Single Amino Acid Substitution in the Putative Transmembrane Helix V in KdpB of the KdpFABC Complex of *Escherichia coli* Uncouples ATPase Activity and Ion Transport†

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ABSTRACT: The KdpFABC complex, found in a variety of prokaryotes, is an emergency potassium uptake system which belongs to the family of P-type ATPases. Site-directed mutagenesis of the charged residues aspartate 583 and lysine 586 in the putative transmembrane helix V of subunit KdpB revealed that these charges are involved in the coupling of ATP hydrolysis and ion translocation. Phenotypic characterization of KdpFABC derivatives carrying alterations at either D583 or K586 demonstrated that only restoration of charges at these positions allowed growth on low potassium concentrations. Substitutions, which eliminated the negative charge at position 583, did not allow growth below 15 mM potassium on solid media. In contrast, substitutions of the positive charge at position 586 allowed growth down to 0.3 mM potassium. Purified KdpFABC complexes carrying these substitutions exhibited ATPase activity, which was, however, found to be comparatively resistant to *o*-vanadate. Furthermore, elimination of the charges led to a complete loss of ion-stimulated ATPase activity, though the rate of hydrolysis was comparable to wild-type activity, indicating an uncoupling between ATP hydrolysis and ion translocation. This fact was substantiated by reconstitution experiments, in which the D583A complex was unable to facilitate ion translocation, whereas the D583E mutant complex still exhibited such activity. On the basis of these results, a new transport model for the Kdp-ATPase is presented here, in which the amino acids D583 and K586 are supposed to play a role in the gating mechanism of the complex. Furthermore, movement of the charged side chains could have a direct influence on the free energy profile within the potassium transporting subunit KdpA, thereby facilitating ion transport against the concentration gradient into the cytosol.

P-type ATPases, an enzyme family ubiquitously found in cells of prokaryotes, plants, and animals, hydrolyze ATP and form a phospho intermediate during the reaction cycle. A series of domain movements couple ATP hydrolysis and ion translocation. The recent elucidation of the structure of the Ca^{2+}-ATPase at various stages of the reaction cycle offers a fascinating insight into these processes (*1*−*5*). Most of the P-type ATPases are composed of a central subunit that mediates both reactions, nucleotide hydrolysis and ion transport (e.g., Na^+,K^+-ATPase and Ca^{2+}-ATPase) (for review see ref *6*). However, the KdpFABC complex of *Escherichia coli* appears to be an exception to this rule. Subunit KdpB mediates ATP hydrolysis and shares the key motifs of P-type ATPases (*7*−*9*), while subunit KdpA, which shows homologies to potassium channels, is required for potassium binding and transport (*7*, *8*, *10*, *11*).

The structures of P-type ATPases are thought to be very similar, although the overall sequence similarity is quite low (*12*). Only key motifs are well conserved throughout the enzyme family (*13*), such as the ATP binding region and the active site of ATP hydrolysis. For KdpB it was shown recently that the catalytic loop located between TM4 and TM5 is required for ATP binding and hydrolysis (*14*). Furthermore, the structure of the nucleotide-binding domain (N-domain) of KdpB was solved using heteronuclear NMR, clearly showing that the nucleotide binding mechanism occurs through cation−π interaction (*15*). Similar mechanisms have been reported for the N-domain of the Na^+,K^+-ATPase (*16*) and for the Ca^{2+}-ATPase (*4*). Therefore, the nucleotide binding mechanism in the KdpFABC complex is similar to that of related P-type ATPases. However, no defined network of stabilizing hydrogen bonds between the protein and the ribose moiety was reported, suggesting that the nucleotide binding mechanism in the Kdp-ATPase is rather simple.

In contrast to other P-type ATPases, large differences exist in the way ion binding occurs by the KdpFABC complex. The ion binding sites of related P-type ATPases were shown to be built up by residues of TM4−6 and TM8 of the large subunit (*1*, *17*−*19*). For the KdpFABC complex a large body of evidence is in favor of subunit KdpA containing the ion binding site and being responsible for the translocation of the ion across the membrane. In addition, mutational analyses revealed that KdpA carries selectivity filter regions similar to that of the KcsA channel (*10*, *11*, *20*, *21*). Therefore, a

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mechanism should exist for the coupling of ATP hydrolysis via KdpB and ion binding and translocation via KdpA. Surprisingly, computer analyses and sequence comparison with other P-type ATPases revealed that KdpB has two highly conserved charged residues (D583 and K586) in its putative transmembrane helix V. The aim of this study was to alter these residues in order to investigate their impact on ATPase activity and ion transport.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Oligonucleotide Primers. All bacterial strains, plasmids, and oligonucleotide primers used in this study are listed in Table S1, Table S2, and Table S3 in the Supporting Information, respectively.

