be maximal in the pH range of 7.3−7.4, whereas at lower and higher pH the pump current decreased significantly. At low pH values, back-binding of protons reduces the electrogenic pump activity, whereas at high pH the lack of protons in the functional sites inhibits the pump current.[6] Furthermore, the detected (net) proton transport in the absence of K^+ was found to be in the opposite direction to the physiologically relevant K^+ transport.[9]

Mg^{2+} is required as an essential cofactor for the phosphorylation of the P-type ATPases. In the case of the Na,K-ATPase, it was reported that it can be replaced by other

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divalent cations, e.g., Ca^{2+}, although none of these was as effective as Mg^{2+}.[10]

KdpFABC transports K^+ ions against their electrochemical potential gradient across the membrane utilizing the energy released during ATP hydrolysis. Products of the ATP hydrolysis, ADP and inorganic phosphate, P_i, are proposed to affect the KdpFABC pumping process. Besides the direct competition between ATP and ADP at their binding site, the presence of ADP in the medium may also reverse the phosphorylation step, $E_1ATP \leftrightarrow E_1\text{-}P + ADP$, thus decreasing the overall turnover rate of the KdpFABC. In the same manner, inorganic phosphate may decelerate the turnover rate by shifting the dephosphorylation equilibrium toward the phosphorylated state, $P\text{-}E_2K_2 \leftrightarrow E_2(K_2) + P_i$.

The phosphate analogue *o*-vanadate is a well-known specific inhibitor of all P-type ATPases. Vanadate binds to the E_2 state of the enzyme, mimicking the phosphorylated E_2 transition state. Among the first suggestions that KdpFABC is indeed a member of the P-type ATPases was the observed inhibition of enzymatic activity by micromolar concentrations of *o*-vanadate.[11,12]

A fluorescence technique employing a potential-sensitive dye $DiSC_3(5)$ was applied to investigate the electrogenicity of the K^+ transport by the KdpFABC reconstituted in lipid vesicles. It was already shown that ATP-induced pumping of K^+ by the inside-out oriented pumps is an electrogenic process.[9,13] The detected fluorescence decrease of the membrane-potential indicator, $DiSC_3(5)$, corresponds to the inside-negative potential as a result of extrusion from the vesicles of net positive charge. In such experiments, the pump current of the reconstituted KdpFABC is activated by extravesicular (equivalent to cytoplasm) ATP, Mg^{2+}, and intravesicular (equivalent to periplasmic) K^+.

Here using $DiSC_3(5)$, the pump activity of the reconstituted inside-out KdpFABC in *E. coli*-lipid vesicles was investigated as a function of the composition of the extravesicular (cytoplasmic) medium. In the first part, the effects of cytoplasmic K^+ and Mg^{2+} on the pump current are presented, followed by the dependences on ADP and inorganic phosphate, which are the products of ATP hydrolysis. The concentration changes of ATP, ADP, and inorganic phosphate during the experiments are considered to be negligible due to the large external medium volume. In the second part, the specific inhibition of enzyme activity, and therefore also of the transport, by *o*-vanadate was tested with both the *β*-DDM-solubilized complex and reconstituted into *E. coli* vesicles. To study the energetics of the enzyme and transport activity of KdpFABC, their temperature dependence was investigated, providing the corresponding activation energies.

MATERIALS AND METHODS

Materials. Total lipid extract from *E. coli* was purchased from Avanti Polar Lipids. The fluorescent dye $DiSC_3(5)$ was ordered from Sigma-Aldrich. Na_2ATP (≥99%) and valinomycin were obtained from Boehringer-Mannheim. *β*-DDM was purchased from Anatrace (Maumee, OH). BioBeads SM-2 were obtained from Biorad. MgATP (≥95%) and all other reagents were purchased from Merck or Sigma-Aldrich at the highest quality available.

Purification of the KdpFABC complex. The C-terminally His_{14}-tagged KdpFABC complex was expressed in *E. coli* as described previously.[9] The enzyme complexes were solubilized in buffer containing 1% (w/v) *β*-DDM for 1 h on ice. Solubilized complexes were collected by centrifugation at 200000*g* at 4 °C for 1 h, and the supernatant was applied to a 5 mL HisTrap column (GE Healthcare), pre-equilibrated with 50 mM Tris-HCl, 20 mM $MgCl_2$, 10% (v/v) glycerol, 150 mM NaCl, 10 mM imidazole, 0.5 mM PMSF (pH 7.5) and 0.2% (w/v) *β*-DDM. The column was connected to a FPLC system (Amersham, Biotech) and washed with the same buffer containing 20 mM imidazole at flow rate 0.5 mL/min. Thereafter, the enzyme was eluted with buffer containing 130 mM imidazole. Samples of the protein-containing fractions were analyzed on a 12.5% SDS-PAGE gel. Afterward, fractions containing the same amount of all subunits were collected and concentrated (Vivaspin, 6 50.000 MWCO).

Reconstitution of the KdpFABC Complex in Lipid Vesicles. An appropriate amount of *E. coli* lipids dissolved in chloroform was vacuum-dried under rotation in a glass flask, and the obtained thin lipid film was solubilized with dialysis buffer containing 25 mM imidazole, 1 mM EDTA, 5 mM $MgSO_4$, 70 mM K_2SO_4 at pH 7.2, 2% *β*-DDM, and 2% $C_{12}E_8$ to the final lipid concentration of 10 mg/mL. Sulfate was chosen because it has a significantly lower membrane permeability than chloride. The lipid/detergent mixture was sonicated under nitrogen atmosphere and kept at room temperature until a clear solution was obtained. Equal volumes of *β*-DDM-solubilized KdpFABC and *E. coli* lipid solution were mixed at a protein/lipid ratio of 1:5 (w/w). Removal of detergent was accomplished by adding 30% (w/v) Bio-Beads followed by constant shaking overnight at 4 °C. Finally, the vesicles were separated from the beads using capillary tips and stored for 3 h on ice before used in fluorescence experiments. Size distribution of the vesicles was determined by dynamic light scattering using a DLS Viscotek 802 spectrometer. The vesicle diameter was found to be 117 ± 21 nm (SD).

