Replacement of Glycine 232 by Aspartic Acid in the KdpA Subunit Broadens the Ion Specificity of the K^+-Translocating KdpFABC Complex

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ABSTRACT Replacement of glycine residue 232 with aspartate in the KdpA subunit of the K^+-translocating KdpFABC complex of *Escherichia coli* leads to a transport complex that has reduced affinity for K^+ and has lost the ability to discriminate Rb^+ ions (Buurman et al., 1995, *J. Biol. Chem.* 270:6678–6685). This glycine residue is the first in a highly conserved GGG motif that was aligned with the GYG sequence of the selectivity filter (P- or H5-loop) of K^+ channels (Jan and Jan, 1994, *Nature.* 371:119–122). Investigations with the purified and reconstituted KdpFABC complex using the potential sensitive fluorescent dye $DiSC_3(5)$ and the "caged-ATP/planar bilayer method" confirm the altered ion specificity observed in uptake measurements with whole cells. In the absence of cations a transient current was observed in the planar bilayer measurements, a phenomenon that was previously observed with the wild-type enzyme and with another *kdpA* mutant (A:Q116R) and most likely represents the movement of a protein-fixed charge during a conformational transition. After addition of K^+ or Rb^+, a stationary current could be observed, representing the continuous pumping activity of the KdpFABC complex. In addition, $DiSC_3(5)$ and planar bilayer measurements indicate that the A:G232D Kdp-ATPase also transports Na^+, Li^+, and H^+ with a reduced rate. Similarities to mutations in the GYG motif of K^+ channels are discussed.

INTRODUCTION

The KdpFABC complex of *Escherichia coli* is a high-affinity K^+ uptake system (for reviews, see Siebers and Altendorf, 1993; Altendorf and Epstein, 1996), a member of the class of P-ATPases. These ion-motive ATPases transport a wide variety of cations and phospholipids in bacteria, plants, fungi, and animal cells (reviewed in Møller et al., 1996; Axelsen and Palmgren, 1998). Kdp is a "back-up" system, expressed only when the major uptake system, Trk, does not suffice because of low medium K^+ or mutation. Hence Kdp can be mutated at will because it is dispensable under most growth conditions.

Among the P-ATPases, the KdpFABC complex has a unique position, because domains that bind the transported ion (K^+) and provide the energy for the transport are located in different subunits, namely KdpA and KdpB, respectively. The largest subunit, KdpB, shares all of the conserved regions of P-ATPases identified by Serrano (1988) (see also the more recent and more extensive examination by Axelsen and Palmgren (1998)), is phosphorylated during the reaction cycle (Siebers and Altendorf, 1989), probably at the highly conserved Asp^{307} residue (Puppe et al., 1992), and is labeled by 2-azido-ATP (Dröse, 1997). The function of the essential KdpC subunit is not established. Recently it was shown that KdpC forms a very stable complex with KdpA, suggesting that these two may have a joint evolutionary origin distinct from that of KdpB (Gassel et al., 1998). The hydrophobic 29-residue KdpF peptide is not essential in vivo, but appears to be important for the stability of the solubilized KdpFABC complex (Altendorf et al., 1998; Gassel et al., 1999).

The analysis of K^+ affinity mutants generated by random mutagenesis revealed that amino acid residues involved in K^+ binding are almost exclusively located in the KdpA subunit that contains 10 membrane-spanning segments (Buurman et al., 1995). The topological arrangement of KdpA illustrated in Fig. 1 *A* was based on the analysis of fusions to alkaline phosphatase and β-galactosidase. According to this topology, the K^+ affinity mutants are clustered in one cytoplasmic and three periplasmic loops. Based on this distribution, Buurman et al. (1995) postulated the existence of an initial high-affinity K^+-binding site on the periplasmic site of KdpA in addition to a low-affinity site on the cytoplasmic site. Moreover, two regions that resemble the M1-H5-M2 region of inwardly rectifying K^+ channels in eukaryotes were postulated for KdpA (Jan and Jan, 1994, 1997). About 50% of the K^+ affinity mutants isolated by Buurman et al. (1995) are clustered in these two H5-like segments (Fig. 1 *A*). An alternative model (Fig. 1 *B*) based on computer modeling was presented very recently by Durell et al. (2000). This model suggests an evolutionary relationship of KdpA with K^+ channels and K^+ symporters. The major difference is that the region containing the third cluster of K^+ affinity mutants is rearranged to the periplasmic side and represents one of four H5(P-loop)-like domains within KdpA.

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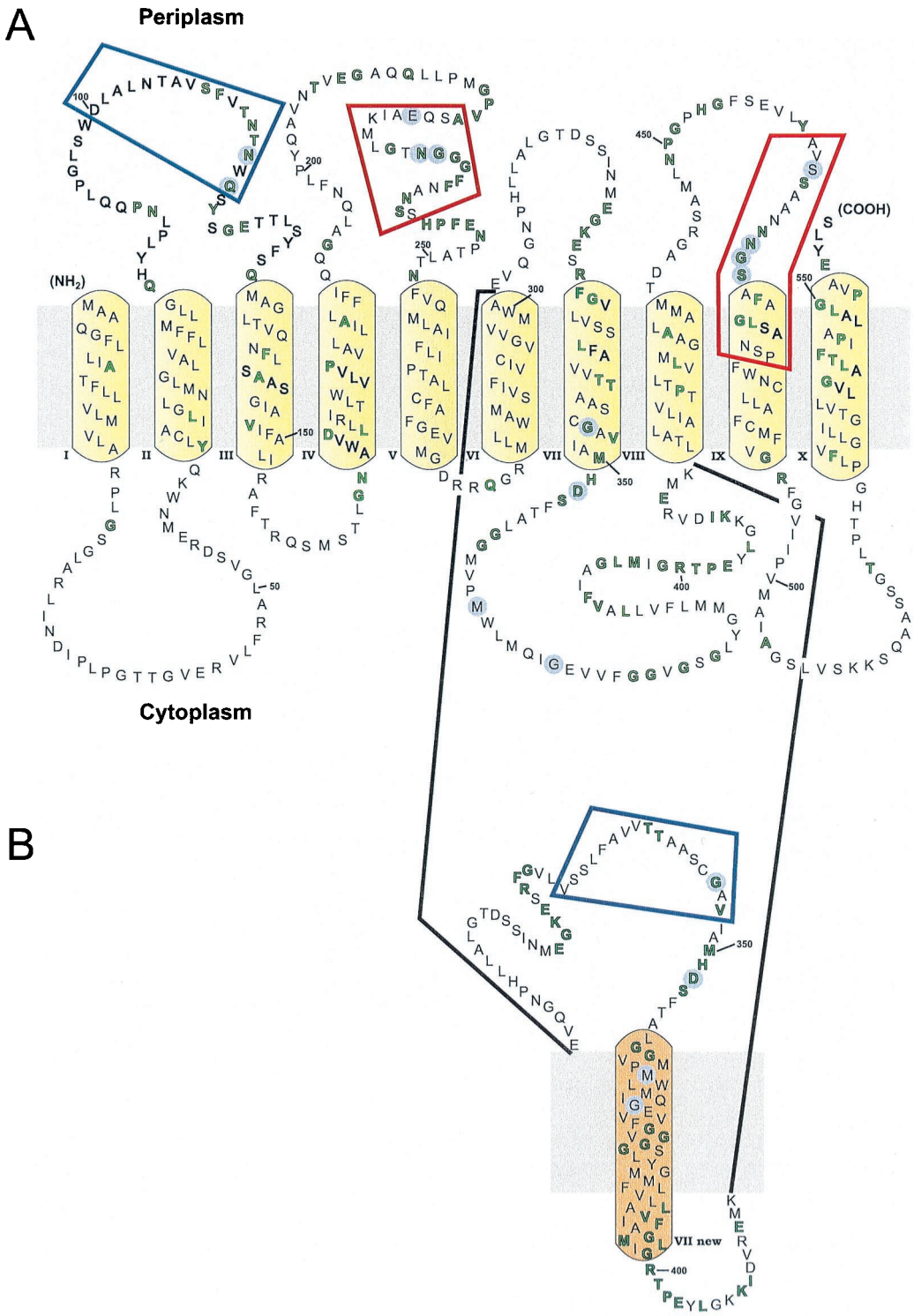