Growth Conditions, Media, and Supplements. E. coli cells were routinely grown in Luria−Bertani (LB) medium (*22*) supplemented with the appropriate antibiotics. For the synthesis of recombinant proteins cells were grown in either LB medium or K115 minimal medium. Strains carrying mutations in the *kdp* operon were grown in KML, K115 minimal medium, or K0 minimal medium as previously described (*23*) with different potassium concentrations. Supplements were added in the following final concentrations: ampicillin, 100 μg mL^{-1}; carbenicillin, 100 μg mL^{-1}; thiamin, 1 mg mL^{-1}; isopropyl β-D-thiogalactopyranoside (IPTG),[1] 1 mM; and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal), 1 mM.

Generation of KdpB Mutants. The *kdpFABC* genes expressed from the pSMC10His vector resulted in a KdpFABC complex, where KdpC carried a C-terminal decahistidinyl epitope. The restriction sites *Bam*HI and *Sal*I were used to introduce the mutated *kdpB* cassette, generated by PCR. The oligonucleotide primer used (see Table S3) changed Asp583 to alanine (D583A), serine (D583S), asparagine (D583N), lysine (D583K), and glutamate (D583E). Lys586 was replaced by alanine (K586A), arginine (K586R), aspartate (K586D), and threonine (K586T). The *kdpB* cassettes containing the mutated codons were first cloned into a pUC18 vector and sequenced (Department of Botany, University of Osnabrück). Constructs were digested with *Bam*HI and *Sal*I, and the resulting fragments were cloned into pSMC10His. Plasmid pSMC10His-D583 and pSMC-10His-K586 derivatives were transformed into TKW3205. This strain was routinely used for expression of *kdp* constructs, since it lacks the chromosomally encoded *kdp*, *atp*, *trkA*, and *trkD* genes. Expression from the pSMC10His vector is under the control of the endogenous *kdpD* and *kdpE* gene products (*11*).

Purification of the KdpFABC Complex. TKW3205 cells carrying the pSMC10His derivatives were grown in K0 medium supplemented with different potassium concentrations. D583 mutants were grown overnight in K5 and transferred to K0 medium. After 1−3 h the *kdpFABC* operon was induced by adding 2.5 mM KCl. When the cell culture reached an optical density around 1.0, cells were harvested in order to prevent degradation of the KdpFABC complex in the stationary phase. The K586 mutants were grown in K1 medium overnight and transferred into K0 medium, and the *kdpFABC* operon was induced with 0.5 mM KCl. The KdpFABC complexes, containing a decahistidinyl fusion at the C-terminus of subunit KdpC, were purified via metal affinity chromatography. For that, after cell lysis using a Ribi cell fractionator, membrane proteins were solubilized using 1% *n*-decyl α-D-maltopyranoside (decylmaltoside, DM) for 60 min on ice, when proteins were used for reconstitution experiments. Protein used for ATPase assays was solubilized with 1% aminoxide for 30 min. Hydrolytic activities of the enzyme complexes were determined to be identical for both detergents. However, aminoxide-solubilized protein could not be used for reconstitution experiments since it leads to leaky proteoliposomes. Solubilized proteins were collected by centrifugation at 100000*g*, 4 °C, for 90 min. The supernatant was applied to Ni-NTA agarose preequilibrated with 25 mM HEPES−Tris, pH 7.8, 5 mM imidazole, 0.5 mM $MgCl_2$, and 0.2% DM. After binding of the protein at 4 °C under constant shaking for 60 min, the Ni-NTA slurry was transferred into a glass column (diameter 1.2 cm) and connected to a fast-performance liquid chromatography (FPLC) device (Amersham-Bioscience, Freiburg, Germany). Elution of KdpFABC complexes from the Ni-NTA resin was performed in the same buffer as described above containing 250 mM imidazole. The peak fraction was collected, and the protein was further purified by size-exclusion chromatography using a Superdex-75 column (Amersham Bioscience). The column was run with 20 mM HEPES−Tris, pH 8.0, 0.5 mM $MgCl_2$, and 0.2% decyl maltoside.