Determination of Protein Concentration and ATPase Activity. The concentration of detergent-solubilized protein was determined by the Lowry assay. The ATPase activity was determined at 37 °C using the malachite-green assay.[9,14] The enzyme activity of the *β*-DDM-solubilized KdpFABC complex was typically ∼0.53 *μ*mol of P_i mg^{-1} min^{-1} in the presence of saturating KCl and ATP concentrations. The ATPase activity of KdpFABC reconstituted in vesicles was determined in the absence of detergents, which means that only the activity of the inside-out oriented KdpFABC fraction complexes was detected due to the membrane impermeability to ATP. The ATPase activity was found to be ∼0.28 *μ*mol of P_i mg^{-1} min^{-1} in the presence of 70 mM K_2SO_4 and 5 mM $MgSO_4$ (pH 7.2). The observation that the enzyme activity of the inside-out oriented ATPases is about half of the activity of the solubilized enzyme indicates that the pumps are reconstituted equally in both orientation. The amount of protein needed for a single measurement was ∼1 *μ*g.

$DiSC_3(5)$ Fluorescence Experiments. The positively charged fluorescent dye $DiSC_3(5)$ has been introduced as an indicator of inside-negative membrane potentials in vesicles, and it has been successfully used to detect the electrogenic pump activity of the reconstituted KdpFABC complex.[9,13] Only the inside-out oriented ion pumps are activated by the addition of ATP to the external medium, and the outward oriented transport of K^+ generates an inside-negative electric potential. The only anion was sulfate to minimize leak current through the vesicle membrane. The intravesicular K^+ concentration was 140 mM in all experiments. To perform an experiment, 1 mL of standard buffer containing 25 mM imidazole, 1 mM EDTA, 5

dx.doi.org/10.1021/bi5008244 | *Biochemistry* 2014, 53, 5674−5682

mM $MgSO_4$ (pH 7.2), and different concentrations of K^+, Mg^{2+}, ADP, and inorganic phosphate, were thermally equilibrated in a fluorescence cuvette in a PerkinElmer LS 50B luminescence spectrometer. Fluorescence was excited at 650 nm (slit 5 nm), and the emission was detected at 675 nm (slit 5 nm). 300 nM $DiSC_3(5)$ and an aliquot of the vesicle suspension containing about 80 μg/mL lipids were added. When a steady-state fluorescence level was obtained, 2.5 mM ATP (pH ~7) were added to trigger the pump activity. The pump activity was reflected in a fluorescence decrease that exponentially approached a steady state level at which the pump current is compensated by the (reverse) leak current due to the membrane conductance. It was shown that there is a linear relation between the fluorescence level and the electric membrane potential, U.[9] Under the steady-state condition the relation holds: $I_{pump} = I_{leak} = \lambda_{leak}U$. Since the leak conductance is a lipid-dependent constant, the pump current is proportional to the steady-state fluorescence level. To allow a comparison between different experiments, the fluorescence changes were normalized with respect to the initial fluorescence level, F_0 (U = 0), before the addition of ATP,

$$F_{norm}(t) = \frac{F(t) - F_0}{F_0} \quad (1)$$

where $F_{norm}(t)$ is the normalized fluorescence amplitude, and $F(t)$ is the measured fluorescence.

The initial slope of the fluorescence change, defined as the derivative of the signal with respect to the time at t = 0, is directly proportional to the initial enzyme activity.[15] In short, the initial slope of the electric membrane potential can be defined as the derivative of the time course of the exponentially decreasing membrane potential, $U(t) = U_\infty(1 - e^{-t/\tau})$, with respect to the time, at t = 0.

$$I(t)_{t=0} \propto \left(\frac{dU}{dt}\right)_{t=0} = U_\infty \frac{1}{\tau} e^{-t/\tau}|_{t=0} = \frac{U_\infty}{\tau} \quad (2)$$

Considering that the normalized fluorescence level is proportional to the transmembrane potential build up by the pump, $F_{norm} = K'U$, their corresponding derivatives with respect to time at t = 0 are also proportional,

$$\left(\frac{dU}{dt}\right)_{t=0} = (K')^{-1}\left(\frac{dF_{norm}}{dt}\right)_{t=0} \quad (3)$$

Taking into account that the maximum amplitude of the normalized fluorescence signal is proportional to the transmembrane potential in the stationary phase, $F_{max} = K'U_\infty$, the initial slope of the normalized fluorescence signal is proportional to the ratio of the maximum amplitude, F_{max}, and the time constant, τ, of the normalized fluorescence signal. All equations taken together, result in

$$\left(\frac{dF_{norm}}{dt}\right)_{t=0} = K^* \frac{F_{max}}{\tau}|_{t=0} = K^{\#} I(t)|_{t=0} \quad (4)$$

where K^* is the proportionality factor that depends only on the properties of each specific vesicle preparation, and $K^{\#} = C_m K^*$, C_m is the membrane capacitance of the vesicle. Therefore, results from the same vesicle preparation can be directly compared, while the proportionality factor may differ between different preparations. The estimation that the initial slope of the fluorescence signal change reflects the initial pump activity is correct under the assumption that the vesicles are of uniform size, and at the initial stage of the experiment when the membrane voltage is still negligible. After longer times of pump activity, the depletion of intravesicular K^+ becomes a limiting factor, and the membrane voltage may affect the turnover rate.