FIGURE 1 (*A*) Topological model of the KdpA subunit according to Buurman et al. (1995). Putative transmembrane α-helices are enclosed in yellow boxes. Some of the more hydrophobic periplasmic and cytoplasmic loops may fold back into the membrane. The sites of K^+ affinity mutants identified by Buurman et al. (1995) are indicated by gray-blue circles. Amino acid residues that are conserved in the predicted KdpA protein sequences of *E. coli*, *Synechocystis sp.* PCC 6803 and *Clostridium acetobutylicum* are shown in green. The postulated P-loop/H5-like regions according to Jan and Jan (1994) are enclosed by red colored lines. (*B*) Topological model of the KdpA subunit according to Durell et al. (2000). Only that part of the model is shown that differs significantly from that by Buurman et al. (1995). The two additional M1-H5-M2 motifs are enclosed by blue colored lines. The rest of the color code is as in *A*.

Møller et al. (1996) suggested that the unusual composition of the KdpFABC complex could represent an intermediate state—a quasi-"missing link"—between the mostly prokaryotic heavy-metal-transporting type I and the mostly eukaryotic type II P-ATPases (split into two groups in the analysis of Axelsen and Palmgren, 1998) that transport cations of lower atomic masses, like Na^+, K^+, Ca^{2+}, and H^+. In this scheme KdpB is homologous to the energy-transducing part of the type I ATPases, which is supported by the phylogenetic analysis of Axelsen and Palmgren (1998), while KdpA forms the K^+-translocating pathway and shares homologous features with K^+ channel proteins (Møller et al., 1996; Jan and Jan, 1994, 1997). If this assumption is correct, the KdpFABC complex is an interesting model system to investigate the evolution and catalysis of ion transport by P-ATPases.

In two preceding publications we showed that the KdpFABC complex is an electrogenic ion pump (Fendler et al., 1996) and that a part of the electrogenic charge movement probably represents the transfer of charged amino acid residues coupled to a conformational transition (Fendler et al., 1999). In transport measurements with whole cells (Buurman et al., 1995), two intrinsic features of the wild-type KdpFABC complex could be observed: K^+ is transported with high affinity (K_m 2 μM) and high selectivity, because Rb^+, with a Pauling radius (1.49 Å) comparable to that of K^+ (1.33 Å) is transported with much lower affinity (K_m 8 mM). Nevertheless, the replacement of Gly^{232} with aspartate in KdpA (A:G232D), the first residue in the highly conserved GGG motif (Fig. 1 *A*), not only reduced the affinity for K^+ from 2 μM to 1.2 mM, but also affected the narrow ion selectivity. This altered KdpFABC complex transports Rb^+ with a comparable K_m (1.4 mM) and v_{max} (Buurman et al., 1995). In this report the transport properties of the purified and reconstituted A:G232D KdpFABC complex were analyzed in more detail. Fluorimetric measurements with the potential-sensitive dye $DiSC_3(5)$ and electrical measurements on planar bilayers confirm that Rb^+ ions are transported by the A:G232D KdpFABC complex. In addition, experimental evidence for the transport of Li^+, Na^+, and H^+ is also provided. The drastic change in the ion selectivity of the A:G232D KdpFABC complex resembles the "unselective mutants" of K^+ channels that arise by substitution of one of the two glycine residues of their highly conserved GYG motif (Heginbotham et al., 1992, 1994; Slesinger et al., 1996; Nakamura et al., 1997). These findings suggest that the mechanism of K^+ selectivity of the KdpA subunit is related to that of K^+ channels.

MATERIALS AND METHODS

Bacterial strains, construction of plasmid pSD126, and growth of bacteria

The *E. coli* K12 strain TKR1000 (*kdpA42* Δ*atp706 nagA trkA405 trkD1 thi rha lacZ*) was transformed with the plasmid pSR5 (Iwane et al., 1996) as described previously (Fendler et al., 1999). Both the chromosomal encoded and the plasmid encoded *kdp* genes carry the *kdpA42* (A:Q116R) mutation (Buurman et al., 1995).

Plasmid pSD126 carries the *kdpA13* mutant (A:G232D) version of the *kdpFABC* operon and its promoter in a derivative of pBR322. The plasmid is 9.7 kb long and very similar in structure to pSR5 (Iwane et al., 1996), except that it carries a different *kdpA* point mutation, and the *Ava*I –*Nru*I region of the vector has been replaced with a ~600-bp fragment from Bluescript plasmid pSK(−) (Stratagene), which has the f1 phage origin of replication. The f1 origin is oriented in the direction opposite that of the *amp* gene of the plasmid.

The plasmid pSD126 was transformed in the *E. coli* K12 strain TKW3205 (Δ*kdpABC5* Δ*atp706 nagA trkA405 trkD1 thi rha lacZ*) (Puppe, 1991). The resulting transductants, TKR1000/pSR5 and TKW3205/pSD126, were grown in minimal medium containing 0.5 mM K^+ and 50 μg/ml ampicillin, as described by Siebers and Altendorf (1988).

Protein purification and preparation of the proteoliposomes

The KdpFABC complex of both strains was prepared as described by Fendler et al. (1996). The resulting KdpFABC pool contained 15 mM HEPES-Tris (pH 7.5), 100 mM NaCl, and 0.2% decylmaltoside. The NaCl was removed by dialysis against 15 mM HEPES-Tris (pH 7.5), 0.2% decylmaltoside. Proteoliposomes were formed as detailed by Fendler et al. (1996). For the fluorimetric measurements the following modifications were made: *E. coli* lipids (10 mg/ml) were solubilized in decylmaltoside (10 mg/ml). After formation of the proteoliposomes they were centrifuged for 45 min at 225,000 × *g*, and the pellet was resuspended in half of the starting volume in 15 mM HEPES-Tris (pH 7.5).

ATPase activity

For the determination of the ATPase activity, inside-out vesicles or the purified KdpFABC complex from strains TKW3205/pSD126 and TKR1000/pSR5 were incubated with different KCl, RbCl, NaCl, CsCl, and LiCl concentrations in the range of 0–50 mM. The ATPase activity was determined using the automated ATPase microassay of Henkel et al. (1988), following the modifications described previously (Altendorf et al., 1998).

Fluorometric measurements

The proteoliposomes were loaded by sonication (two times 10 s) with 50 mM KCl, 50 mM RbCl, 50 mM NaCl, and 50 mM LiCl. The fluorometric measurements were carried out at room temperature (~24°C) in 1 ml solution containing 15 mM HEPES-Tris (pH 7.5), 2 mM $MgSO_4$, 1 μM $DiSC_3(5)$, 20 μl of the proteoliposomes, and, as indicated, 50 mM of the corresponding salt. The settings of the fluorometer (RF-5001 PC; Shimadzu Europe) were as follows: excitation wavelength, 650 nm; emission wavelength, 675 nm; band-pass, in both cases, 5 nm; integration time, 1 s.

