The Conserved Dipole in Transmembrane Helix 5 of KdpB in the *Escherichia coli* KdpFABC P-Type ATPase Is Crucial for Coupling and the Electrogenic K^+-Translocation Step†

Doris Becker,‡ Klaus Fendler,§ Karlheinz Altendorf,‡ and Jörg-Christian Greie*,‡

Abteilung Mikrobiologie, Fachbereich Biologie/Chemie, Universität Osnabrück, 49069 Osnabrück, and Max Planck Institut für Biophysik, Max-von-Laue-Strasse 3, 60438 Frankfurt/Main, Germany

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ABSTRACT: The KdpFABC complex of *Escherichia coli*, a high-affinity K^+-uptake system, belongs to the group of P-type ATPases and is responsible for ATP-driven K^+ uptake in the case of K^+ limitation. Sequence alignments identified two conserved charged residues, D583 and K586, which are located at the center of transmembrane helix 5 (TM 5) of the catalytic KdpB subunit, and which are supposed to establish a dipole involved in energy coupling. Cells in which the two charges were eliminated or inverted by mutagenesis displayed a clearly slower growth rate with respect to wild-type cells under K^+-limiting conditions. Purified KdpFABC complexes from several K586 mutants and a D583K:K586D double mutant showed a reduced K^+-stimulated ATPase activity together with an increased resistance to orthovanadate. Upon reconstitution into liposomes, only the conservative K586R mutant was able to facilitate K^+ transport, whereas the elimination of the positive charge at position 586 as well as inverting the charges at positions 583 and 586 (D583K:K586D) led to an uncoupling of ATP hydrolysis and K^+ transport. Electrophysiological measurements with KdpFABC-containing proteoliposomes adsorbed to planar lipid bilayers revealed that in case of the D583K:K586D double mutant the characteristic K^+-independent electrogenic step within the reaction cycle is lacking, thereby clearly arguing for an exact positioning of the dipole for coupling within the functional enzyme complex. In addition, these findings strongly suggest that the dipole residues in KdpB are not directly responsible for the characteristic electrogenic reaction step of KdpFABC, which most likely occurs within the K^+-translocating KdpA subunit.

Ion-translocating P-type ATPases can be found in different organisms belonging to all kingdoms of life (*1*). These enzymes catalyze active ion transport across biological membranes at the expense of ATP, thereby forming a high-energy aspartyl phosphoryl enzyme intermediate (*2*). During the so-called post-Albers reaction cycle, P-type ATPases undergo large domain movements, which lead to different binding affinities for both ATP and the transported substrate (*3*). Detailed insights into the molecular transport mechanism were already obtained from structures of the sarcoplasmic Ca^{2+}-ATPase at different stages of catalysis (*4*−*8*). Most members of the P-type ATPase superfamily share numerous stretches of homologous amino acid sequences with respect to the sites of nucleotide binding and (de)phosphorylation and mainly consist of only one polypeptide, which forms both the catalytic and the ion-translocating domain. In contrast, the KdpFABC complex of *Escherichia coli* as a prokaryotic member of the P-type ATPase family is composed of four different subunits, with each subunit exhibiting its own function. This unique bacterial P-type ATPase is expressed under K^+ limitation or high osmolality in the medium. The active enzyme is composed of four membrane-bound subunits. The KdpB subunit (72 kDa) is the catalytic subunit mediating ATP hydrolysis and shares all homologies with other P-type ATPases (*9*, *10*). K^+ binding and transport are mediated by the KdpA subunit (59 kDa), which shows strong silimarities to K^+ channels of the MPM type such as KcsA, KtrB, and HKT (*8*, *11*, *12*). The KdpC subunit (21 kDa) was shown to be involved in the assembly of the complex (*13*), and moreover, it was recently demonstrated that the C-terminal part of the polypeptide is most likely also involved in catalysis (*3*). The small hydrophobic peptide KdpF (3 kDa) was shown not to be essential in vivo, but was demonstrated to stabilize the complex in vitro (*14*). For all other P-type ATPases, the sites of ATP hydrolysis and ion transport are located within the central subunit. In the case of the KdpFABC complex, nature has evolved a mechanism that allows energy transmission from one subunit to the other, i.e., from the site of ATP hydrolysis to the site of K^+ transport.

A first hint for specific residues that could be involved in this unique coupling mechanism was obtained by computational sequence comparison of KdpB with other P-type ATPases, which identified two charged residues (D583 and K586) located at the center of TM[1] 5 (compare Figure 1). These residues could not be identified within other P-type

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* To whom correspondence should be addressed. Phone: +49 541 9692809. Fax: +49 541 9692870. E-mail: greie@biologie.uni-osnabrueck.de.

‡ Universität Osnabrück.

§ Max Planck Institut für Biophysik.

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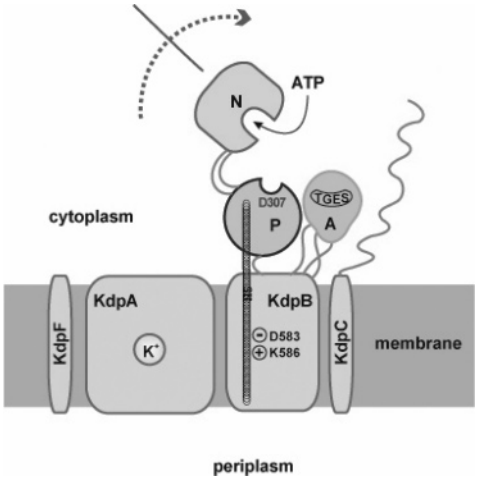

FIGURE 1: Subunit organization within the KdpFABC complex. Whereas K^+ translocation is mediated by the KdpA subunit, KdpB comprises all signature motifs of P-type ATPases with corresponding N-, P-, and A-domains as pictured. The essential phosphorylation site D307 and the conserved TGES motif in the P- and A-domains, respectively, are indicated as well as the conserved dipole residues D583 and K586 in the transmembrane helix 5.