RESULTS

Effect of Extravesicular/Cytoplasmic K^+ Concentration on the Pumping Activity of the KdpFABC Complex. Calibration of the fluorescence responses as a function of the membrane potential were described previously.[9] It was performed by measuring (normalized) steady-state fluorescence signals at different ratios of internal and external K^+ concentrations in the presence of valinomycin and calculating the respective Nernst potential. Control experiments with 140 mM K^+ on both sides of the membrane showed no significant fluorescence change upon addition of valinomycin, in agreement with the absence of a membrane potential. A linear relationship between the fluorescence response and the corresponding membrane potential was observed in the range between −40 and −120 mV. The transport activity of the reconstituted KdpFABC complexes was investigated with respect to different K^+ concentrations present in the external medium. Vesicles were prepared with 140 mM K^+ inside at pH 7.2. The concentration of K^+ in the standard buffer was varied in the range from 0.14 to 140 mM, and the ionic strength of the solution was kept constant by addition of $Tris^+$. Activation of the KdpFABC pump was induced by the addition of 1 mM Mg-ATP (pH ≈ 7), and the respective fluorescence decrease was a result of K^+ extrusion from the vesicles. The fluorescence decrease (Figure 1, inset) was fitted with a monoexponential function, and the initial slope was calculated as the time derivative at $t \rightarrow 0$ (eq 4). The initial (maximal) fluorescence

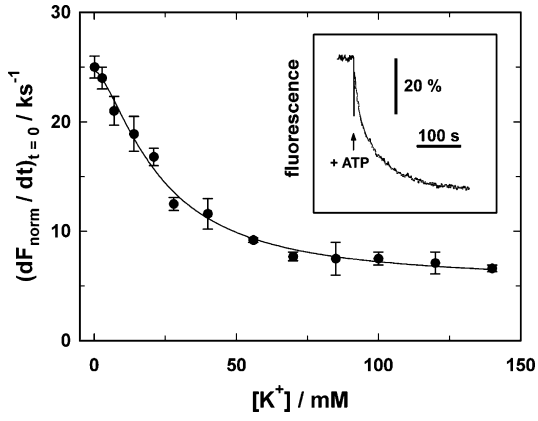

Figure 1. Initial slopes of the ATP-induced $DiSC_3(5)$ fluorescence decrease as a function of K^+ concentration present in the external medium. The initial slope reflects the KdpFABC pump activity. The intravesicular K^+ concentration was constant 140 mM at pH 7.2. The concentration dependence was fitted with a Hill function, with a $K_i(K^+_{ex})$ = 23 ± 2 mM and a Hill coefficient of n = 1.5 ± 0.2 (s.e.m.). The inset shows an example of a fluorescence trace of an ATP-induced generation of a membrane potential built up across the vesicle membrane. The vesicles were added to standard buffer containing 140 mM K^+. Upon addition of 1 mM MgATP an exponential drop of the fluorescence was observed. The time course was fitted by a single exponential, $F(t) = F_{max}(1 - \exp(-t/\tau))$, with a time constant of τ = 48.2 s and a fluorescence amplitude, F_{max} = 0.35 (gray line). According to eqs 2 and 3, this results in an initial slope of the fluorescence decrease, $|dF_{norm}/dt| = F_{max}/\tau$, of 0.0072 s^{-1} = 7.2 ks^{-1}.

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decrease per time, reflecting the maximal pump current, was found to be $-6.6 \pm 0.3\ s^{-1}$ in extravesicular 140 mM K^+ and 5 mM Mg^{2+}. The calculated initial slopes were plotted as a function of the external K^+ concentration (Figure 1). It was observed that the initial pumping activity of the reconstituted KdpFABC complex decreased with increasing K^+ concentration of the external medium, indicating an inhibitory effect of the extravesicular (cytoplasmic) potassium concentration. The concentration dependence of the initial slope was fitted with a Hill function, and an inhibition constant, $K_i(K^+)_{ex}$, was determined as the K^+ concentration at which the inhibition of the KdpFABC pumping activity is half-maximal. The obtained value was found to be 23 ± 2 mM with a Hill coefficient of $n = 1.5 \pm 0.2$.

Effect of Extravesicular Mg^{2+} Concentrations on the Pumping Activity. In the second set of experiments, the Mg^{2+} concentration in the external medium was varied. To investigate the effect of magnesium on the pumping activity of the reconstituted KdpFABC, vesicles were prepared as described above. The same buffer (25 mM imidazole, 1 mM EDTA, 140 mM K^+, pH 7.2) was used as external medium with different $MgSO_4$ concentrations between 0 and 20 mM. The pump activity was induced by addition of 1 mM Na_2-ATP (pH ≈ 7). In the absence of magnesium, no pump activity was measured as expected. The fluorescence decrease in the presence of respective Mg^{2+} concentrations was fitted with a monoexponential function, and the pump current represented by the initial slope was determined as a characteristic parameter. The dependence of the initial slope on the free magnesium concentration is shown in Figure 2. The free Mg^{2+}

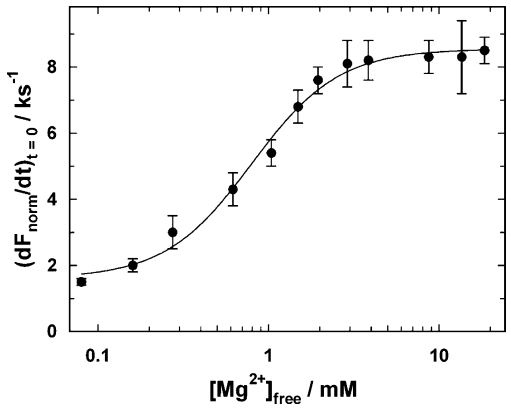

Figure 2. Calculated initial slopes, reflecting the initial KdpFABC pump activity, as a function of external Mg^{2+} concentration at pH 7.2. The K^+ concentration of 140 mM was the same inside and outside of the vesicles. The data were fitted with a Hill function, providing the $K_{1/2}(Mg^{2+})$ of 0.8 ± 0.1 mM, and a Hill coefficient, n, of 1.7 ± 0.2 (s.e.m.).

concentrations were calculated from the total Mg^{2+} concentration and the buffer composition using the program winmaxc32 (C. Patton, Stanford University, Palo Alto, CA). Data were fitted with a Hill function, and the corresponding $K_{1/2}(Mg^{2+})$ was found to be 0.8 ± 0.1 mM. The Hill coefficient, n, of 1.7 ± 0.2 indicates binding of more than one Mg^{2+} ion. The presented results reveal that the pumping activity of the reconstituted KdpFABC increased with increasing Mg^{2+} concentration up to 20 mM.