Reconstitution of the KdpFABC Complex. Reconstitution of the KdpFABC complex into proteoliposomes was carried out as described by Gassel (*24*) on the basis of established protocols (*25*, *26*). The KdpFABC complex purified as described above was mixed with *E. coli* lipids (5 mg mL^{-1}) (Avanti Polar Lipids, Alabaster, AL) in a protein/lipid ratio of 1/20. The liposomes were partially solubilized by addition of Triton X-100 (0.16%). Detergents were removed by successive addition of pretreated Bio-Beads SM-2 (*27*). The proteoliposomes were spun down at 100000*g* in order to remove unincorporated protein. The proteoliposomes were washed twice with 20 mM HEPES−Tris, pH 7.8, and 0.5 mM $MgCl_2$ and, subsequently, loaded with the appropriate ions by addition of salt (usually 50 mM KCl) to the reconstitution buffer followed by three cycles of freezing, thawing, and sonication.

ATPase Assay. ATPase activity of the purified KdpFABC complex was determined using the microtiter plate assay of Henkel et al. (*28*) following the modifications described previously (*8*). Half a microgram of purified protein was routinely used for a single measurement.

Fluorometric Measurements. The fluorometric measurements with reconstituted KdpFABC protein were carried out as described (*8*, *24*−*26*). The measurements were performed in 20 mM HEPES−Tris, pH 7.8, 2 mM $MgCl_2$, and 50 mM KCl in a 1 mL reaction volume using a SLM-Aminco 8100 spectrofluorometer (SLM-Aminco, Rochester, MN) with the following parameters: excitation, 650 nm; emission, 675 nm; bandwidth of monochromators, 4 nm; integration time, 1 s. The reaction mixture contained 1 μM $DiSC_3(5)$, and the reaction was started by adding 1 mM Na_2ATP. Inhibitors (*o*-vanadate) and ionophores (valinomycin) were added in 100 and 1 μM concentrations, respectively.

[1] Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

Table 1: Complementation[a]

	WT	D583A	D583S	D583N	D583K	D583E	K586A	K586T	K586D	K586R	D583A/K586A
K115	++	++	++	++	++	++	++	++	++	++	++
K15	++	0	0	0	0	++	++	++	++	++	−
K10	++	−	−	0	−	++	++	++	++	++	−
K5	++	−	−	−	−	++	++	++	++	++	−
K2.5	++	−	−	−	−	++	++	++	++	++	−
K1	++	−	−	−	−	++	++	++	++	++	−
K0.5	++	−	−	−	−	++	+	+	+	++	−
K0.3	++	−	−	−	−	++	0	0	0	++	−
K0.1	++	−	−	−	−	++	−	−	−	++	−

[a] Complementation tests with TKW3205 carrying pSMC10His plasmids. Plasmids code for KdpFABC-His_{10} with mutations in KdpB as indicated. Cell growth was tested on minimal medium agar plates supplemented with different potassium concentrations (mM). Plates were incubated at 37 °C for 24 h. Key: (−) no growth; (0) poor growth; (+) moderate growth; (++) optimal growth.

RESULTS

Complementation Assays of KdpB D583 and K586 Variants. Growth of the strains was tested on minimal media containing different concentrations of potassium. Wild-type cells synthesizing the native KdpFABC complex were able to grow on minimal medium without added potassium (K0 medium according to ref *23*). Due to chemical impurities, traces of K^+ (20 μM) are present in K0 medium, which are sufficient to promote growth of wild-type *E. coli*. Complementation tests with strain TKW3205 carrying the pSMC10His derivatives are listed in Table 1 (wild-type: KdpFABC-His_{10} complex encoded by pSMC10His).

Conservative replacements that maintain the charges at positions 583 and 586, namely, D583E and K586R, exhibited a growth phenotype comparable to that of the wild type. However, D583 substitutions that did not restore the negative charge led to a drastically altered phenotype. The TKW3205 strains carrying the D583A, D583S, D583N, and D583K mutations were all unable to grow on medium containing less than 15 mM KCl, and even on K15 medium only poor growth was observed. To test whether the two charges at positions D583 and K586 form a salt bridge, a double mutant was constructed (D583A/K586A). The phenotype of this double mutant was identical to that in which the charge at position 583 was eliminated. These results, together with the observation that the substitution of residue D583 gave rise to a different phenotype than replacement of K586, made it unlikely that the amino acid pair D583 and K586 form a salt bridge.

Localization of KdpB Mutant Complexes. Since replacements of amino acids within transmembrane helices of proteins may cause incorrect folding followed by their loss of function, it was determined whether the KdpFABC mutant complexes were properly inserted into the cytoplasmic membrane. Therefore, membranes of *E. coli* TKW3205 synthesizing the different mutant complexes were purified, subjected to SDS−PAGE analysis, and immunoblotted using anti-KdpB antibodies and anti-penta-His antibodies. All KdpFABC complex variants were found to be integrated in the cytoplasmic membrane, and protein levels were comparable (data not shown). Consequently, we conclude that the mutant complexes are correctly folded and that the phenotypes observed are not due to misrouting of the protein variants.