ATPases and are conserved among all KdpB subunits sequenced so far. Mutational studies leading to conservative, polar or nonpolar amino acid replacements already revealed significant effects on the growth phenotype of the resulting mutant cells. In the case of D583, it could be shown that in isolated KdpFABC complexes the K^+-stimulated ATPase activity and ion transport rates are drastically reduced when the negative charge is eliminated (*15*, *16*). In addition, it was already demonstrated that the catalytic cycle of the KdpFABC complex comprises a K^+-independent electrogenic partial reaction step (*17*), which was most likely attributed to intrinsic charge movements within the transport complex, with the conserved dipole in TM 5 of KdpB being the best candidate for the displaced charges.

In this paper, we have further investigated the role of the two residues, especially with respect to the positive charge K586, focusing on the effect of different mutations on the growth phenotype, ATPase activity, and coupled ion transport in more detail. Moreover, BLM measurements with reconstituted KdpFABC complexes were carried out to analyze the effects on the ATP-dependent, but K^+-independent, electrogenic partial reaction. On the basis of our findings, we suggest that both the presence and the exact positioning of residues D583 and K586 play a crucial role in the coupling between KdpB and KdpA, but that the residue(s) which is/are responsible for the K^+-independent charge displacement during the catalytic cycle is/are most likely located within the KdpA subunit.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions. *E. coli* K12 strain TKW3205 (Δ*kdpFABC* Δ*atp thi rha lacZ nagA trkA405 trkD1*) (*19*) was transformed with either plasmid pSMC10His (*15*) or plasmid pGS4 (*3*) or derivatives thereof generated in this study. Resulting transformants carry the *kdpFABC* genes under control of the native *kdp* promoter and comprise either His_{10}-tagged KdpC or His_{14}-tagged KdpA subunits, respectively. Cells were grown in KML or minimal medium with different potassium concentrations according to ref *20* supplemented with ampicillin (50 μg/mL).

Construction of Plasmids. The construction of KdpB mutants with mutations at either position 583 or position 586 in the *kdpFABC* operon on plasmid pSMC10His is already described in ref *15*. To generate a double mutation (D583K:K586D) in the *kdpB* gene, standard two-step PCR was used to amplify a *Bam*HI/*Sal*I-flanked *kdpB* cassette with pSMC10His as the template, which was subsequently digested with *Bam*HI and *Sal*I and reintroduced into properly digested pSMC10His. Plasmid pGS4 encodes the *kdpFABC* genes together with 14 histidine codons at the 3′ end of the *kdpA* gene. A KdpA-Q116R mutant was generated in a pGS4 background by the standard two-step PCR technique by use of an *Eco*RI/*Nco*I-flanked *kdpA* cassette. To generate the KdpA-Q116R/KdpB-D583K:K586D triple mutant, plasmids pSMC10His(KdpB-D583K:K586D) and pGS4(KdpA-Q116R) were doubly digested with *Bam*HI and *Sal*I, and the resulting *kdpB* fragment from pSMC10His(KdpB-D583K:K586D) was ligated into pGS4(KdpA-Q116R), resulting in pGS4(KdpA-Q116R/KdpB-D583K:K586D). All mutations were confirmed by sequencing (Abteilung Botanik, Universität Osnabrück).

Complementation Assay of KdpFABC Wild-Type and Mutant Cells. Growth phenotypes were tested in liquid minimal medium containing an initial potassium concentration of 1 mM KCl. Cells were adapted stepwise from KML-rich medium to lower K^+ concentrations by 24 h of precultivation according to ref *20* and then freshly inoculated at an optical density (OD_{600}) of 0.1. Each strain was subsequently again cultivated for 24 h with monitoring the initial K^+ concentration, exponential growth rate and generation time, optical density, and residual K^+ concentration of the medium in the stationary growth phase.

Expression and Purification of the KdpFABC Complex. TKW3205 cells carrying plasmid pSMCHis10 or pGS4 derivatives were grown in minimal medium and stepwise adapted to limited K^+ concentrations by the successive reduction of KCl according to refs *9* and *15* with the following cultivation pattern. Wild-type cells were precultured overnight in K0.5 medium and finally also grown in K0 medium. Upon entering the stationary growth phase due to K^+ depletion of the medium, *kdpFABC* gene expression was induced by the addition of 45 μM KCl. KdpB-K586, KdpB-D583K:K586D, and KdpA-Q116R/KdpB-D583K:K586D mutants were precultured overnight in K5 medium and finally grown in K4 medium with the addition of 0.5 mM KCl to induce *kdpFABC* expression. KdpA-Q116R mutant cells were precultured overnight in K2 medium and finally grown in K1 medium with the addition of 0.1 mM KCl. Cells were harvested at an optical density (OD_{600}) of around 1.0 by centrifugation (10000*g*, 4 °C, 15 min) and suspended in 50 mM Tris−HCl, pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 10% (v/v) glycerol, 0.5 mM PMSF. Inverted membrane vesicles were prepared by passage through a cell fractionator (Basic Z cell disrupter, Constant Systems Ltd.). Cell debris was removed by low-speed centrifugation

[1] Abbreviations: BLMs, planar lipid bilayers ("black lipid membranes"); DDM, dodecyl maltoside; FEP, flame emission photometry; NTA, nitrilotriacetic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TM helix, transmembrane helix.