Effect of ADP and Inorganic Phosphate on the Pumping Activity. At the beginning of the experiments, the internal and external K^+ concentration was 140 mM with different aliquots of concentrated stock solutions of ADP or P_i added to the external medium. When a stable fluorescence signal was achieved, pump activity was triggered by addition of 1 mM Mg-ATP (pH ≈ 7), followed by a fluorescence signal decrease to a new steady-state state. Corresponding time-dependent fluorescence traces of ATP-driven K^+ extrusion at different ADP concentrations in the external medium are shown in Figure 3. Fluorescence decreases were fitted with a

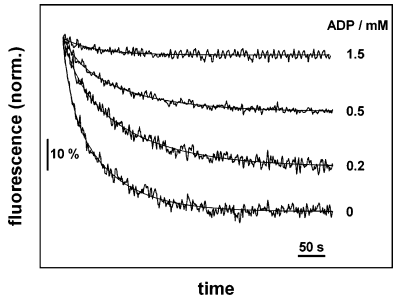

Figure 3. ATP-induced K^+ transport at different ADP concentrations in the external medium. The K^+ concentration was 140 mM (inside an outside of the vesicles) at pH 7.2. The external buffer contained 5 mM $MgSO_4$. Addition of ADP prior ATP addition did not produce a significant $DiSC_3(5)$ fluorescence change. The maximal fluorescence decrease shows a clear decrease with increasing ADP concentration. The lines through the fluorescence traces are fits with a monoexponential function.

single exponential function, and initial slopes were plotted against the corresponding ADP or P_i concentrations. Data were fitted with a Michaelis–Menten function, and calculated half-maximal inhibiting concentrations of ADP and P_i were $K_i = 151 \pm 18\ \mu M$ and $K_i = 268 \pm 75\ \mu M$, respectively (Figure 4). The amplitudes of the fluorescence responses upon ATP activation decreased with increasing both ADP and P_i concentrations (Figure 3, shown for ADP). In comparison with the results

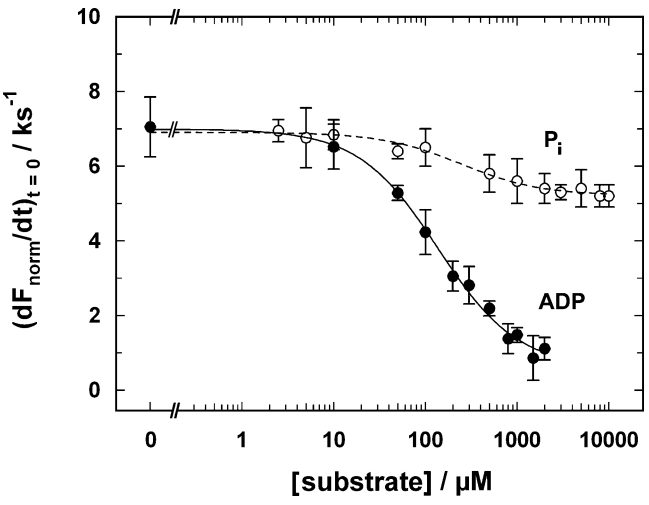

Figure 4. Initial slope, $[(dF_{norm}/dt)_{t=0}]_{max}$, of the $DiSC_3(5)$ fluorescence as a function of ADP (filled circles) and P_i (open circles). The ATP concentration was 1 mM, whereas internal and external K^+ concentration was 140 mM. Data were fitted with a Hill function, with K_i(ADP) of 151 ± 18 µM and a Hill coefficient of 0.88 ± 0.09, and $K_i(P_i)$ of 268 ± 75 µM and a Hill coefficient of 0.66 ± 0.13 (s.e.m.).

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obtained for the Na,K-ATPase, it was found that the kinetics of inhibition seems to be similar for ADP, because the half-maximal inhibitory concentrations are at the same order of magnitude. On the other hand, inhibition of the Na,K-ATPase by inorganic phosphate was found to be only weak, whereas a much stronger inhibitory effect was observed for the reconstituted KdpFABC complex.

Inhibition by *o*-Vanadate. The first set of experiments consisted of the determination of the enzyme activity by the malachite green ATPase assay of β-DDM-solubilized enzyme in the presence of different *o*-vanadate concentrations between 0 and 1 mM. The ATP concentration was kept constant at saturating 667 μM. For each *o*-vanadate concentration, three independent measurements were performed, and the average enzyme activity was calculated. The *o*-vanadate concentration dependence was fitted with a Hill function yielding a half-inhibitory concentration, K_i, of 1.03 ± 0.08 μM, and a Hill coefficient of 1.5 ± 0.1 (data not shown). At 1 mM *o*-vanadate, the enzyme activity was reduced to 0.02 μmol of P_i (mg of protein)$^{-1}$ min^{-1}, which corresponds to ∼4% of the initial value of 0.53 μmol of P_i (mg of protein)$^{-1}$ min^{-1}.

The inhibitory effect of the *o*-vanadate was investigated also with KdpFABC reconstituted in vesicles. The malachite-green assay was performed with vesicles in the absence of detergent as described above. The concentration of *o*-vanadate was varied between 0 and 5 mM, while the ATP concentration was kept constant at 667 μM. The experiments were performed in triplicate for each *o*-vanadate concentration, and the average ATPase activity was calculated (Figure 5A). In the absence of *o*-vanadate, the ATPase activity of the reconstituted KdpFABC used in this series of experiments was found to be 0.175 ± 0.002 μmol of P_i (mg of protein)$^{-1}$ min^{-1} in buffer containing 140 mM K^+ and 5 mM Mg^{2+}. The *o*-vanadate concentration dependence was fitted with the Michaelis–Menten function, with a K_i of 62 ± 7 μM. The enzyme activity was reduced to 0.065 ± 0.002 μmol of P_i (mg of protein)$^{-1}$ min^{-1} at 5 mM *o*-vanadate. This means that about 37% of the enzyme activity could not be inhibited by 5 mM *o*-vanadate in the reconstituted preparation.