Purification of KdpB Mutant Complexes. Figure 1 shows a typical purification scheme of the D583A mutant complex, which was representative for all KdpFABC variants with the following exceptions. In the case of the D583K and K586D complexes, the KdpB polypeptide was not present in the final elution step. It is most likely that the KdpB mutant subunit was degraded, as we could not observe the protein on immunoblots using anti-KdpB antibodies in any of the fractions including the flow-through fraction. However, we cannot completely exclude the possibility that the mutations led to weakened subunit interactions leading to the loss of KdpB. Furthermore, purification of the K586A mutant complex resulted in a reduced amount of KdpB. However, purification of the K586R mutant complex was still achieved.

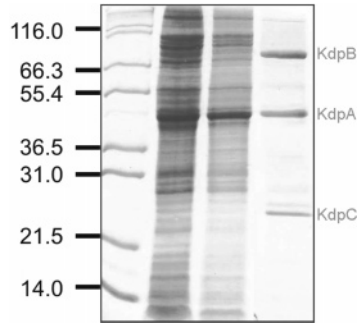

FIGURE 1: Purification of the KdpFABC-His_{10} mutant complex D583A. The KdpFABC-His_{10} complex was purified using Ni-NTA affinity chromatography. Aliquots from different purification steps were subjected to SDS−PAGE. Each lane was loaded with approximately 10 μg of protein. Lanes (from left to right): (1) molecular mass standard (kDa); (2) whole cell extract of induced cells; (3) flow-through fraction of Ni-NTA; (4) elution fraction. The gel was stained with Coomassie blue.

Biochemical Properties of KdpB Mutants. All three tested KdpFABC complex variants (D583A, D583E, and K586R) exhibited ATPases activity, while major differences were observed in ion stimulation of ATP hydrolysis. The D583A enzyme complex exhibited an ATPase activity which was not stimulated by potassium, although the overall rate was comparable to the maximal wild-type level (Figure 2). The ATPase activity of D583E and K586R mutant complexes was stimulated by K^+. However, the affinity for potassium seems to be reduced in the K586R mutant (Figure 2).

Influence of o-Vanadate on ATPase Activities. To test whether the observed ATPase activities of the Kdp-ATPase mutants are based on an impaired E1/E2 transition, the influence of *o*-vanadate on the ATPase activity was tested. In comparison to the wild-type enzyme, the D583A, D583E, and K586R mutant complexes were found to be less sensitive to vanadate (Figure 3), although higher concentrations were inhibitory. This indicates a shift of the E1/E2 equilibrium,

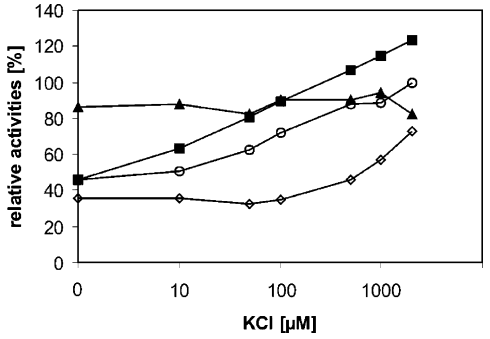

FIGURE 2: Potassium-stimulated ATPase activity of solubilized KdpFABC-His_{10} complexes. The KdpFABC complexes were solubilized with aminoxide and purified as described. The maximal activity for the wild-type KdpFABC-His_{10} complex was 4.95 μmol mg^{-1} min^{-1} (set to 100%). Key: (○) KdpFABC-His_{10}; (■) KdpFABC-D583E-His_{10}; (▲) KdpFABC-D583A-His_{10}; (◇) KdpFABC-K586R-His_{10}. Data are the mean of three experiments with less than 10% variation.