(10000*g*, 4 °C, 20 min), and membranes were harvested by high-speed centrifugation (150000*g*, 4 °C, 1.5 h). The membrane pellet was suspended in 50 mM Tris−HCl, pH 7.5, 10 mM $MgCl_2$, 10% (v/v) glycerol, 0.5 mM PMSF, adjusted to a protein concentration of 5 mg/mL, and solubilized by the addition of 1% (w/v) DDM for 60 min on ice with gentle stirring. Solubilized proteins were separated by centrifugation (150000*g*, 4 °C, 1.5 h), and the KdpFABC complexes were subsequently purified by applying the supernatant to affinity chromatography. Ni^{2+}-NTA agarose (Qiagen) was equilibrated with 50 mM Tris−HCl, pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl, 10% (v/v) glycerol, 0.5 mM PMSF, 0.2% (w/v) DDM, 10 mM imidazole. His-tagged KdpFABC complexes were bound to the resin by applying 25 mL of supernatant/mL of equilibrated resin with gentle shaking for 1 h on ice. The complexed resin was then transferred to a glass column and developed on an Äkta-FPLC instrument (Amersham Biosciences) at a flow rate of 0.5 mL/min. The KdpFABC complex was eluted with 130 mM imidazole, and the corresponding fractions were concentrated by use of Amicon Ultra-4 Ultracell 30K centrifugal filter devices (Millipore) at 3000*g* and 4 °C for subsequent size exclusion chromatography. Samples were loaded onto a 10/30 Superdex-S200 column (Amersham Biosciences) equilibrated with 50 mM Tris−HCl, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 0.5 mM PMSF, 0.2% (w/v) DDM and developed at a flow rate of 0.5 mL/min. Protein-containing peak fractions were again pooled and concentrated as described above.

Reconstitution of KdpFABC Complexes. Preformed liposomes for the reconstitution of KdpFABC were prepared according to ref *21* with the following modifications. *E. coli* lipids (10 mg/mL chloroform solution, Avanti Polar Lipids) were evaporated under a gentle stream of argon and redissolved in a concentration of 5 mg/mL in detergent buffer. Preformed liposomes were finally dialyzed against 15 mM Tris−HCl, pH 7.5, 2 mM $MgSO_4$ and sized through 0.4 μm polycarbonate membranes (Whatman). For reconstitution, liposomes were partially solubilized by the addition of 0.3% (v/v) Triton X-100 with gentle mixing for 5 min. Subsequently, 150 μg of purified protein was added to the mixed micelles at a protein:lipid ratio of 1:20, and the sample was adjusted to a standard volume of 600 μL with buffer and kept at room temperature for 30 min under gentle agitation. In the case of subsequent BLM measurements, 15 mM Tris−HCl, pH 7.5, was used as the buffer. If proteoliposomes were taken for fluorometric transport measurements, 15 mM Tris−HCl, pH 7.5, 50 mM KCl was used. Detergent was removed by the successive addition of Bio-Beads SM-2 (Biorad) pretreated as described by Holloway (*22*). After reconstitution, the supernatant containing the proteoliposomes (lipid concentration 5 mg/mL with 0.25 mg/mL protein) was separated from the Bio-Beads, and the proteoliposomes were washed twice in the corresponding buffer (100000*g*, 4 °C, 45 min) to remove residual protein. Prior to BLM or fluorometric analyses, proteoliposomes were sonicated for 10 s by use of a sonifier bath (Branson B-220, 50/60 Hz).

Fluorometric Transport Measurements. The flourometric transport measurements with reconstituted KdpFABC complexes were carried out in 1 mL of 15 mM Tris−HCl, pH 7.5, 50 mM KCl, 2 mM $MgCl_2$ as described (*10*, *18*) by use of an SLM-Aminco 8100 spectrofluorometer (SLM-Aminco) at an excitation wavelength of 650 nm, an emission wavelength of 675 nm, a monochromator bandwidth of 4 nm, and an integration time of 1 s. The reaction mixture containing 10 μL of proteoliposomes was supplied with 1 μM $DiSC_3(5)$. ATP (1 mM) was added to the sample to start the transport reaction. Orthovanadate (100 μM) served as a specific P-type ATPase inhibitor, whereas 1 μM valinomycin was used as a K^+-specific uncoupling ionophore.

BLM Analysis. The preparation of planar bilayers, the electrical recording instrumentation, photolysis of caged ATP, the buffer composition, and measurements of conductivity were essentially performed and utilized as described (*10*, *17*, *18*). Planar lipid bilayers were formed in a thermostated Teflon cuvette, with the two cuvette compartments filled with 1.5 mL of electrolyte containing 50 mM Tris−HCl, pH 7.5, 2 mM $MgSO_4$, 1 mM DTT. A 20 μL portion of KdpFA(Q116R)BC-containing proteoliposomes or 25 μL of KdpFA(Q116R)B(D583K:K586D)C-containing proteoliposomes was added to one side of the compartment and allowed to adsorb to the planar membrane by gentle stirring of the solution for 1 h. Caged ATP (30−60 μM) was used in the experiments, and the ATP was photolytically released by use of an excimer laser flash at 308 nm. To ensure a virtually K^+-free buffer system, flame emission photometry (FEP) was used to determine residual potassium concentrations.

ATPase Activity Assay. ATPase activities of purified KdpFABC complexes were determined in triplicate using the 96-well microtiter plate assay described in ref *23* with the modifications described in ref *10*. A 0.5 μg portion of purified protein was routinely used for a single measurement.

Standard Assays. Protein concentrations were assayed with the bicinchoninic acid assay used as recommended by the supplier (Pierce) with bovine serum albumin as the standard or by the method of Hartree (*5*). Proteins were separated by SDS−PAGE using 16.5% T/6% C separating gels together with 4% T/3% C stacking gels (*25*) and stained with Coomassie Brillant Blue G250. Concentrations of medium K^+ were measured by FEP using an Eppendorf ELEX 6361 flame photometer in a buffer containing 5 mM CsCl, 1.5% (w/v) TCA.