Another approach to investigate the inhibitory effect of *o*-vanadate was carried out by measuring the K^+ transport out of the vesicles with $DiSC_3(5)$ in the presence of various concentration of the inhibitor. Vesicles were formed and studied in the presence of 70 mM K_2SO_4 (inside and outside). K^+ export was triggered by addition of 1 mM Mg-ATP (pH ≈ 7). After a stable fluorescence signal was obtained, corresponding to the maximal negative membrane potential, aliquots of *o*-vanadate stock-solution were added, resulting in a fluorescence signal increase to a new steady-state. Because of the partial inhibition of the pump activity, a lower steady-state membrane potential was maintained that met the condition that pump and leak current compensated each other again. The amplitudes of the steady-state fluorescence levels were plotted against the respective *o*-vanadate concentrations (Figure 5B). The data could be fitted by a Hill function with a half-maximal inhibition constant of K_i = 58 ± 4 μM and a Hill coefficient n = 0.47 ± 0.11. At 3 mM *o*-vanadate, the remaining steady-state fluorescence was reduced to ∼32% of the level in absence of the inhibitor. This number agrees with the fraction of uninhibited KdpFABC complexes obtained from the activity measurements.

Temperature Dependence of the KdpFABC Pumping Activity. To gain insight into the energetics of the KdpFABC

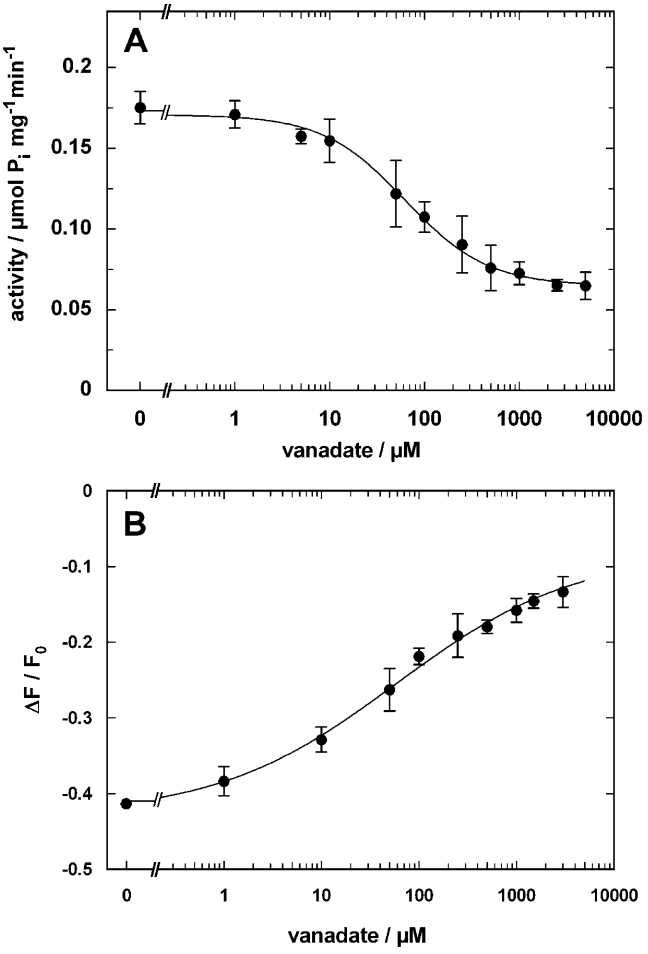

Figure 5. Inhibition of the ATPase activity of reconstituted KdpFABC into vesicles by *o*-vanadate. (A) The malachite-green assay performed at different *o*-vanadate concentrations. The data were fitted with a Hill function, and the respective half-maximal inhibitory concentration K_i was 62 ± 7 μM. (B) $DiSC_3(5)$ fluorescence increase upon addition *o*-vanadate in small aliquots. The data were fitted with a Hill function and a K_i of 58 ± 4 μM was determined.

complex, the temperature dependence of its enzymatic and transport functions was determined. The malachite-green assay was performed with the β-DDM solubilized KdpFABC complex at various temperatures to determine the activation energy under turnover conditions and nonlimiting substrate conditions. Aliquots were incubated for 30 min at temperatures between 6 and 40 °C in buffer containing 50 mM Tris-HCl, 3.3 mM KCl, 2 mM $MgCl_2$, 0.67 mM Na_2-ATP at pH 7.8. At higher temperatures (>40 °C), the ATPase activity strongly decreased, probably due to denaturation of the KdpFABC complex. For each temperature, three independent measurements were averaged (data not shown). Presenting the enzyme activities as an Arrhenius plot allowed the determination of the activation energy, E_a = 55 ± 1 kJ/mol, which is in good agreement with recent experiments.[6]

To determine the activation energy of ATP-driven K^+ transport across the membrane, experiments with reconstituted vesicles were performed, and the initial pump current was calculated from the $DiSC_3(5)$ fluorescence decrease. Vesicles were prepared in buffer of 25 mM imidazole, 1 mM EDTA, 5 mM $MgSO_4$, and 70 mM K_2SO_4 at pH 7.2. Experiments were performed at various temperatures between 12 and 29 °C, three

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at each temperature. The ATP-induced fluorescence decrease was fitted with a monoexponential function, the initial slope was calculated using eq 4, and the average from the three experiments was determined. According to eq 4, the initial slope of the fluorescence decrease is proportional to the rate constant of the rate-limiting reaction step of the pump cycle, $k = 1/\tau$, and therefore, may be represented as an Arrhenius plot (Figure 6). The activation energy calculated from the linear