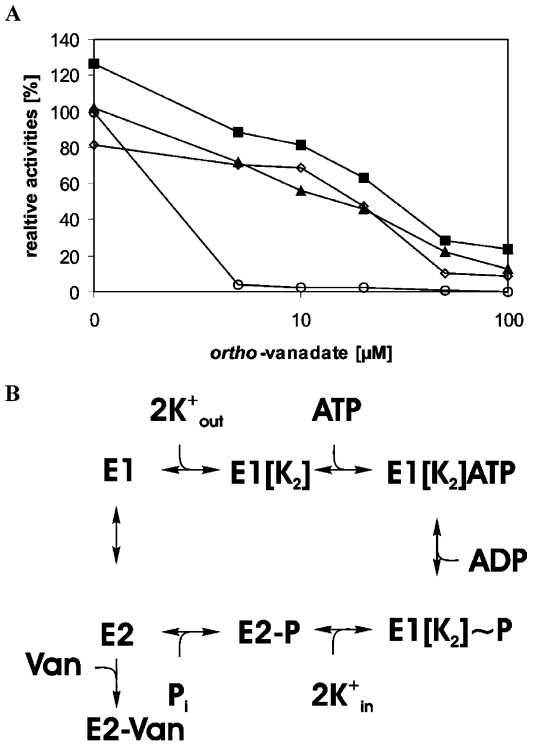

FIGURE 3: (A) *o*-Vanadate-inhibited ATPase activity of solubilized KdpFABC-His_{10} complexes. The KdpFABC complexes were solubilized with aminoxide and purified as described. The maximal activity for the wild-type KdpFABC-His_{10} complex was 4.37 μmol mg^{-1} min^{-1} (set to 100%). Key: (○) KdpFABC-His_{10}; (■) KdpFABC-His_{10}-D583E; (▲) KdpFABC-His_{10}-D583A; (◇) KdpFABC-His_{10}-K586R. Data are the mean of three experiments with less than 10% variation. (B) Simplified reaction cycle of the Kdp-ATPase. The KdpFABC complex presumably binds two potassium ions in the E1 state (*25*). ATP is bound to this complex and is subsequently hydrolyzed, thereby forming the energized E1 state. During the conformational change from E1 to E2 the potassium ions are deliberated into the cytosol. After dephosphorylation, the enzyme returns to its E1 conformation. The P-type ATPase inhibitor *o*-vanadate (Van) binds to the E2 state, thereby mimicking an E2-P transition state.

suggesting that the E1/E2 transition could be accelerated. Consequently, the observed ion independent ATPase activity of the D583A mutant is not due to hydrolysis occurring in an E1 + ATP → E1~P + ADP → E1 + P_i manner, but rather the full reaction cycle is accomplished.

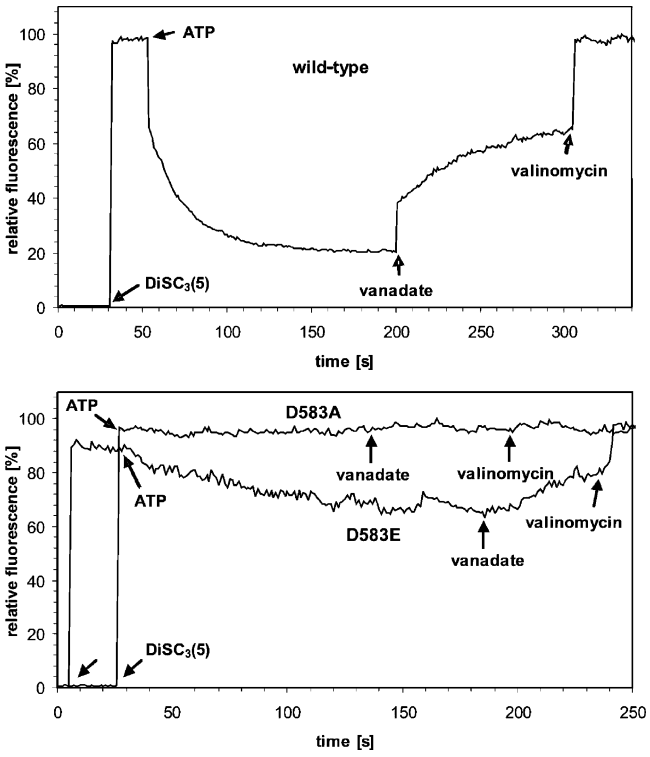

FIGURE 4: Electrogenic K^+ transport of reconstituted KdpFABC-His_{10} wild-type, -D583E, and -D583A complexes. Reconstitution of the KdpFABC complexes was performed as described in Experimental Procedures. $DiSC_3(5)$ fluorescence measurements were performed as described using 20 μL of proteoliposomes in a 1 mL reaction volume. The KdpFABC-His_{10} (WT), -D583A, and -D583E complexes were solubilized with decyl maltoside. The buffer contained 50 mM KCl, both inside and outside the proteoliposomes. Substances were added in the following concentrations: 1 mM ATP, 100 μM *o*-vanadate, and 1 μM valinomycin. The maximal fluorescence was set to 100%.