RESULTS

Complementation Analysis of KdpB TM 5 Mutants. Previous experiments have already shown that mutations of the conserved dipole D583/K586 in TM 5 of KdpB have strong effects on the growth yield on agar plates under potassium limitation (*15*). These growth effects were now analyzed in a more quantitative manner with respect to K_M and v_{max} of the resulting KdpFABC complexes. In the case of a v_{max} effect, a slower growth rate is expected under conditions of K^+ limitation, with the stationary phase growth yield and final medium K^+ concentration remaining unaffected with respect to those of the wild-type cells. In turn, for a K_M effect, the growth rate is supposed to be unaffected, but the cells should enter the stationary growth phase at a lower optical density and higher external K^+ concentrations. The growth phenotype of *E. coli* TKW3205 strains harboring either wild-type or mutant KdpFABC complexes was tested in liquid minimal medium with limiting potassium concentrations of

Table 1: Complementation Analysis of KdpB-D583/K586 Exchanges[a]

E. coli TKW3205 harboring	μ (h^{-1})	final OD (600 nm)	residual K^+ concn (mM)
KdpFABC wild type	0.52	2.08	0.8
D583E	0.52	2.13	0.4
D583K	0.17	1.90	0.5
D583A	0.14	1.79	0.5
K586R	0.52	2.03	0.5
K586D	0.35	1.99	0.5
K586A	0.38	1.99	0.5
D583K:K586D	0.22	1.96	0.5

[a] *E. coli* strain TKW3205 (Δ*kdpFABC*) was complemented with plasmids coding for wild-type and mutant KdpFABC complexes with exchanges of the dipole residues D583 and K586 in KdpB as indicated. Cell growth was tested in liquid minimal medium supplemented with 1 mM KCl. The growth rate of the log phase, the optical density of the stationary phase, and the residual potassium concentration in the stationary growth phase medium were recorded.

1 mM (Table 1). To exclude artificial growth effects possibly caused by the cultivation history or traces of K^+ from the starting culture, cells were adapted stepwise from KML-rich medium to minimal medium with 1 mM K^+ as described in the Experimental Procedures. To ensure that the effects on the growth phenotype are related to the mutations generated and not due to a possible disintegration of mutant KdpFABC complexes, all enzymes were shown to be present in equal amounts in the membrane by SDS−PAGE (data not shown). The amount of K^+ which has not been taken up by the cells during growth was analyzed by FEP as residual potassium concentration in the medium of the stationary growth phase. All strains tested exhibited a typical growth curve under these conditions with almost identical optical densities of around 2.0 in the stationary growth phase, arguing in favor of the notion that the mutations do not affect the final K^+ uptake level. This notion is further supported by the observation that, in a similar experiment, a KdpA mutant (KdpA-Q116R), for which a significantly reduced affinity for K^+ is reported (*26*), already enters the stationary growth phase at an optical density of around 1.5 (data not shown). All of the mutants tested exhibited a residual K^+ concentration in the medium of ∼0.5 mM in the stationary growth phase, which is also quite close to that of the wild-type strain (0.8 mM). In contrast, the growth rates of the different mutants could be subdivided into three different categories. Whereas the wild type and the conservative amino acid exchanges D583E and K586R led to an identical growth rate of 0.52 h^{-1}, the elimination of the negative charge at position 583 (mutations D583K, D583A, and D583K:K586D) led to drastically reduced growth rates. Substitutions of the positive charge at position 586 had much less severe effects, resulting in growth rates of around 0.36 h^{-1}. First, this observation emphasizes the dominant role of the negative charge at position 583, which can be neither replaced nonconservatively (D583K, D583A) nor moved to position 586 (D583K:K586D). Second, these findings strongly suggest a v_{max} effect caused by the mutations rather than an alteration of K_M. Since all strains entered the stationary phase at the same optical density and external K^+ concentration while exhibiting altered growth rates, this reflects a decreased K^+ uptake efficiency during K^+-limited growth conditions.

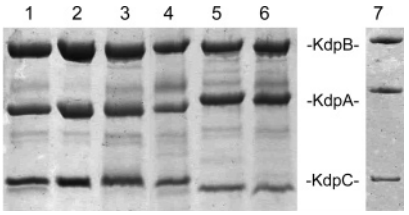

FIGURE 2: Isolated wild-type and mutant KdpFABC complexes. KdpFABC complexes were isolated as described, subjected to SDS−PAGE, and stained with Coomassie Brilliant Blue. Key: lanes 1−6, 5 μg of isolated protein; lane 7, 3 μg of isolated protein; lane 1, wild-type KdpFABC; lane 2, KdpFAB(K586R)C; lane 3, KdpFAB(K586D)C; lane 4, KdpFAB(D583K:K586D)C; lane 5, KdpFA(Q116R)BC; lane 6, KdpFA(Q116R)B(D583K:K586D)C; lane 7, KdpFAB(K586A)C. The higher apparent molecular weights of the KdpA subunits in lanes 5 and 6 and the KdpC subunits in lanes 1−4 and 7 are due to the presence of the additional His tags.

ATPase Activities of Purified KdpFABC Complexes. To gain more insights into the catalytic and transport properties of the enzyme, isolated KdpFABC complexes were tested for differences in ATPase and K^+-transport activity. All complexes were isolated from an *atp* deletion strain lacking the genes coding for F-ATPase to avoid contaminating background activity. All isolated KdpFABC complexes were revealed to be sufficiently pure and stoichiometric (Figure 2), thus demonstrating structural integrity. First, wild-type and mutant KdpFABC complexes were tested for K^+-stimulated ATP hydrolysis, a key feature of this P-type ATPase. In the case of D583 it has already been shown that only the conservative exchange D583E behaved like the wild type and that, in the case of nonconservative exchanges, ATPase activity is no longer stimulated by K^+ (*15*, *16*). Corresponding experimental data on residue K586 are still lacking except for a K586R mutant, which behaved like the wild type (*15*). Thus, all K586 mutants were tested against wild-type KdpFABC with respect to ATP hydrolysis in the presence of stimulating 2 mM KCl (Figure 3A). For a direct comparison, the ATP hydrolysis activity of wild-type KdpFABC was set to 100%. Isolated KdpFABC complexes were then assayed for ATP hydrolysis gradually stimulated by K^+ (Figure 3B). ATPase activity in the presence of 2 mM KCl was again set to 100%. From all mutants tested, only the conservative K586R mutant exhibited comparable K^+-stimulated rates of ATP hydrolysis with respect to the wild type, whereas mutants K586A and K586D displayed only a residual hydrolytic activity, which was, however, still stimulated by increasing K^+ concentrations. Interestingly, the double mutation D583K/K586D led to an intermediate ATP hydrolysis rate of about 40% compared to that of wild-type KdpFABC complexes, which was found not to be stimulated by KCl. As a control for the electrophysiological analyses, the KdpA-Q116R mutant and the KdpA-Q116R/KdpBD583K:K586D triple mutant were also included in the assay, both displaying also intermediate rates of ATP hydrolysis like the D583K/K586D mutant. In contrast, only the KdpA-Q116R mutant was stimulated by KCl (data not shown). In summary, all mutants except for D583K/K586D were stimulated by K^+, with K586A and K586D showing only residual rates of ATP hydrolysis.