Bilayer measurements

The preparation of bilayers, the electrical recording, photolysis of caged ATP, and measurements of conductivity were performed as described (Fendler et al., 1996). The electrolyte contained 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM DTT, and various amounts of caged ATP and other salts. The proteoliposomes were adsorbed to the planar bilayer and activated by photolytic release of ATP.

Caged ATP, P^3-1-(2-nitro)phenylethyladenosine 5′-triphosphate Na^+ salt, was purchased from Calbiochem. For experiments that required the absence of Na^+, the $(C_2H_5)_3NH^+$ salt of caged ATP was kindly supplied

by E. Grell (Max-Planck-Institut für Biophysik, Frankfurt am Main, Germany).

RESULTS

ATPase activity of A:G232D KdpFABC complex

Because the ion selectivity of the KdpFABC complex identified by transport measurements is in good agreement with the stimulating effect of these cations on the ATPase activity (Epstein et al., 1978; Siebers and Altendorf, 1989), we analyzed the effect of increasing concentrations of KCl, RbCl, LiCl, CsCl, and NaCl on everted vesicles of strain TKW3205/pSD126 (A:G232D) (Fig. 2 *A*). The strain carries a partial deletion of the *atp* operon and therefore possesses no functional ATP synthase. The residual ATPase activity in the absence of cations could be completely inhibited by 200 μM *ortho*-vanadate (data not shown), a specific inhibitor of P-ATPases. In addition, vesicles from strain TKW3205 had no measurable cation-stimulated ATPase activity under identical experimental conditions (data not shown; data for a *recA* derivative is in Puppe et al., 1992). Consequently, all of the measurable activity could be attributed to the KdpFABC complex.

With vesicles from strain TKW3205/pSD126 (A:G232D), a stimulation of ATP hydrolysis by K^+, Rb^+, Li^+, and Na^+ was observed (Fig. 2 *A*), with apparent affinities of $\sim$2 mM for K^+ and Rb^+. The apparent affinities of Li^+ and Na^+ are difficult to determine, because the curves show no saturation. The altered ion affinity and selectivity of the A:G232D KdpFABC complex are preserved during solubilization and subsequent purification (Fig. 2 *B*). As a control we used the A:Q116R KdpFABC complex because its low affinity for K^+ with high specificity for K^+ would reveal any nonspecific effects. As expected, the A:Q116R was stimulated only by K^+ ion (apparent affinity 6 mM) and not by Li^+, Na^+, or Rb^+ ions (Fig. 2 *C*). Cs^+ ions in concentrations up to 50 mM stimulate neither the activity of the A:G232D nor that of the A:Q116R KdpFABC complex (data not shown).

$DiSC_3(5)$ fluorescence measurements

To answer the question of whether overall transport is electrogenic in the A:G232D KdpFABC complex, as shown for the wild type (Fendler et al., 1996) and the A:Q116R KdpFABC complex (Fendler et al., 1999), the purified enzyme was reconstituted into proteoliposomes and the electrogenic properties were analyzed with the potential-sensitive fluorescence dye $DiSC_3(5)$ (Figs. 3 and 4). The fluorescence experiments were performed at high cation concentrations to minimize the effect of ion gradients created by the pump. Before activation of the reconstituted KdpFABC complex with 1 mM ATP, the K^+ or Rb^+ concentrations inside and outside the proteoliposomes were 50 mM. In the case of the A:G232D KdpFABC complex, the generation of a negative potential inside the proteoliposomes, which could be monitored with the dye $DiSC_3(5)$ (Hoffmann and Laris, 1974), could be observed after the addition of ATP, when KCl or RbCl was present (Fig. 3, *A* and *B*). This demonstrates that the A:G232D KdpFABC complex could transport K^+ as well as Rb^+ ions in an electrogenic manner. In contrast, the reconstituted A:Q116R KdpFABC complex catalyzes only the electrogenic transport of K^+ (Fig. 3 *C*; Fendler et al., 1999) but not that of Rb^+ (Fig. 3 *D*). In all cases the fluorescence intensity decays slowly after the addition of 100 μM *ortho*-vanadate. This shows that the proteoliposomes were relatively permeant for ions. Addition of the K^+ and Rb^+ ionophore valinomycin allows the fast back-flow of the expelled K^+ or Rb^+ ions and, consequently, abolishes the potential generated by the KdpFABC complex. The addition of the electroneutral K^+/H^+ antiporter monensin had no effect on the negative potential generated by the A:G232D and A:Q116R KdpFABC complexes (data not shown).

When ATP is added to proteoliposomes in the absence of alkali metal cations, there is a slow, linear decrease in fluorescence (Fig. 4 *A*). When either NaCl or LiCl is present, ATP produces a more rapid decrease in fluorescence (Fig. 4, *B* and *C*). The effect on fluorescence is much less pronounced than in the case of KCl or RbCl, in good agreement with the graded effect of these cations on the ATPase activity of the A:G232D KdpFABC complex (Fig. 2, *A* and *B*).

Current measurements at the planar bilayer

Proteoliposomes were adsorbed to a planar bilayer as described before (Fendler et al., 1996, 1999). Only the inside-out fraction of incorporated KdpFABC complexes is activated by caged ATP. Upon irradiation of caged ATP with an excimer laser flash, ATP is generated within 45 ms under the conditions of these experiments (Fendler et al., 1996).

The ion pumps in the liposome membrane are capacitively coupled to the measuring system via the capacitances of the liposome and of the planar bilayer. After photolytic ATP release a transient current is observed (Fig. 5), rising with a time constant of $\sim$15 ms and decaying with one of 150 ms (0 K^+, 0 Rb^+) to 700 ms (5 mM Rb^+). A rapidly decaying transient current in the absence and a slowly decaying current in the presence of K^+ has previously been found in the wild-type KdpFABC complex and in the A:Q116R KdpFABC complex (Fendler et al., 1996, 1999). In addition, in the A:G232D KdpFABC complex an increase in the transient current is also found in the presence of Rb^+. As in the case of the A:Q116R KdpFABC complex (Fendler et al., 1999), we tentatively assign the rising phase of the signal ($\tau \approx 15$ ms) to the release of ATP and the decaying phase measured in the presence of K^+ and Rb^+