Transport Activities of the Reconstituted D583A and D583E Complexes. Reconstitution of the altered complexes into proteoliposomes was performed in order to test whether the D583A and D583E derivatives were able to transport K^+ ions. Addition of the potential sensitive dye $DiSC_3(5)$ to proteoliposomes increased the fluorescence, which was calibrated to 100%. Transport of K^+ from the lumen of the proteoliposome into the medium was mediated by inside-out reconstituted Kdp-ATPase molecules and was started by addition of 1 mM Na_2ATP. The loss of positive charges in the lumen of the proteoliposomes led to a fluorescence decrease. Addition of 100 μM *o*-vanadate completely inhibited the Kdp-ATPase and resulted in a slow backflow of K^+, since the proteoliposomes were not tightly sealed (Figure 4). The K^+ ionophore valinomycin completely abolished the potential by transporting K^+ along the gradient into the proteoliposomes, thus showing that the potential was indeed generated by K^+ transport. In contrast, the D583A complex did not show any transport activity at all (Figure 4), whereas the D583E enzyme did show transport activity, although to a much smaller extent as compared to the wild-type Kdp-FABC complex (Figure 4). To ensure that the reconstituted protein complexes were still hydrolyzing ATP, ATPase activities were checked for all proteoliposome preparations. The results clearly demonstrate that abolishing the charge

at residue Asp583 led to an uncoupling of K^+ transport and ATP hydrolysis, while the conservative exchange D583E still allows K^+ translocation, although to a much smaller extent.

DISCUSSION

It is still unknown how the KdpFABC complex, a unique member of the P-type ATPase family, couples ATP hydrolysis to ion transport. A large body of evidence suggests that KdpA forms a KcsA-like potassium channel (*10*, *11*, *29*). However, the Kdp system does not work as an ion channel but rather forms a powerful ion pump. The transport is energized by KdpB, which comprises a P-type ATPase. However, we have no evidence that KdpB is directly involved in ion binding. Intriguingly, KdpB has only seven predicted transmembrane helices, thus lacking helix VIII which participates in ion binding of the Na^+,K^+-ATPase (*17*) and the Ca^{2+}-ATPase (*1*). To discern the differences between KdpB and other P-type ATPases, we found that KdpB contains two charged amino acids, Asp583 and Lys586, within the proposed helix V. These amino acids are conserved throughout the KdpB polypeptides but have no counterpart in other P-type ATPases. Therefore, these residues were subjected to site-directed mutagenesis. The fact that the K586 and D583 complexes exhibited different phenotypes with respect to growth on potassium-limited media made it very unlikely that D583 and K586 form a salt bridge. It was important to exclude formation of a salt bridge, since examples of salt bridges within other P-type ATPases demonstrate the importance of such bonds for a correct membrane insertion and routing of the enzymes. The yeast plasma membrane H^+-ATPase contains a salt bridge between transmembrane segments 5 and 6 (*30*). Site-directed mutagenesis at the residues R695 and D730 was performed where positively and negatively charged residues were swapped or eliminated (R695D/D730R and R695A/D730A). The results revealed that the double mutants retain their correct membrane insertion and pumping activity (*30*). In the case of the charged residues within the putative TM5 of KdpB, a double mutant (D583A/K586A) exhibited the same phenotype as observed for those D583 mutant complexes, where the negative charge was removed. This result indicates that the D583 residue has a dominant effect and plays a major role in the function of KdpB. Furthermore, all mutant complexes were found to be membrane integral, suggesting a correct routing and assembly of the Kdp complex.