Another key feature of the KdpFABC complex as a P-type ATPase is its inhibition by orthovanadate in the E2 state of the reaction cycle (*20*, *27*). The ATPase activities of all mutants except for K586A were shown to be sensitive to

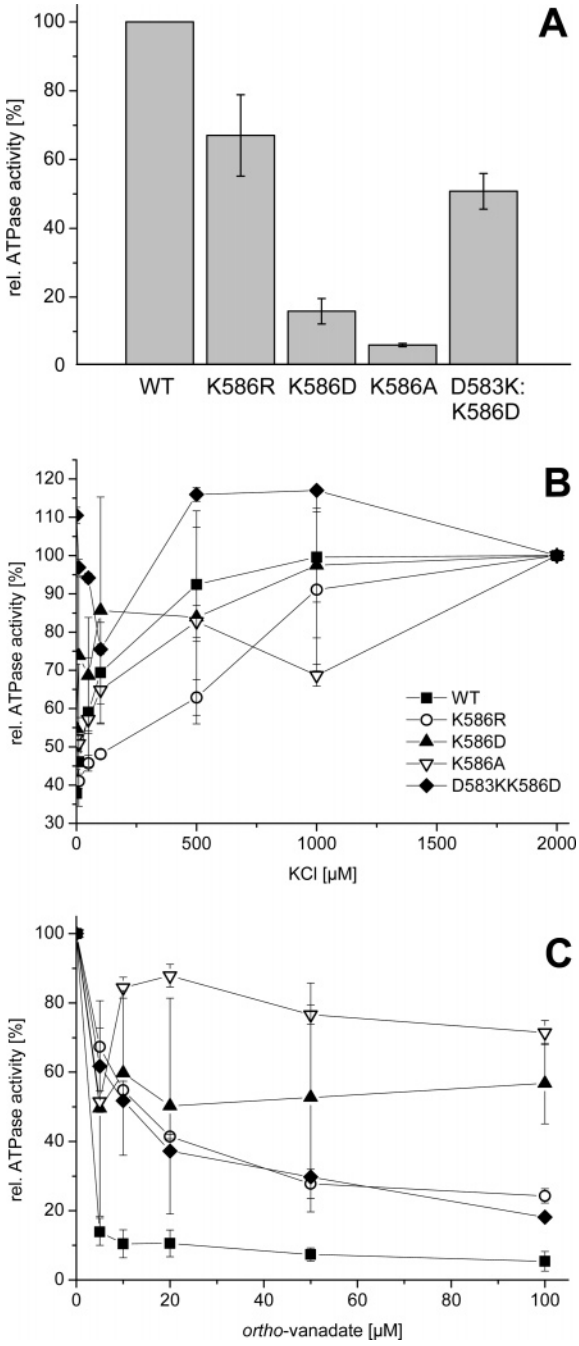

FIGURE 3: K^+-stimulated and orthovanadate-inhibited ATPase activity of isolated wild-type and mutant KdpFABC complexes. Amino acid exchanges at positions D583 and K586 in KdpB were tested as indicated. WT = wild type. Enzymes were isolated in 0.2% (w/v) dodecyl maltoside. (A) Relative maximal K^+-stimulated ATPase activities in the presence of 2 mM KCl. The ATP hydrolysis activity of wild-type KdpFABC was 1.16 μmol mg^{-1} min^{-1} and set to 100%. (B) K^+ stimulation of ATP hydrolysis. Maximal ATPase activies in the presence of 2 mM KCl were set to 100% and correspond to the values shown in (A). (C) Inhibition of ATP hydrolysis activity by orthovanadate. Noninhibited ATPase activities in the presence of 2 mM KCl were set to 100% and correspond to the values shown in (A). Use of symbols as in (B). All measurements were done in triplicate. The apparently large error bars of, for example, K586D in panels B and C are due to the low overall activity of the corresponding enzyme complex, which also enlarges the systemic error when set to 100%.

orthovanadate (Figure 3C), although less pronounced as observed for the wild type. This increased resistance toward the inhibitor might indicate that the equilibrium of the E1/E2 transition in the reaction cycle of the resulting KdpFABC complexes is shifted more to the E1-P state. The K586A mutant again displayed only a residual ATPase activity, which could not be further inhibited by orthovanadate. The same was observed, although to a lesser extent, for the K586D mutant, in which the overall hydrolytic activity is near the detection limit of the inhibition assay.

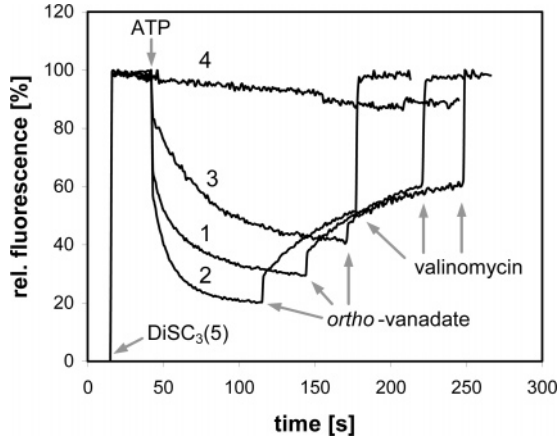

FIGURE 4: Electrogenic K^+ transport of reconstituted wild-type and mutant KdpFABC complexes. Flourometric measurements were carried out in the presence of 50 mM KCl by use of 1 μM $DiSC_3$-(5). The transport reaction was started by the addition of 1 mM ATP at the time indicated. The additions of 100 μM orthovanadate and 1 μM valinomycin are also indicated by arrows. The maximal fluorescence upon $DiSC_3$(5) addition was set to 100%. Four different fluorescence traces are shown: 1, wild-type KdpFABC; 2, KdpFA(Q116R)BC; 3, KdpFAB(K586R)C; 4, no detectable transport activity in the case of KdpFAB(K586D)C, KdpFAB-(K586A)C, KdpFAB(D583K:K586D)C, and KdpFA(Q116R)B-(D583K:K586D)C.