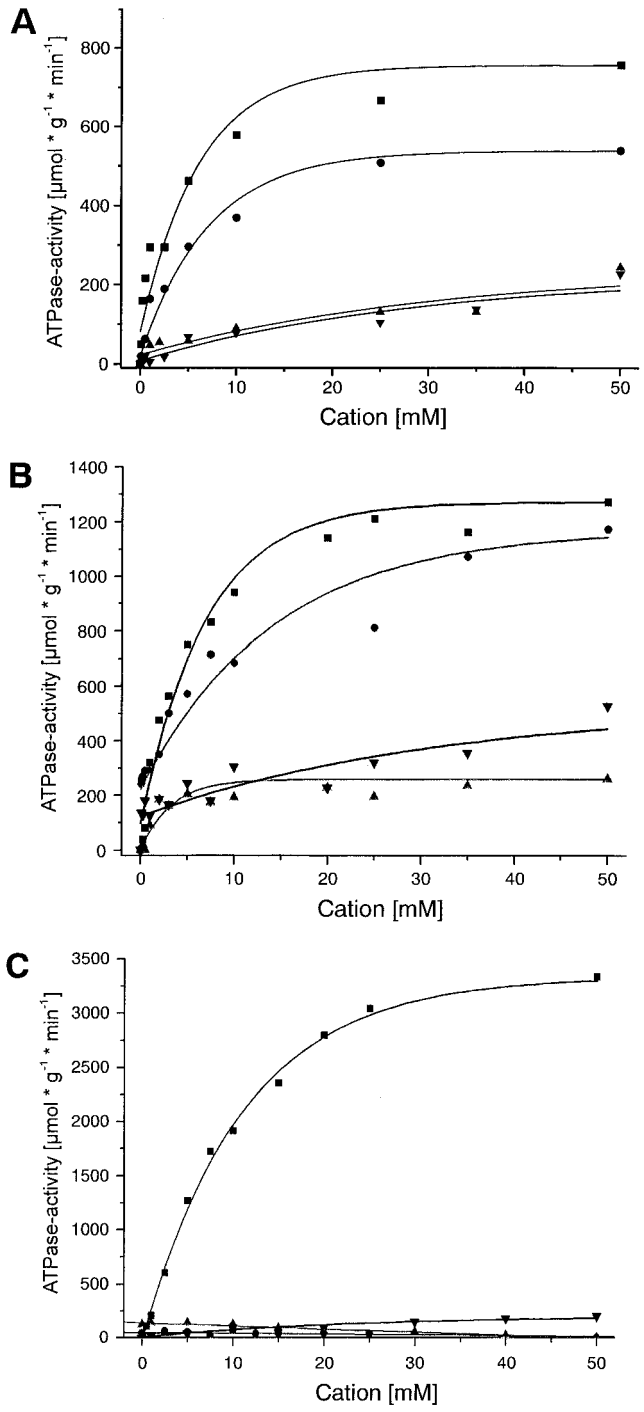

FIGURE 2 Stimulation of the ATPase activity of the A:G232D and the A:Q116R Kdp-ATPase by monovalent cations. The residual ATPase activity in the absence of monovalent cations (up to 10% of the maximal activity) was subtracted from each curve. The ATPase activity was analyzed with the microtiter plate assay of Henkel et al. (1988) as detailed under Materials and Methods. Buffer: 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM ATP. Monovalent cations were added as indicated: ■, KCl; ●, RbCl; ▲, NaCl; ▼, LiCl. The mean values of three assays are shown. To each cavity, right-side-out vesicles with a protein content of (*A*) 5 μg of TKW 3205/pSD126 (A:G232D) and 0.5 μg of the purified Kdp complexes of (*B*) TKW 3205/pSD126 (A:G232D) or (*C*) TKR 1000/pSR5 (A:Q116R) were added, respectively.

(500–700 ms) to the charging of the proteoliposomes and to the build-up of concentration gradients.

Caged ATP acts like a competitive inhibitor for the KdpFABC complex (Fendler et al., 1996, 1999). The same characteristic is observed for the A:G232D KdpFABC complex. Binding constants for ATP and caged ATP have been determined in the absence and presence of K^+ and Rb^+, according to Fendler et al. (1996). In the absence of cations the binding constants were 2.7 mM (caged ATP) and 0.22 mM (ATP). In the presence of 3 mM K^+ we found 0.60 mM (caged ATP) and 0.10 mM (ATP), and with 6 mM Rb^+ 0.61 mM (caged ATP) and 0.11 mM (ATP). Only the affinity for the caged ATP is significantly different in the presence and absence of cations. However, no significant difference between K^+ and Rb^+ was detected. The binding constants in the A:G232D KdpFABC complex agree well with the values obtained for the wild-type KdpFABC complex of 0.63 mM (caged ATP) and 0.07 mM (ATP) in the presence of K^+ (Fendler et al., 1996).

Transient currents at different K^+ and Rb^+ concentrations

To determine the cation affinity of the A:G232D KdpFABC complex we measured the transient electrical currents at various concentrations of KCl and RbCl. The dependence of the peak values of the transient currents at different cation concentrations is shown in Fig. 6. The figure shows data from different measurements that have been normalized to their value in the absence of the cation. The Rb^+ and K^+ concentration in the nominal absence of these cations was always lower than 10 μM and was arbitrarily set at 1 μM. As mentioned above, a current in the absence of cations is observed. The addition of K^+ and Rb^+ increases the peak current by approximately the same amount, but with a different concentration dependence. Half-saturation constants for this activation were ~0.3 mM for K^+ and ~1 mM for Rb^+. This is slightly lower than the 2 mM obtained from the ATP hydrolysis experiments described above.

At high cation concentrations ($K_{0.5} \approx 40$ mM) a decrease in the peak current is observed. This effect has previously been assigned to a Hoffmeister effect of the Cl^- cations (Fendler et al., 1996). The fact that it is not observed in the hydrolysis measurements shows that it does not affect the rate constant of the rate-limiting step or that this effect acts on the stoichiometry of transport rather than on a rate constant.

Stationary currents in the absence and presence of different cations

Continuous pumping activity of the enzyme can only be demonstrated by stationary currents. Under normal conditions the bilayer measurement system is capacitively cou-