To characterize the altered KdpFABC complexes biochemically, TKW3205 cells carrying the corresponding pSMC10His derivatives were grown in minimal medium supplemented with 0.5 mM (for K586 mutant complexes) or 5 mM KCl (for D583 mutant complexes), and subsequently, the complexes were purified. The overall ATPase activities of the purified complexes were similar between mutant and wild-type enzyme complexes. However, the ATPase activity of the D583A complex was not stimulated by potassium. In addition, the sensitivity against *o*-vanadate was drastically reduced (Figure 3). Two plausible explanations are possible: (i) The mutant enzyme complex is disturbed in the intrasubunit communication of KdpB. It is well established that P-type ATPases undergo domain movements during the reaction cycle where movements of the cytoplasmic domains, e.g., the phosphorylation domain and nucleotide-binding domain, induce movements in the transmembrane segments (*2*, *4*, *5*). (ii) The function of KdpB is not affected, but the hydrolysis of ATP is no longer coupled to ion transport. Reduced vanadate sensitivity cannot differentiate between these models. If the conformational changes between the E1∼P state and the E2-P state are disturbed, accumulation of E1∼P would occur. Since vanadate is proposed to bind to the E2 state, it is consistent with the observation that the inhibitory effect is less pronounced. On the other hand, it is well established that ion binding and transport are necessary for a proper reaction cycle of P-type ATPases. A typical example is the Na^+-dependent ATPase activity and the K^+-dependent pNPPase activity of the Na^+,K^+-ATPase. In the case of a direct involvement of D583 in KdpB on transport activity, accumulation of an E1 state could also be possible as a result of a faster transition from the E2 to the E1 state. Reconstitution of the altered complexes into proteoliposomes revealed that the D583A mutant has lost the ability to transport potassium ions (Figure 4), although the ATPase activity was not affected (Figure 2). In contrast, the D583E complex was still able to facilitate ion translocation, although the transport activity was less compared to the wild-type KdpFABC complex. Therefore, the D583 residue is likely to be involved in ion translocation, although the question remains whether this interaction is direct or indirect. Electrophysiological measurements with KdpFABC complex-containing proteoliposomes, adsorbed to black lipid membranes (BLM), suggested that a protein-bound negative charge might move relative to the membrane during the reaction cycle (*21*, *25*). Localization of this charge has hitherto not been possible. However, it is likely that this charge is located within KdpB and not in KdpA, since the channel-like KdpA structure seems to be more rigid than KdpB. Major vertical movements are not observed in potassium channels. The D583 residue in KdpB might be a good candidate for such a role, since this residue is membrane embedded and conserved throughout the KdpB polypeptides of different organisms. Yet, the crystal structures of the Ca^{2+}-ATPase do not show a major vertical movement of the corresponding helix V. Moreover, this helix is bent and swiveled. However, these small movements may be sufficient to generate a switch in the adjacent subunit KdpA.

Ion transport in other P-type ATPases is understood in detail, because either amino acids contributing to the ion translocation have been localized by mutational analysis or, in the case of the Ca^{2+}-ATPase, high-resolution structures with bound substrates are available. With the exception of the Kdp-ATPase, all other enzymes of the P-type ATPase family couple ATP hydrolysis and domain movement directly to ion transport. These enzymes share an ion binding pocket within the membrane and exhibit an occluded state as a logical consequence. The example of the well-studied sarcoplasmic reticulum Ca^{2+}-ATPase shows that the ion binding pocket is formed by TM4−TM6 and TM8. The helices TM4 and TM6 are unwound at the level of the calcium binding sites (*1*). A series of structures showing the Ca^{2+}-ATPase in various states demonstrated how the ions get access to their binding sites (*5*), and mutational analysis (*19*) confirmed the exclusive role of glutamate 309 as the capping residue which leads to occlusion of the calcium ions. In fact, sequence alignments seem to substantiate the idea that all P-type ATPases of the type II−V classes share an overall topology, including an access channel for ions (*12*).