Transport Activities of Reconstituted KdpFABC Complexes. Electrogenic K^+ transport activities were analyzed with wild-type and mutant KdpFABC complexes reconstituted in liposomes to examine whether the ATP hydrolysis activity observed in the ATPase assay is coupled to active K^+ transport. From the analysis of mutant KdpFABC complexes with amino acid exchanges at position D583 it is already known that even the conservative exchange of this residue uncouples ATP hydrolysis from substrate translocation (*15*). Proteoliposomes were loaded with 50 mM KCl, and ATP-driven export of K^+ out of the proteoliposome by inside-out reconstituted KdpFABC complexes was started by the addition of 1 mM ATP. The translocation of K^+ imposes a membrane potential which is readily detected by the potential-sensitive fluorophor $DiSC_3$(5) (*28*) (Figure 4, curve 1). The specific inhibition of transport activity by the addition of orthovanadate served as a control for active transport by the P-type ATPase, whereas the final addition of valinomycin served as a control of K^+ specificity of the signal. Furthermore, similar relaxation rates upon the addition of orthovanadate clearly indicate that the differences in the fluorescence traces are due to transport activities and not due to differences in the overall conductance of the vesicles. From all of the mutants tested, only the exchange Q116R in KdpA affecting K^+ affinity exhibited active transport rates comparable to that of the the wild type (curve 2), which argues in favor of the notion that mutations in the selectivity filter region of KdpA do not affect the energy transmission between KdpA and KdpB. In contrast, even the K586R mutant, which is still carrying the positive charge at this

residue, shows a significantly reduced transport activity of about 50% from that of the wild type (curve 3). Considering the wild-type-like ATP hydrolysis rates measured above, these findings support the notion that even conservative exchanges at this position at least partially uncouple ATP hydrolysis from K^+ translocation as in the case of D583 (*15*). In contrast, all mutants in which the positive charge at position 586 (K586D, K586A) is eliminated and even the mutants with restored but inverted charges (D583K:K586D, KdpA-Q116R/KdpB-D583K:K586D) exhibited no detectable K^+ transport in this assay (curve 4). These results not only further underline the importance of the mere presence of a positive charge at residue 586 in KdpB, but also emphasize the mandatory exact positioning of the side chain carrying the positive charge to functionally couple ATP hydrolysis to K^+ translocation.

Analysis of the Substrate-Independent Electrogenic Reaction Step of KdpFABC. One unique feature of the KdpFABC complex among the P-type ATPases is the presence of a substrate-independent electrogenic reaction step (*17*, *18*). This reaction step has been initially attributed to a movement or alteration of the accessibility of the charges established by the conserved D583/K586 dipole within TM 5 of KdpB. However, experimental evidence for this assumption is still lacking. Therefore, the KdpA-Q116R/KdpB-D583K:K586D triple mutant KdpFABC complexes were reconstituted in liposomes and adsorbed to planar lipid bilayers. The KdpA-Q116R KdpFABC complex was used as a wild-type-like control. By use of the KdpA-Q116R background with a decreased affinity for K^+ (*26*), any artifacts due to possible intrinsic K^+ impurities in the buffer solutions on the substrate-independent reaction step are effectively prevented (*17*, *29*). As shown above, the KdpA-Q116R KdpFABC complex exhibits substantial K^+-stimulated ATPase activity as well as wild-type-like K^+ translocation rates and, thus, constitutes a suitable wild-type-like reference. In this background, the analysis of the D583K:K586D exchange, in which the dipole is still present but inverted, provides information not only about the effect of the mere presence or absence of the charged residues as single exchange mutants would do, but also with respect to alterations in the positioning and/or orientation of the dipole in the membrane.

Transient or stationary electrical currents generated during the reaction cycle of the KdpFABC complex upon energization of transport by the release of caged ATP were measured in the absence and presence of K^+. Prior to the analysis, all buffers were checked by FEP for traces of K^+ impurities, which were found to be at a concentration of 2 μM, far below the K^+ affinity of the KdpA-Q116R KdpFABC complex, which is reported to be 6 mM (*26*). Figure 5A shows the electrical current traces of the reconstituted KdpA-Q116R KdpFABC complexes in proteoliposomes adsorbed on a lipid bilayer. In the case of the presence of 10 mM KCl together with the uncoupler monensin, there is a rather strong current of about 0.04 nA, which is only slowly decaying and related to K^+ transport from the bulk solution into the lumen of the adsorbed proteoliposomes. Without the K^+-uncoupling agent, the decay of the stationary current was found to be much faster due to the opposing electrochemical potential imposed on the membrane by the electrogenic transport of potassium ions. In contrast, in the case of zero potassium, i.e., the absence of transport substrate, there is only a short and transient current of 0.05 nA originating from intrinsic protein charge movements. Together, these findings are in good accord with data already published (*17*, *18*, *29*). In contrast, in the case of the reconstituted KdpA-Q116R/KdpB-D583K:K586D KdpFABC complex, no electrical current could be observed, neither in the absence nor in the presence of K^+ (Figure 5B). The absence of even the transient current demonstrates that even a mere inversion of the charged residues within the dipole fails to promote active electrogenic K^+ transport. Furthermore, it shows that the dipole within TM 5 of KdpB is indeed responsible for the electrogenic partial reaction step observed before (*15*, *16*). However, since the membrane-intrinsic charges are still present in the KdpA-Q116R/KdpB-D583K:K586D mutant but only inverted, the dipole residues cannot be held directly responsible for the observed charge displacements.