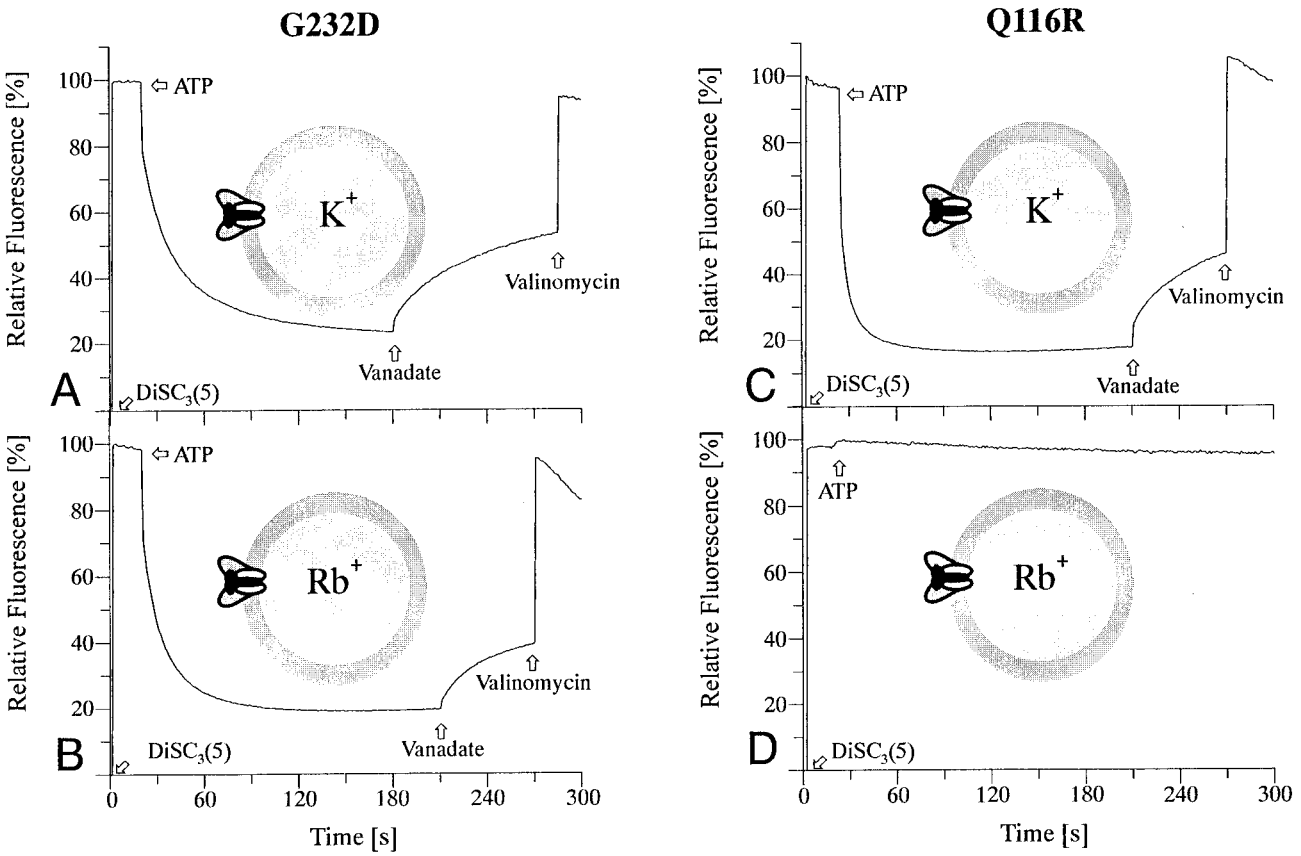

FIGURE 3 $DiSC_3(5)$ fluorescence measurements with the A:G232D and A:Q116R KdpFABC complexes reconstituted into proteoliposomes. Preloaded proteoliposomes (20 μl) containing 50 mM KCl or RbCl as indicated, were diluted into 1 ml 15 mM HEPES-Tris (pH 7.5), 2 mM $MgCl_2$, supplemented with 50 mM of the corresponding cation chloride. Additions to 1 μM of the fluorescence dye $DiSC_3(5)$, 1 mM ATP, 1 μM valinomycin, and 100 μM *ortho*-vanadate are indicated by arrows.

pled and does not allow the recording of stationary currents. However, with the use of appropriate ionophores, the membranes can be made conductive for the transported ions and a stationary current can be measured. We have used a combination of the electroneutral ionophore monensin that exchanges Na^+, K^+, and Rb^+ ions against H^+ and the H^+ ionophore 1799. Together they form a H^+-, Na^+-, K^+-, and Rb^+-transporting system. Stationary currents can be recorded under these conditions, as shown in Fig. 7.

Each of these current traces corresponds to a separate experiment. The magnitude of the signals varies between experiments, because different amounts of protein are adsorbed to the planar lipid membrane. Therefore, a direct comparison between different experiments is not possible. However, judging from a number of measurements performed in the presence of ionophores, we find that the stationary current at the cation concentration of maximum peak current (2 mM KCl or 5 mM RbCl; see Fig. 6) is approximately the same. This is consistent with the hydrolysis measurements that show approximately the same activity at the corresponding Rb^+ and K^+ concentrations. Stationary currents in the presence of ionophores that could be inhibited by *ortho*-vanadate were also observed with Li^+ (using the Li^+ ionophore A23187 instead of monensin) and Na^+ (data not shown).

Surprisingly, also in the complete absence of K^+ or Rb^+, a stationary current is observed, with an amplitude of 30–50% of that in the presence of the cations. This current —like the currents in the presence of K^+ or Rb^+—can be completely inhibited by 1 mM *ortho*-vanadate, a specific inhibitor of P-ATPases. To confirm that the signal is not due to K^+ contamination of the electrolytes, we determined the K^+ concentration at the end of the experiment in the solution containing the buffer, caged ATP, and the protein. A K^+ contamination of ~6 μM was obtained, which can be neglected compared to the K^+ affinity of the enzyme of 300 μM. These results show that in the absence of other cations the A:G232D KdpFABC complex probably transports H^+ ions at a somewhat lower rate.

Additional support for the transport of H^+ ions comes from experiments in which only the protonophore 1799 was present (Fig. 7 *B*, *upper trace*). Under these conditions a stationary current in the absence of K^+ and Rb^+ was observed. These experiments were performed at low buffer

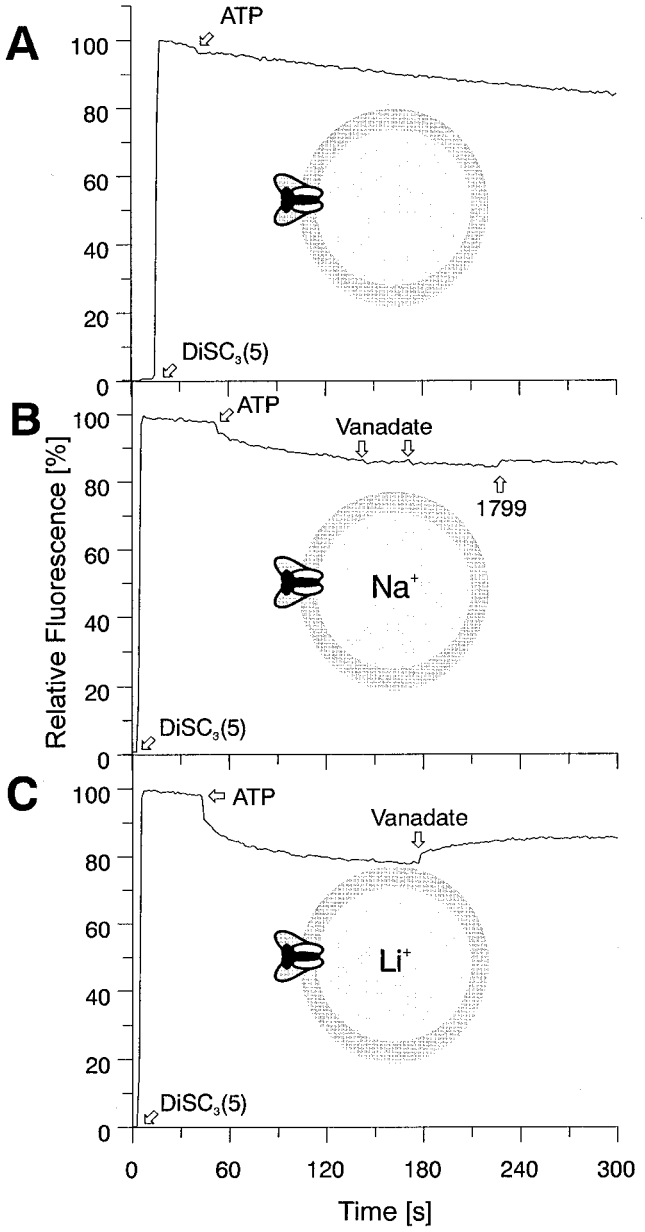

FIGURE 4 $DiSC_3(5)$ fluorescence measurements with the reconstituted A:G232D KdpFABC complex. General conditions were as described in the legend of Fig. 3. Proteoliposomes and the buffer contained either no monovalent cations, 50 mM NaCl, or 50 mM LiCl, as indicated in the figure. The protonophore 1799 was added to a final concentration of 1 μM.

concentration (2.5 mM Tris-HCl, pH 7.5, instead of 50 mM) to exclude charge compensation by H^+. Charge compensation takes place when an ion other than H^+ is transported out of the liposome and H^+ ions enter the liposome via the planar lipid membrane. In this way the measured current is carried by H^+ ions, while the transported ion is a different one. This process will acidify the interior of the liposome, at a rate depending on the buffer concentration; acidification will ultimately reduce the current to zero. Therefore, at high

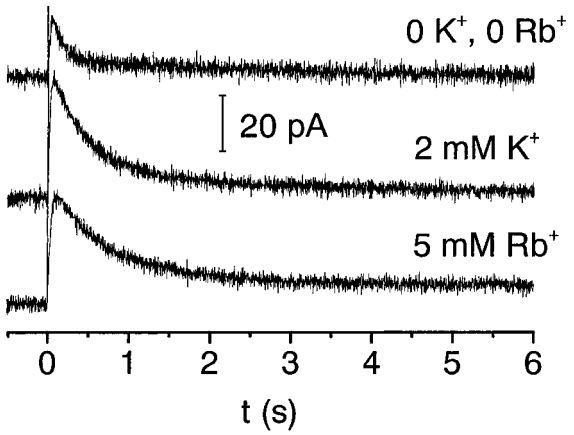

FIGURE 5 Transient currents generated by the reconstituted A:G232D KdpFABC complex after photolytic release of ATP from caged ATP. The electrolyte contained 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM DTT, 200 μM caged ATP, and cations as indicated.

buffer concentration a stationary current could be measured for a considerable time, although the actively transported ion is not H^+. Fig. 7 *B* demonstrates that even at low buffer concentrations a stationary current can be measured for many seconds. When monensin is added, the current remains unchanged, as shown in Fig. 7 *B* (*lower trace*).