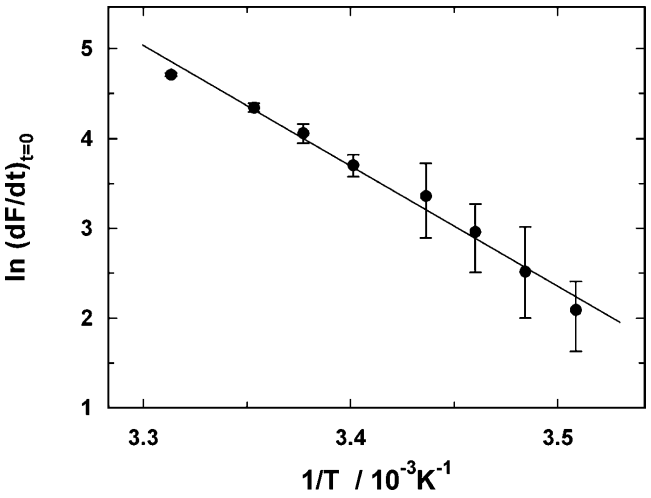

Figure 6. Arrhenius plot of the K^+ pumping activity of the KdpFABC complex reconstituted in *E. coli* lipid vesicles. The initial pump current is directly proportional to the detected $DiSC_3(5)$ fluorescence decrease, $(dF/dt)_{t=0}$. The experiments were performed in the temperature range between 12 and 29 °C. From the regression line, an activation energy of 111 ± 5 kJ/mol was calculated.

slope was 111 ± 5 kJ/mol. The 2-fold higher activation energy indicates a significant difference in the rate-limiting step when compared to the value obtained from the DDM-solubilized enzyme.

DISCUSSION

To obtain more detailed functional properties of the KdpFABC complex, the K^+ pump was reconstituted in *E. coli* lipid vesicles. The advantage of this approach is that on the one hand the protein complex is embedded in a lipid bilayer formed with a lipid mixture isolated from its native host organism. On the other hand, reconstitution allows the separation of substrate-enzyme interactions for both sides of the membrane. The disadvantage of vesicle experiments is that the transport activity of the ion pump can be detected only by the amount of ions transferred across the membrane. Individual reaction steps of the pump cycle cannot be resolved directly. This restriction limits the functional details that may be obtained in experiments with vesicles. In the presented experiments, the fluorescence dye $DiSC_3(5)$ was used as a membrane voltage sensor. It was applied already previously to monitor the ion-translocating activity of KdpFABC.[9,13,16] As explained above, the slope of the initial fluorescence decrease upon ATP addition is proportional to the pump current, and this method allows a direct comparison of the substrate-dependence of the pump activity in experiments from the same vesicle preparation.[9] The KdpFABC complexes become inserted into the membrane in either direction during the reconstitution procedure. This fact is, however, without consequences for the experiments performed since ATP is required for activity and was added to the external medium and does not diffuse through the vesicle membrane. Therefore, only inside-out oriented ATPases contributed to the detected transport activity. This assay was used to study the effect and concentration dependence of those substrates that interact with the KdpFABC complex from the cytoplasmic side.

Cytoplasmic K^+-Concentration Dependence. When the K^+-concentration dependence of the pump current was studied in vesicles, an inhibitory effect of K^+ was detected (Figure 1) with a K_i of 23 mM. During the physiological function of the pump, potassium ions are transported into the cytoplasm. They have to be released at the cytoplasmic side of the protein.[17,18] In terms of the Post−Albers scheme of the KdpFABC protein,[6,9] this reaction step is denoted by $K_2E_1 \leftrightarrow E_1 + 2K^+$. Therefore, K_i may be interpreted as an apparent $K_{1/2}$ value of the reverse reaction, K^+-binding to the E_1 conformation. At enhanced K^+ concentrations, the equilibrium of this reaction will be shifted to the left side by a process called K^+-back binding. Consequently, the flow through subsequent reaction steps and the pump current are reduced. In *E. coli* the cytoplasmic K^+ concentration is on the order of 180 mM under physiological conditions;[19] therefore, only ∼10% of the pumps in the E_1 conformation are free of K^+ and available for progress through the pump cycle, i.e., phosphorylation by ATP as next step. This indicates that in *E. coli* the cytoplasmic K^+ concentration has an inhibitory effect on the KdpFABC complex and that the pump does not gain its maximal turnover rate even at physiological Mg^{2+} concentrations. According to Figure 1, the pump current at 140 mM K^+ is about 25% of the maximal value. When the ion-binding sites are accessible from the extracellular side in the P-E_2 conformation, the half-saturating K^+ concentration was found to be on the order of 2−10 μM.[9,11,20] When compared to K^+-back binding in the E_1 conformation, the effective affinity of the binding sites changes by a factor of more than 2000 between both principal conformations of the ion pump.

Cytoplasmic Mg^{2+}-Concentration Dependence. Mg^{2+} ions are an essential cofactor for KdpFABC phosphorylation, needed to form the enzymatically active MgATP complex as in the case of other P-type ATPases.[12,21] Correspondingly, no ion transport was observed in the absence of Mg^{2+}. Taking into account a dissociation constant of the MgATP complex of 0.1 mM, the concentration of MgATP is saturating (>0.4 mM) in the Mg^{2+}-concentration range covered by the experiments (>0.5 mM) and in the presence of 1 mM ATP. Accordingly, the increase of pump activity by a factor of ∼6 between 0.5 mM and 20 mM Mg^{2+} has to be assigned to a different interaction of Mg^{2+} with the protein. High-affinity K^+ binding in the P-E_2 conformation has also been found to be affected by Mg^{2+}, and the observed effect could be explained by the Gouy−Chapman effect at the entrance of the extracellular access channel to the ion-binding sites.[9] Magnesium ions adsorb to negatively charged lipid head groups or amino-acid side chains in the proximity of the access channel, and thus reduce locally the K^+ concentration, which generates in turn an apparently lower binding affinity for K^+. The increase of the pump current with increased Mg^{2+} concentrations on the cytoplasmic side (Figure 2) may be explained accordingly. Binding of Mg^{2+} close to the cytoplasmic access channel generates a Gouy−Chapman effect that reduces the local K^+ concentration, and thus K^+-back binding. The determined Hill coefficient on the order of 2 indicates the contribution of more than one Mg^{2+} ion, and at 5 mM Mg^{2+} present in the buffer almost all Mg^{2+}-binding sites