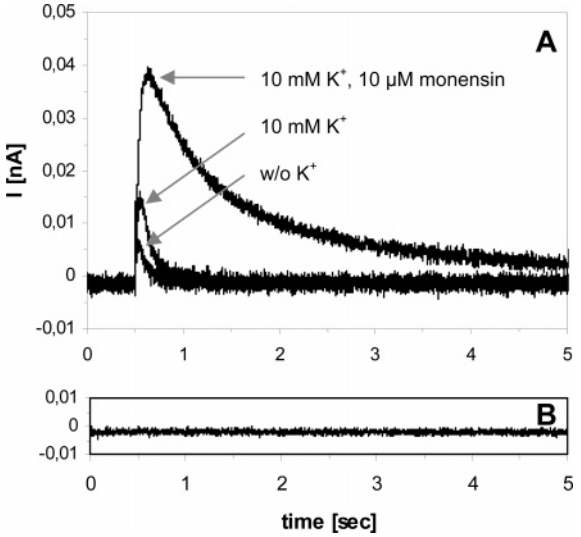

FIGURE 5: BLM analysis of wild-type and mutant KdpFABC complexes. KdpFABC complexes were reconstituted into liposomes and adsorbed to planar lipid bilayers. (A) Currents generated by the KdpA-Q116R KdpFABC complex after the photolytic release of 300 μM caged ATP at $t = 0.5$ s. Current traces were recorded prior to the addition of K^+ (without K^+), after the addition of K^+ (10 mM K^+), and in the presence of 10 mM K^+ together with 10 μM monensin as an uncoupler. (B) No currents could be observed with the KdpA-Q116R/KdpB-D583K:K586D KdpFABC complex, either with or without potassium.

DISCUSSION

P-type ATPases usually consist of only one central subunit, which mediates both ATP hydrolysis and substrate transport. The sites of ATP binding and hydrolysis are located in the cytosolic domains, whereas the sites for ion binding and translocation are found within the transmembrane portion of the enzyme. Thus, to generate and maintain a functional reaction cycle, the cytosolic domains and the transmembrane helices have to work in concert like a mechanical pump. In contrast to other P-type ATPases, the prokaryotic KdpFABC complex of *E. coli* is composed of four single subunits, and moreover, the sites of ATP hydrolysis (KdpB) and substrate transport (KdpA) are spatially separated on two different subunits. Thus, the enzyme has evolved a mechanism in which the energy derived from ATP hydrolysis in KdpB is efficiently transmitted to the site of K^+ translocation in the KdpA subunit. In the case of the well-studied Ca^{2+}-ATPase of the sarcoplasmic reticulum, a distinct set of residues

located in the transmembrane helices 4−6 and 8 are held responsible for the binding and translocation of the Ca^{2+} ions (*30*−*32*). For KdpB, there is yet no such clear evidence that residues of the transmembrane domain are directly involved in K^+ binding and translocation, which is mediated by KdpA. However, earlier studies identified two conserved residues within TM 5 of KdpB (D583 and K586), which most likely form a dipole within the membrane, and which do not have any homologous counterparts in other P-type ATPases. These findings suggest a special role of these residues for the energy coupling between the KdpB and KdpA subunits. Strong effects on KdpFABC ATPase and K^+-transport activity have already been shown for the D583 residue (*15*, *16*). To further elucidate whether both dipole-forming residues are involved in energy coupling, this study concentrated on residue K586. Furthermore, it was tested whether the exact orientation of the dipole is critical to maintain a coupled enzyme complex. Therefore, the double mutant D583K:K586D was analyzed, in which only the positions of both charges were exchanged.

The complementation analysis revealed that all mutants led to the formation of a functional KdpFABC complex properly assembled in the membrane. All constructs tested comprised an almost identical stationary phase optical density after growth for 24 h together with almost identical external K^+ concentrations, thereby strongly arguing in favor of the notion that exchanges of the dipole residues have an effect on the turnover number of the transport complex, but not on the affinity, the latter determining the final amount of potassium which can be taken up by the cells. This is further supported by the decreased growth rates in the case of nonconservative exchanges. In this context it should be mentioned that exchanges of the D583 residue had much more severe effects on doubling time than in the case of alterations at position K586, which is in accord with former studies already pointing toward the notion that the negative charge has a dominant function within the dipole (*15*, *16*). However, the finding that alterations of the dipole charges result in v_{max} effects, but do not affect the K_M value of the transport complex, is in good agreement with a coupling role of these residues rather than these residues being directly involved in the establishment of the K^+ binding site.

Only the isolated KdpFABC complexes comprising the conservative K586R exchange displayed K^+-stimulated ATPase activities comparable to that of the wild type, which is in good agreement with the growth experiments. This effect has already been shown for exchanges of residue D583 (*15*, *16*). In contrast, nonconservative exchanges of K586 led to a significant drop in both growth rate and ATPase activity, although the latter was still inducible with K^+. This argues in favor of the fact that indeed the catalytic turnover is affected, but not the signal transmission from the K^+ binding site to the sites of ATP hydrolysis. The inversion of both charges in the double mutant D583K:K586D led to a less severe drop in ATPase activity, but this activity was not stimulated by K^+ anymore. From these results two conclusions can be drawn. First, a regulatory K^+ binding site is likely located in the KdpA subunit and is most probably identical with the K^+ binding site responsible for substrate translocation. Second, the transmission of energy from KdpB to KdpA on one hand and the K^+ stimulus from KdpA to KdpB on the other are both connected to the presence of a functional dipole, but most likely in two distinct ways. Whereas the single exchanges K586A and K586D still comprise K^+ stimulation of only a very residual ATPase activity and, thus, K^+ signaling from KdpB to KdpA, the inversion of the dipole charges (D583K:K586D) led to a significantly higher ATP hydrolysis rate but without the stimulation by K^+, which is loss in the transmission of the K^+ signal. It is tempting to speculate that this K^+ signal transmission is more related to residue D583, since a similar phenotype of higher ATPase activity together with the loss of K^+ stimulation has previously been observed in the case of a D583A exchange (*15*). Further support for the fact that the regulatory K^+ binding site is identical to the one responsible for K^+ translocation can be drawn from recent mutagenesis studies of the KdpA subunit, in which isolated KdpFABC complexes with amino acid exchanges in the potential selectivity filter regions of KdpA also lack K^+ stimulation of ATPase activity (*8*, *19*, *29*). However, all of the mutants exhibited a reduced rate of ATP hydrolysis with respect to wild-type KdpFABC, but displayed significantly higher resistances toward the specific inhibitor orthovanadate. Since orthovanadate is known to inhibit P-type ATPases in the E2 state of the reaction cycle, these findings suggest that alterations in the transmembrane dipole might have an effect on the E1-P/E2-P transition preceding the E2 state, thereby shifting the catalytic equilibrium away from the E2 conformation.