Because a stationary hydrolytic activity of the A:G232D KdpFABC complex is found with Na^+ and Li^+, we also tested these ions for electrical activity. The peak current at different concentrations of NaCl and LiCl is shown in Fig. 8. The currents were normalized as in Fig. 6, and the concentration in the absence of Li^+ or Na^+ was arbitrarily set at 10 μM. An activation of the transient currents by Na^+ is apparent, while they are independent of Li^+. The Na^+ affinity is ~2 mM, which is somewhat lower than that of

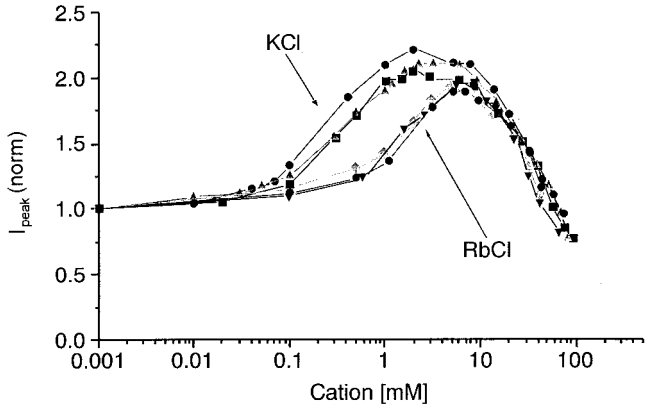

FIGURE 6 Peak currents of the reconstituted A:G232D KdpFABC complex at different K^+ and Rb^+ concentrations. Measurements on three different bilayer preparations with K^+ and three with Rb^+ are shown. The peak currents are normalized to their value in the nominal absence of K^+ and Rb^+. This concentration was arbitrarily set at 1 μM. Electrolyte composition was as in Fig. 5.

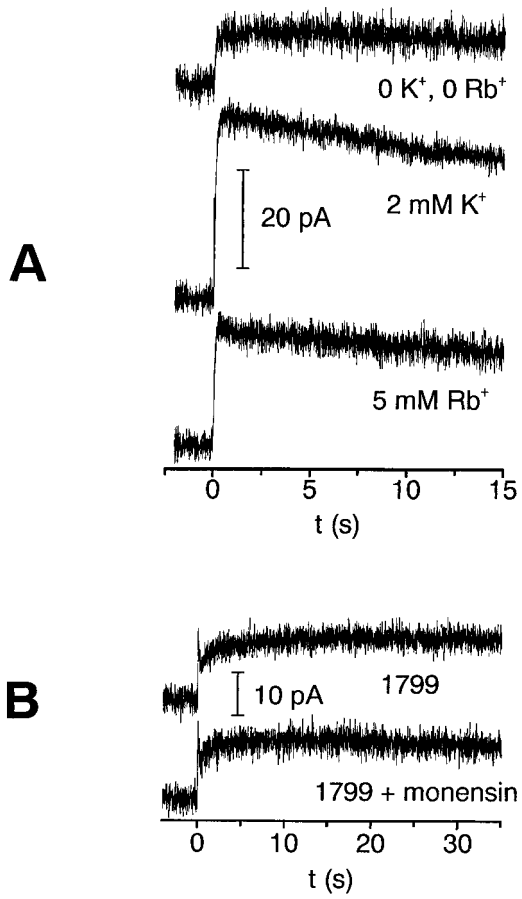

FIGURE 7 Stationary currents of the reconstituted A:G232D KdpFABC complex in the presence of ionophores. (*A*) Stationary currents in the absence and presence of 2 mM K^+ or 5 mM Rb^+. Electrolyte composition: 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM DTT/200 μM caged ATP; ionophores: 10 μM monensin and 3 μM 1799. (*B*) Stationary currents in the absence of K^+ and Rb^+. Electrolyte composition: 2.5 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$/1, mM DTT/200 μM caged ATP; ionophores: 5 μM 1799 (*upper trace*), 10 μM monensin and 5 μM 1799 (*lower trace*).

Rb^+. The lack of activation with Li^+ seems to be at odds with the stimulation of ATP hydrolysis, the reduction of $DiSC_3(5)$ fluorescence (see Fig. 2 and 4 *C*, respectively), and the observation of a stationary current. However, the stationary behavior is related to the rate-limiting step, while the transient currents probably depend on early processes in the reaction cycle. They might be stimulated by a specific cation in a different manner.

DISCUSSION

One of the most striking features of the KdpFABC complex of *E. coli*, a bacterial member of the P-ATPases (Altendorf and Epstein, 1996; Møller et al., 1996), is its high affinity (K_m 2–10 μM; Rhoads et al., 1976; Siebers and Altendorf, 1988, 1989) and selectivity for K^+ ions (Siebers and Altendorf, 1993; Buurman et al., 1995). In contrast to other K^+ transporters and K^+ channels, the KdpFABC complex discriminates markedly against Rb^+ ions (K_m for transport, 8 mM; Buurman et al., 1995) that have a Pauling radius (1.49 Å) comparable to that of K^+ ions (1.33 Å).

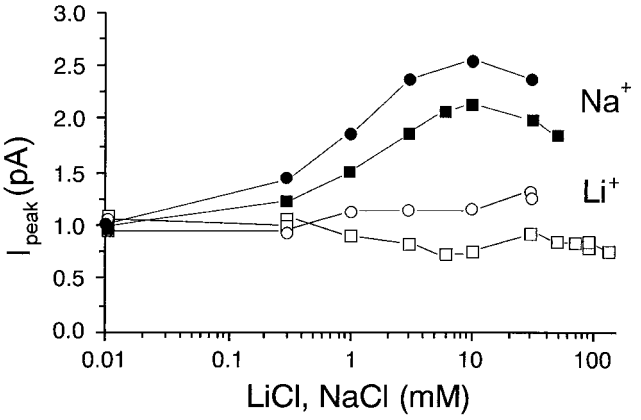

FIGURE 8 Peak currents of the reconstituted A:G232D KdpFABC complex at different Na^+ and Li^+ concentrations. Measurements on two different bilayer preparations with Na^+ and two with Li^+ are shown. The peak currents are normalized to their value in the nominal absence of Na^+ and Li^+. This concentration was arbitrarily set at 1 μM. Electrolyte composition was as in Fig. 5.