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are occupied. A rough estimate based on the Gouy−Chapman theory, on the assumption that two Mg^{2+} ions are located at a distance of ∼4 Å from the entrance, results in a reduction of the local K^+ concentration from 140 mM to 5 mM, a value where K^+-back binding is less strong. In this way, Mg^{2+} ions bound close to the entrance of the access channel can significantly reduce K^+-back binding under physiological Mg^{2+} concentrations and enable a more effective pump process (Figure 2). However, specific Mg^{2+} sites on the cytoplasmic side of the KdpA subunit have not been identified so far. The half-saturating constant of 1.73 mM obtained for Mg^{2+} influence on the KdpFABC complex is almost three times higher than the value reported in the case of the Na,K-ATPase.[22]

Nucleotide-Concentration Dependence. Both products of the ATP hydrolysis, ADP and P_i, were shown to have an inhibitory effect on the enzyme activity[12] and the pump current (Figures 3 & 4). This phenomenon was also observed with other P-type ATPases, e.g., the Na,K-ATPase.[23−25] The inhibitory effect of ADP was explained by the reversal of the phosphorylation reaction, $E_1ATP \leftrightarrow E_1\text{-}P + ADP$. With increasing ADP concentrations, the reaction equilibrium is shifted to the left, and the reaction flow through this partial reaction of the pump cycle becomes rate limiting. The inhibitory constant, K_i, of 151 μM is in agreement with respective published data for the Na,K-ATPase.[25] Correspondingly, the inhibition of the pump current by inorganic phosphate is explained in terms of back-door phosphorylation, $E_2(K_2) + P_i \rightarrow P\text{-}E_2K_2$, which is the reversal of the dephosphorylation step in the Post−Albers scheme. This process was investigated in great detail for the Na,K-ATPase.[25,26] Under comparable experimental conditions, i.e., in the presence of saturating ATP concentrations, the half-maximal inhibiting P_i concentration was found to be ∼14 mM for the Na,K-ATPase, while the KdpFABC complex showed an approximately 50-fold higher sensitivity (K_i = 268 μM). To find an explanation for this difference, one can consult experiments in which back-door phosphorylation of the Na,K-ATPase was measured in the absence of ATP. Under this condition, the half-saturating P_i concentration was 23 μM.[26] This observation indicates that in the applicable reaction sequence of the pump cycle, $P\text{-}E_2K_2 \leftrightarrow E_2(K_2) \leftrightarrow E_2(K_2)ATP$, the presence of ATP is able to drain the $E_2(K_2)$ state of the pump, when it binds in the E_2 conformation to the so-called low-affinity binding sites, and thus, an apparently lower binding affinity for P_i is measured. According to this consideration, the detected high P_i affinity of the KdpFABC complex may be explained by a lower binding affinity for ATP to the $E_2(K_2)$ state than in the case of the Na,K-ATPase. A conspicuous difference between the inhibiting action of ADP and P_i is obvious at the limit of high concentrations. ADP was able to abolish the pump current almost completely, while P_i reduced the pump current only by ∼20%.

***o*-Vanadate Inhibition.** Many ATPases and especially P-type ATPases are inhibited by *o*-vanadate, which acts as a transition-state analogue of phosphate. *o*-Vanadate binds in the presence of Mg^{2+} to the dephosphorylated E_2 state with a high affinity in the micromolar concentration range.[27] The inhibitory effect was also shown for the KdpFABC complex.[11−13,28] While the inhibition constant obtained from the enzyme activity, K_i, was 1 μM in the case of the solubilized enzyme complex, it was 62 μM for the reconstituted KdpFABC complex (Figure 5A). When the effect of *o*-vanadate on the pump current across the vesicle membrane was determined, a comparable K_i of 58 μM was found under the same conditions. In the case of the solubilized KdpFABC complex, 93% of the enzyme activity was inhibited by 1 mM *o*-vanadate, while in the case of the reconstituted enzyme only ∼65% could be inhibited with 5 mM *o*-vanadate. Because the data could be fit satisfactorily by a Michaelis−Menten approach, the remaining enzyme activity seems to be inert against inhibition, and this indicates a severe modification of a fraction of the KdpFABC complexes with respect to its interaction with *o*-vanadate. This significantly different effect of the inhibitor on both the solubilized and reconstituted form may have two different reasons: (1) The environment of the complex is different; in one case, it is surrounded by a narrow and strongly curved annulus of β-DDM molecules and lipids. In the other case, the complex is embedded in a lipid bilayer of *E. coli* lipids with a very low detergent concentration. (2) The K^+ concentration is saturating in both cases, but in the experiments with solubilized enzyme it is significantly lower (3.3 mM) than with vesicles (140 mM). The half-saturating K^+ concentration on the cytoplasmic side of the ion pump was determined to be 23 mM. The K_i value of *o*-vanadate obtained for the complex solubilized in β-DDM is in agreement with the value of 1.5 μM obtained for complexes solubilized in a different detergent, Aminoxide WS-35.[11] Investigations of the K_i value in native cells are not available. From studies with other P-type ATPases, it is known that *o*-vanadate is a potent inhibitor with K_i values <10 μM.[29−31] Therefore, it is reasonable to propose that the values of K_i obtained from the vesicle preparations were distorted by the experimental conditions.

The reduced affinity for *o*-vanadate became visible in both sets of experiments with the reconstituted KdpFABC complex, in the enzyme activity as well as in the stationary membrane potential during pump activity. It has to be mentioned, however, that the stationary membrane potential is an indirect approach because the observed effect is coupled to the voltage-dependent leak currents through the membrane. The influence of other experimental conditions is reflected in the reduced Hill coefficient of ∼0.5.