However, the ATPase activity analysis revealed that both the conservative K586R exchange and the D583K:K586D double mutant exhibit significant rates of ATP hydrolysis, with the K586R mutant being comparable to wild-type KdpFABC. In contrast, from the K^+ transport analysis it became clear that even the conservative exchange from K to R at position 586 led to a significantly reduced K^+ transport activity, whereas the double mutant, in which both charges are still present but shifted with respect to their positioning within TM 5 of KdpB, displayed no detectable K^+ transport like the other mutants in which the positive charge is eliminated. On the basis of previous mutagenesis studies on residue D583, it has become apparent that exchanges of this residue result in only marginal transport activity of isolated KdpFABC complexes (*15*, *16*). These findings clearly argue in favor of the notion that the exact positioning of the charges is mandatory for energy coupling within the assembled enzyme. The mere exchange from the native K to R already resulted in a loss of about 50% of translocation activity, thereby indicating that the spatial arrangement of the dipole residues within the transmembrane coupling domain is delicately balanced and that coupling does not rely on the mere presence of a salt bridge formed by the dipole residues.

Despite the fact that the two charges D583 and K586 are shown to be involved in ion translocation, it is still unclear how the coupling of energy and K^+ transport is mediated in the KdpFABC complex. In contrast to other P-type ATPases such as the Ca^{2+}-, the Na^+,K^+-, and the H^+-ATPase, the KdpFABC complex has to couple energy and transport across two different subunits. Furthermore, in KdpFABC, no counterion is so far known to be translocated in exchange with K^+ like in the other P-type ATPases. Thus, although all of these enzymes belong to the family of P-type ATPases with respect to the generation of energy, the mechanism by which the transport substrate is translocated through the

KdpFABC complex is distinctly different. In this context, earlier studies on the electrogenicity of reconstituted KdpFABC in black lipid membranes demonstrated that the transporter exhibited a transient current upon the activation of transport even in the absence of K^+, which was attributed to an electrogenic conformational transition, which at least alters the *cis*/*trans* accessibility of intrinsic charges (*17*, *18*, *29*). Localization of these charges has hitherto not been possible, although the conserved D583/K586 residues within TM 5 of KdpB were initially supposed to be good candidates because of the strong effects of corresponding mutations on ATPase activity and transport. In this study, we clearly demonstrated that this unique transient electrogenic translocation step is strictly connected to the presence of the dipole residues at their native positions. The double mutant D583K:K586D in the KdpA-Q116R background did not show any electrogenic transport activity, neither in the presence nor in the absence of substrate. In particular, the absence of even the transient substrate-independent current further underlines the conclusion that not the mere presence of a dipole at a corresponding position in TM 5 of KdpB is responsible for coupling, but that the charged residues involved in energy transmission from KdpB to KdpA have to be in an exact position with respect to their spatial arrangement. In addition, these results suggest that in contrast to our earlier assumption (*17*) the dipole D583/K586 is most probably not identical with the charges displaced during the K^+-independent conformational transition. This view is further supported by the notion that a direct electrostatic action of the dipole residues on the K^+ bound and translocated by the KdpA subunit can most likely be ruled out, since the potassium ions are supposed to be shielded by the surrounding transmembrane helices of KdpA within the putative filter region (*8*, *11*, *12*, *19*). Together with the lack of K^+-stimulated ATPase activity, our findings demonstrate that, in the D583K:K586D double mutant, the KdpB/ KdpA communication is severely hindered in both the direction of K^+ stimulation from KdpA to KdpB and the direction of energy transmission from KdpB to the catalytic charge displacement in KdpA. Although the understanding of the coupling mechanism in this P-type ATPase is just at the beginning, it is tempting to speculate about the mechanism of uncoupling in the D583K:K586D double mutant. On the basis of the observed phenotype of a persisting K^+-insensitive ATPase activity together with the lack of transport, orthovanadate sensitivity, and electrogenicity, one possible hypothesis could be that the complex is stabilized in the E1-P conformation, where it rapidly hydrolyzes ATP. This scenario would imply that the dipole residues control the E1-P/E2-P conformational transition and, thus, stabilize the E1-P state of the enzyme.

However, further charged residues are supposed to be involved in energy transmission in this unique transport system, especially at the so far noncharacterized KdpA/KdpB interface. On the basis of the electrostatic interaction model, the conserved residue R493 in KdpA is yet another possible coupling partner in this scenario. R493 is located on one of the helices, which, according to the current model of KdpA, are not part of the modules forming the putative K^+ channel (*12*). Most interestingly, KdpFABC complexes in which the arginine residue is eliminated resulted in a complete nonfunctional phenotype in our analyses. The corresponding *E. coli* cells did not grow under K^+-limiting conditions, nor did the isolated enzyme complexes exhibit any ATP hydrolysis or K^+ transport activity (data not shown). On the one hand, this further demonstrates that the K^+ binding site responsible for K^+-stimulated ATP hydrolysis is located within KdpA. On the other hand, this residue is most likely the interaction partner for the dipole residues in KdpB during functional coupling. A more detailed investigation of this interaction is, therefore, the current primary target for further mutagenesis and analysis of the KdpFABC complex in our laboratory.

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