Using the same technique as in this publication, we have shown that the KdpFABC complex transports K^+ ions in an electrogenic manner (Fendler at al., 1996). We also investigated the KdpFABC complex purified from an *E. coli* strain harboring the Q116R substitution in KdpA (Buurman et al., 1995) that has drastically reduced affinity for K^+ ions (K_m 6 mM) but still discriminates against Rb^+ ions. With this enzyme complex we observed an electrogenic, ATP-dependent, but K^+-independent step (Fendler et al., 1999). On the basis of our results we proposed a model in which the first electrogenic step is associated with the transport of one protein-bound negative charge (e.g., an ionized carboxyl group of an amino acid residue) to the extracellular side of the ion pump, while the second electrogenic step represents the transport of two K^+ ions together with the negative charge to the intracellular side (Fendler et al., 1999). The possibility of detecting the K^+- (in general ion-) dependent steps with the caged ATP/planar bilayer method encouraged us to investigate the electrical properties of the G232D mutant because it has lost the ability to discriminate K^+ and Rb^+ ions (Buurman et al., 1995).

The A:G232D KdpFABC complex has relaxed substrate specificity

The mutant *kdpA13*, encoding the substitution G232D in KdpA, was isolated during an attempt to identify K^+ affinity mutants of the KdpFABC complex of *E. coli* by random mutagenesis (Buurman et al., 1995). In contrast to the other 14 mutants located in *kdpA*, this mutant transports Rb^+ ions

with K_m and v_{max} values (1.4 mM, 60 μmol g^{-1} (dry weight of cells) min^{-1}) similar to those for K^+ ions (1.2 mM, 120 μmol g^{-1} (dry weight of cells) min^{-1}) (Buurman et al., 1995). Although there are other mutants that show a reduced discrimination between K^+ and Rb^+, some of the reported K_m values for Rb^+, especially those with a low v_{max}, are probably not correct, because the transport measurements were not corrected for the residual TrkF system that does transport Rb^+ (see footnote in Fendler et al., 1999).

In the present publication we present evidence that the A:G232D KdpFABC complex is indeed stimulated by Rb^+, and to a lesser extent by Na^+ and by Li^+. And, based on the generation of a stationary current in the absence of any alkali metal cations, we believe this complex also transports protons. The stimulating effects of K^+ and Rb^+ on the ATP hydrolysis of membrane-bound and purified A:G232D KdpFABC complex occurred at a comparable concentration (K_m ~2 mM; Fig. 2). In contrast, the ATPase activity of the A:Q116R KdpFABC complex is stimulated only by K^+ ions (Fig. 2).

In general, the K_m values obtained from ATPase activity measurements of KdpFABC complexes are in good agreement with those obtained from uptake measurements with whole cells (Epstein et al., 1978; Siebers and Altendorf, 1989; Dröse and Altendorf, unpublished observations). In addition, in $DiSC_3(5)$ measurements with the purified and reconstituted A:G232D KdpFABC complex, the generation of a negative potential inside the proteoliposomes was observed in the presence of K^+ and Rb^+ ions, indicating that this altered ion pump transported both cations in an overall electrogenic reaction. In contrast, there are no indications for Rb^+ transport catalyzed by the A:Q116R KdpFABC complex (Fendler et al., 1999; Fig. 3 *D*). Further evidence comes from electrical measurements with the purified, reconstituted A:G232D KdpFABC complex adsorbed to a planar bilayer. In the absence of cations and ionophores, a transient current was detected that increased after the addition of K^+ or Rb^+ ions. A comparable behavior was observed with the wild-type and the A:Q116R KdpFABC complex only in the presence of K^+ ions (Fendler et al., 1996, 1999). Therefore, the A:G232D KdpFABC complex possesses a K^+-/Rb^+-independent electrogenic step in addition to a K^+-/Rb^+-dependent one. While the transient current in the absence of cations is most probably due to the stimulation of a single turnover charge translocation (Fendler et al., 1999), continuous K^+ and Rb^+ pumping of the A:G232D KdpFABC complex is demonstrated by the experiments in the presence of ionophores; after the addition of the protonophore 1799 together with monensin (Fig. 7 *A*), a stationary current was observed in the presence of K^+ and Rb^+ that represents the continuous pumping activity of the enzyme. The measurements demonstrate that the A:G232D KdpFABC complex has the same electrical transport properties as the wild-type enzyme and the A:Q116R KdpFABC complex, with the subtle distinction that the former KdpFABC complex also transports Rb^+ ions. Therefore, we apply the kinetic model described for the A:Q116R KdpFABC complex (Fendler et al., 1999) and make the following assignments for the A:G232D KdpFABC complex: the first step represents the movement of a protein-bound negative charge (e.g., an acidic amino acid residue) toward the extracellular side of the protein, while the second step corresponds to the transport of two K^+ or Rb^+ ions, respectively.

The change in substrate specificity, seen dramatically in the case of Rb^+, extends to Na^+, Li^+, and H^+. The ATPase activity of the A:G232D complex is stimulated by Na^+ and by Li^+ (Pauling radii of 0.95 and 0.6 Å, respectively), although to a lower extent than by K^+ or Rb^+ (Fig. 2). In addition, these results were confirmed by $DiSC_3(5)$ fluorescence measurements and the observation of stationary currents in the presence of ionophores. The fact that Li^+ ions failed to increase the magnitude of the transient current (Fig. 8) can probably be explained by the different kinetic origins of transient and stationary behavior. An activation by Cs^+ ions was not observed either in ATPase activity or in $DiSC_3(5)$ measurements.

We have found that in the absence of other cations, even H^+ can be transported by the A:G232D KdpFABC complex. The first indications of this came from a small hydrolytic activity in the absence of cations. While this may be alternatively explained by hydrolysis uncoupled from transport, we have shown that under these conditions charge is still transported by the KdpFABC complex (Fig. 7 *B*). That this activity is clearly due to the KdpFABC complex is demonstrated by its inhibition by *ortho*-vanadate. Stationary currents observed in the presence of the protonophore 1799 lend further support to the notion that H^+ ions are transported by the A:G232D KdpFABC complex. A direct comparison of the transport activity of the A:G232D KdpFABC complex in the absence and presence of K^+ shows that the turnover in the absence of K^+ is ~30–50% of that in its presence. This is much more than expected from the residual hydrolytic activity of the enzyme in the absence of K^+ (10% of that in its presence; data not shown). However, this comparison may be misleading, because build-up of capacitive voltages that inhibit the stationary current may be larger for metal cations than for H^+, even in the presence of ionophores.

Whether H^+ transport takes place in the wild-type KdpFABC complex is difficult to judge. Although a small transient current is observed in the absence of K^+, this could be due to K^+ contamination of the buffers, which is impossible to avoid, given the high K^+ affinity (2–10 μM) of the wild-type enzyme. Production of a stationary current by the wild-type complex in the absence of cations was not tested for. A different situation exists in the A:Q116R KdpFABC complex. Here the cation specificity is preserved while the K^+ affinity drops to 6 mM (Buurman et al., 1995; Fendler et al., 1999). Again, a transient current in the

absence of K^+ is observed (Fendler et al., 1999). However, even if ionophores are added, no continuous pumping activity can be recorded. Only the A:G232D KdpFABC complex sustains continuous H^+ pumping. In conclusion, the A:G232D KdpFABC complex has lost most of its cation specificity and is no longer able to discriminate between K^+, Rb^+, H^+, Li^+, and Na^+.