The high K^+ concentration in the vesicle experiments may be excluded as a cause of the reduced K_i value because 140 mM K^+ would promote K^+-back binding (Figure 1), and thus increase the population of the $E_2(K_2)$ state that would in turn enhance *o*-vanadate binding, and apparently, increase binding of the inhibitor. This is contrary to the experimental finding.

At the present state, the reduced capacity of *o*-vanadate to inhibit the enzymatic and transport activity in reconstituted vesicles is a promising hint at the affected mechanism. At 1 mM *o*-vanadate only ∼63% of the inside-out oriented KdpFABC complex is inhibited (compared to >90% of the solubilized enzyme). This observation indicates that embedding of the complex into the lipid bilayer may modify the moiety to which the inhibitor has to bind such that it becomes much less attractive in a way (or is even disabled). This hypothesis would be supported by the observation that in the presence of even 10 mM P_i, which binds in the same state to the same site, the pump current is also reduced not more than 24%; in contrast, 2 mM ADP that binds to the $P\text{-}E_1$ state reduces the pump current more than 85% (Figure 4). To reveal the true origin of this phenomenon, an elaborate study of the lipid dependence on this effect has to be conducted.

Activation Energy of the Rate-Limiting Steps. The activation energy of the KdpFABC complex reconstituted was determined from the enzyme activity and the initial pump

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current through the vesicle membrane. In both approaches, the KdpFABC complex works under turnover conditions. Nevertheless, the activation energy was higher by a factor of 2 in the case of the pump current (111 kJ/mol vs 55 kJ/mol). To determine the origin of this significant effect, the differences in both assays have to be considered as in the case of the *o*-vanadate inhibition. The activation energy is a characteristic parameter of the rate-limiting reaction step in the reaction sequence observed in the experiment. In both cases, the enzymes work under turnover conditions, and in previous investigations it was found that the rate-limiting reaction steps of the solubilized complex are the conformation transitions with activation energies of up to 61 kJ/mol under the various conditions.[6] The large difference observed between the solubilized and reconstituted complex have to be caused, therefore, by either a different conformation transition that comes into play upon reconstitution or by the same transition whose activation energy has been doubled in the lipid bilayer environment.

A direct comparison of the enzyme activity of the solubilized and reconstituted KdpFABC complex is impossible since the precise distribution of the inside and right-side out oriented proteins is unknown. Assuming an equal distribution of the pumps in the vesicle membrane, the turnover number of the reconstituted pumps would be 20% higher at 37 °C as can be calculated from the data in the absence of vanadate (see above). At ∼24 °C, however, the turnover rate would be slower by a factor of 2 on the basis of the determined activation energies.

The same possibly relevant differences between both preparations have to be discussed as in the case of the *o*-vanadate inhibition. First, the amount of detergent molecules encasing the membrane domain of the KdpFABC complex was high for the solubilized and low for the reconstituted protein. Second, the experiments with the solubilized enzyme were performed in the presence of 3.3 mM K^+ and 2 mM Mg^{2+}, pH 7.8, while the pump current was studied in the presence of 140 mM K^+ and 5 mM Mg^{2+}, pH 7.2. On the basis of the magnitude of the activation energy, a modification of ion-binding and release reactions may be excluded as origin of the effects because changes of ∼55 kJ/mol require major changes in the reaction mechanism. To propose a modified interaction between the KdpA and KdpB subunit is a reasonable working hypothesis. However, similar to the analysis of the *o*-vanadate effects, further detailed studies on the lipid dependence of activation energy are required to gain further mechanistic insights.

■ CONCLUSIONS

The investigation of the KdpFABC complex reconstituted in lipid vesicles allowed the analysis of substrate interactions specifically with binding sites on the cytoplasmic side of the ion pump. In the E_1 conformation the ion-binding sites, assumed to be located in the KdpA subunit, are accessible from the cytoplasm and have a K^+-binding affinity about 2000-fold lower than when accessible from the opposite side of the membrane. Nevertheless, significant back binding of K^+ would occur and effectively reduce K^+ transport by the pump at physiological K^+ concentrations in the cytosol (>100 mM). This effect is, however, counteracted by binding of Mg^{2+} ions close to the terminus of the cytoplasmic access channel, which reduces significantly the local K^+ concentration due to the Gouy−Chapman effect. Accordingly, binding of Mg^{2+} avoids the necessity of an even larger shift of the binding affinity of the K^+ sites and ensures that K^+ release from their binding sites is less affected by back binding under physiological conditions.

A second class of substrate interaction occurs at the cytoplasmic surface of the KdpB subunit. Higher concentrations of ADP, as product of the phosphorylation reaction, E_1 + ATP ↔ P-E_2 +ADP, affect this reaction step in the expected manner by reversing this step and reducing the reaction flow appropriately. More complex were the findings with the reconstituted complex concerning the dephosphorylation of the enzyme, P-E_2K_2 → $E_2(K_2)$ + P_i. When this reaction is reversed in a so-called backdoor phosphorylation by addition of inorganic phosphate, the pump current could be reduced by only a small fraction. The inhibition by *o*-vanadate that takes place at exactly the same reaction step, in which it replaces phosphate, shows a similar behavior. It seems that the process of binding of phosphate (or its transition-state analogue) and the concomitant conformational rearrangement were affected by the environment created by the lipids present in the vesicle membrane. This hypothesis is supported by the recent finding that K^+-binding affinity and the enzyme activity are also extremely sensitive to the detergent in which the KdpFABC complex was purified.[9] It was proposed that a so-far unidentified component in the mixture of detergents used there was crucial for reproducing best the in vivo properties of the enzyme.

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Notes

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■ ABBREVIATIONS

$DiSC_3(5)$, dipropylthiadicarbocyanine iodide; β-DDM, *n*-dodecyl β-D-maltoside; $C_{12}E_8$, octaethylene glycol monododecyl ether

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