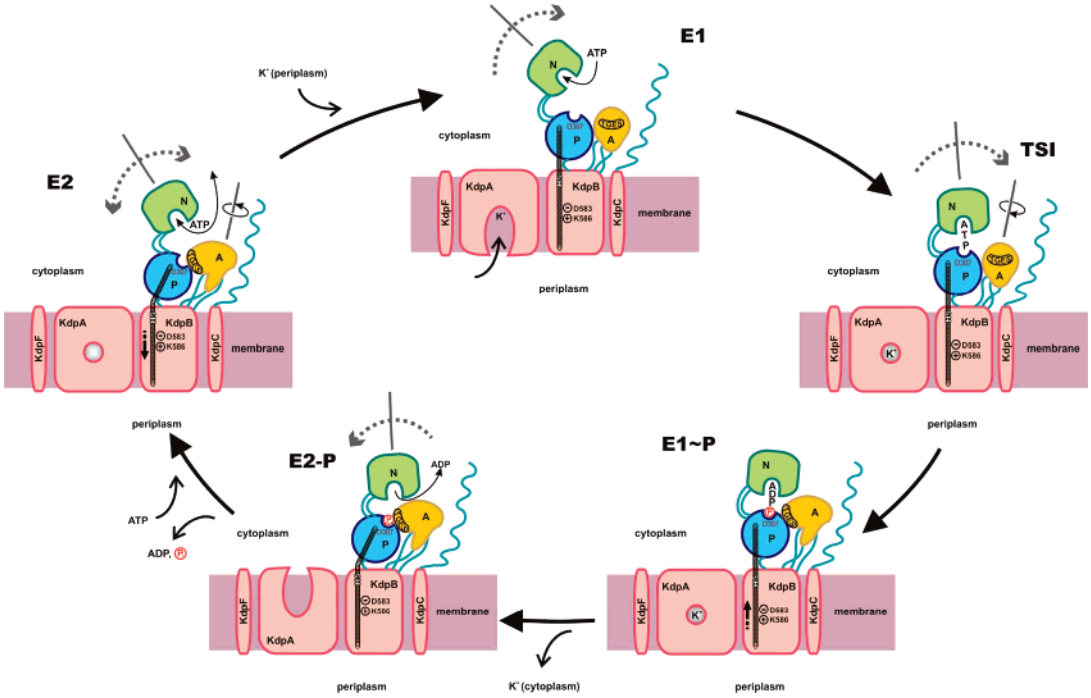

FIGURE 5: Reaction cycle of the KdpFABC complex modified according to ref *15*. Binding of potassium ion to the E2 state of the KdpFABC complex promotes high-affinity ATP binding to the N-domain of KdpB. Subsequently, the KdpFABC complex is transformed to its E1 state, and the potassium ion is able to enter the now energetically favorable binding site in the center of the selectivity filter of KdpA. Upon reaching the transition state TSI followed by phosphorylation, the potassium ion becomes occluded. The subsequent E1 to E2 transition, accompanied by major conformational changes in KdpB, alters the dipole formed by D583 and K586. This makes the place of the potassium ion in the center of the selectivity filter of KdpA unfavorable and, consequently, pushes the ion toward the cytoplasmic side of the membrane, where the ion is finally released. Dephosphorylation of the complex regenerates the system.

However, the Kdp-ATPase must have a different ion translocation mechanism. The interplay between KdpB and KdpA can either be explained by an ion channel containing residues from both subunits or an inductive transport mediated by a counterion within KdpB. With the emerging high-resolution structures of ion pumps it becomes clear that these enzymes follow a general principle. To overcome the costs of energy for bringing ions from the high dielectric medium into the low dielectric environment of the membrane, ions form pairs within the membrane (for a recent review, see ref *31*). In KdpB the two charged residues, D583 and K586, located in the middle of the membrane are potentially forming a strong dipole. Movement or reorientation of such a dipole within KdpB upon ATP hydrolysis could have effects on the free energy surface in the selectivity filter of KdpA, forcing the potassium ion to leave at the cytoplasmic side of the membrane. A general principle of potassium channels is the removal of the hydration shell of the ion. The water molecules are stripped of the ion by oxygen atoms within the selectivity filter, where it passes a bottleneck. The filter is proposed to contain two ions at a time, separated by two water molecules. The two ions move in a concerted fashion between the two configurations, $K^+-H_2O-K^+-H_2O$ (1,3 configuration) and $H_2O-K^+-H_2O-K^+$ (2,4 configuration), until a third ion enters, displacing the ion on the opposite side of the queue (*32*−*34*). The Kdp-ATPase needs to "push" the ion through the bottleneck of the selectivity filter into a putative, water-filled cavity of KdpA where the potassium ion is rapidly rehydrated and released into the cytosol. This process is energy dependent, since a high electrochemical gradient is generated (extracellular potassium concentrations are in the low micromolar range while the cytoplasm contains up to molar levels of potassium). It has been suggested previously that long-distance coupling mechanisms, for example, due to ATP binding, could perturb the free energy profile governing the ion translocation (*35*, *36*). In addition, a gating mechanism is likely to exist in KdpA, which prevents backflow of the ions when the enzyme is undergoing the E2−E1 transition. Movements of transmembrane domains, which might be close to the central cavity within KdpA from the cytosolic side, could mediate the gating. This transport model is presented in Figure 5. However, it should be mentioned that gating mechanisms do not necessarily need large domain movements. The example of the bacterial chloride channel Clc from *E. coli* shows that a single glutamate side chain could fulfill the gating process (*37*). Furthermore, the example of the Clc channel illustrates that the distinction between channels, pumps, and secondary carriers may be not as clear as previously thought (*38*). Thus, it is possible that the residue KdpB-D583 is involved in the gating mechanism, rather than in the actual transport process. Therefore, a major challenge for future work on the Kdp-FABC complex is the elucidation of the contact sites between the different subunits.

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SUPPORTING INFORMATION AVAILABLE

Comprehensive tables containing strains (S1), plasmids (S2), and oligonucleotides (S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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