Comparison with affinity/selectivity mutants in K^+ channels and other P-ATPases

The occurrence of a quasi-"nonselective" mutant of the KdpFABC complex is reminiscent of mutations in the highly conserved GYG motif of the selectivity filter (part of the P-loop or H5 motif) of K^+ channels that is absolutely required for K^+ selectivity (Doyle et al., 1998). Mutations in this motif produce nonselectivity for other monovalent cations (Heginbotham et al., 1992, 1994; Slesinger et al., 1996; Nakamura et al., 1997). Moreover, the highly conserved GGG motif, which is present in all of the 16 available KdpA sequences in the National Center for Biotechnology Information data base of microbial genomes (http://www.ncbi.nlm.nih.gov/BLAST/unfinishedgenome.html), can be aligned with the GYG motif of K^+ channels (Jan and Jan, 1994, 1997). Jan and Jan (1994, 1997) concluded from their analyses that KdpA appears to contain two motifs with limited sequence similarity to the M1-H5-M2 hydrophobic domain of inwardly rectifying K^+ channels or the S5-H5-S6 region of voltage-gated K^+ channels. The GGG motif is part of the first H5-like motif within KdpA (Jan and Jan, 1994; see Fig. 1 *A*). While mutations of each of the two glycine residues of the GYG motif always produced a nonselective voltage-gated *Shaker* (Heginbotham et al., 1992, 1994: Nakamura et al., 1997) or inwardly rectifying K^+ channel (Slesinger et al., 1996), the substitution of the conserved tyrosine did not result in the total loss of K^+ selectivity (Heginbotham et al., 1994; Slesinger et al., 1996).

Doyle et al. (1998) were able to explain the structural basis of the K^+ selectivity after determination of the high-resolution structure of the KcsA K^+ channel of *Streptomyces lividans*, which shows a striking sequence similarity to all known K^+ channels. The narrow selectivity filter is formed by the K^+ signature sequence TVGYG of four identical subunits. The side chains of the VGYG sequence point away from the pore and undergo specific interactions with amino acids from the tilted pore helix. These interactions stabilize the filter, in which dehydrated K^+ ions fit precisely. To compensate for the energetic cost of dehydration, the carbonyl oxygen atoms of the signature sequence amino acids must replace the water oxygen atoms. In addition, the spacing of the four parts of the selectivity filter is such that it cannot accommodate the Na^+ ion of smaller radius (Doyle et al., 1998).

The structure of KcsA and the analysis of mutants from eukaryotic K^+ channels further revealed that there are other essential structural elements required to build a functional K^+ channel—for example, the inner and outer helices (M1, M2; S5, S6) that build the "teepee" architecture of the channel (Doyle et al., 1998). It is hard to imagine that those elements are present in the KdpA subunit of the KdpFABC complex. However, Durell et al. (1999) recently presented the hypothesis that there is an evolutionary relationship between K^+ channels and most bacterial K^+ symporters that were also included in the primary analysis by Jan and Jan (1994, 1997). According to their extensive sequence alignments (Durell et al., 1999) and three-dimensional computer modeling (Durell and Guy, 1999), a similar mechanism of K^+ selectivity seems to be present in three different symporter families and in K^+ channels. Their computer modeling postulated a related selectivity filter as well as M1 and M2 segments for the three K^+ symporter families (Durell and Guy, 1999).

A selectivity filter in the KdpA subunit of the KdpFABC complex, similar to that of K^+ channels, is supported by the results presented in this report and by earlier results of Buurman et al. (1995): 1) The substitution of the first glycine residue of the highly conserved GGG motif of KdpA that could be aligned with the GYG motif of K^+ channels (Jan and Jan, 1994, 1997) causes nonselectivity. Similar results were obtained after replacement of the analogous glycine residue of K^+ channels (Heginbotham et al., 1994; Slesinger et al., 1996). 2) About 50% of the substitutions that caused a reduced affinity are located in the two H5-like structures within KdpA (see Fig. 1 *A*). 3) Substitutions that obviously alter the ratio of K^+ and Rb^+ affinity, and have a high v_{max}, are all located within three periplasmic loops, while those in the cytoplasmic loop are less affected (Buurman et al., 1995). This cytoplasmic loop is a candidate for a K^+ occlusion site (Buurman et al., 1995).

Very recently, Durell et al. (2000) presented a model of KdpA, based solely on computer modeling, implying an evolutionary relation of KdpA, K^+ symporters, and K^+ channels. The topology of KdpA predicted by Durell et al. (2000), shown in Fig. 1 *B*, differs from that presented by Buurman et al. (1995), shown in Fig. 1 *A*. Durell et al. (2000) postulate two additional M1-H5-M2 motifs within a single KdpA subunit. In this altered model, the four clusters of K^+ affinity mutants lie within or close to the four P-loops. This model is consistent with the fourfold symmetry of the selectivity filter of K^+ channels (Doyle et al., 1998). However, the altered topology of KdpA is inconsistent in part with the experimental data derived from LacZ and PhoA fusions (Buurman et al., 1995). The cytoplasmic cluster of K^+ affinity mutants is rearranged to or near a P-loop region adjacent to the periplasm (Durell et al., 2000). Furthermore, Epstein and Davis (1970) reported that two different nonfunctional *kdpA* mutants can build a complex with wild-type activity when they are coexpressed in an *E.*

coli cell. This early observation suggests an oligomeric organization of the transport complex in which two KdpA subunits, each with two M1-H5-M2 motifs, form one K^+ selectivity filter.

A K^+ selectivity filter resembling that of K^+ channels in the KdpA subunit of the KdpFABC complex underlines the exceptional position of this ion pump within the P-ATPases (Altendorf and Epstein, 1996; Møller et al., 1996). In other P-ATPases examined so far, e.g., the Ca^{2+}-ATPase from sarcoplasmic reticulum (SR) and the Na^+,K^+-ATPase, the binding of the transported ions occurred in a site between the transmembrane helices 4, 5, and 6 of the same subunit that binds ATP and is phosphorylated during the reaction cycle. The Na^+ and K^+ or the Ca^{2+} ions, respectively, are supposed to be coordinated by conserved residues with oxygen-containing side chains (D, E, T, S) (Andersen and Vilsen, 1995, 1998; Nielsen et al., 1998; MacLennan et al., 1998; Jørgensen et al., 1998; Argüello and Lingrel, 1995), forming an occluded state with no access to the aqueous medium on either side of the membrane (Vilsen and Andersen, 1992a,b; Karlish et al., 1990, 1993; Nielsen et al., 1998). These residues are not conserved in their counterparts of the KdpB subunit, while all other important regions and residues (e.g., phosphorylation site, ATP-binding site, etc.) are present (Siebers and Altendorf, 1993; Møller et al., 1996; Axelsen and Palmgren, 1998). In the topological model for the KdpB subunit proposed by Siebers and Altendorf (1993), there is only one negatively charged residue in transmembrane helix 6 of KdpB; however, the topology of the C-terminal part of KdpB encompassing transmembrane helices 5 and 6 is still uncertain (Lutsenko and Kaplan, 1995). Furthermore, within the K^+ affinity mutants obtained by random mutagenesis, there was only one mutant (*kdpB57*) outside the KdpA subunit that had a transport rate comparable to that of the wild-type enzyme (Buurman et al., 1995). The other two mutants in *kdpB* are more consistent with allosteric mutants, while the one reported for *kdpC* encodes a truncated subunit and not a point mutant (M. Gassel, K. Altendorf, and W. Epstein, unpublished results).

In conclusion, the KdpFABC complex may represent a unique K^+ uptake system that evolved by the assembly of an energy-providing (probably truncated type I) P-ATPase and a K^+ channel or related K^+ symporter, that determines the K^+ specificity and harbors the K^+ transport channel. The additional small subunits KdpC and KdpF may have only structural functions.

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