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Prokaryotic Kdp-ATPase: Recent Insights into the Structure and Function of KdpB

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Abstract
P-type ATPases are amongst the most abundant enzymes that are responsible for active transport of ions across biological membranes. Within the last 5 years a detailed picture of the structure and function of these transport ATPases has emerged. Here, we report on the recent progress in elucidating the molecular mechanism of a unique, prokaryotic member of P-type ATPases, the Kdp-ATPase. The review focuses on the catalytic parts of the central subunit, KdpB. The structure of the nucleotide-binding domain was solved by NMR spectroscopy at high resolution and a model of the nucleotide-binding mode was presented. The nucleotide turned out to be 'clipped' into the binding pocket by a π-π interaction to F377 on one side and a cation-π interaction to K395 on the other. The ^{395}KGXXD/E motif and thus the nucleotide-binding mode seems to be conserved in all P-type ATPases, except the heavy metal-transporting (class IB) ATPases. Hence, it can be concluded that KdpB is currently misgrouped as class IA. Mutational studies on two highly conserved residues (D583 and K586) in the transmembrane helix 5 of KdpB revealed that they are indispensable in coupling ATP hydrolysis to ion translocation. Based on these results, two possible pathways for the reaction cycle are discussed.

Introduction

Ion transport across biological membranes is one of the most frequent energy-consuming reactions in living cells. Typically these transport processes are catalyzed by P-type ATPases. This enzyme family transports ions (and phospholipids) against a concentration gradient at the expense of ATP hydrolysis and forms a phospho intermediate (hence P-type ATPase) during the reaction cycle. Members of P-type ATPases are found in all kingdoms of life. The family of P-type ATPases was divided into different groups related to their substrate specificity [Axelsen and Palmgren, 1998; Lutsenko and Kaplan, 1995]. The heavy metal-transporting P-type ATPases belong to the

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type I class, while the mono- and divalent cation-transporting P-type ATPases were grouped into the type II class consisting of different subgroups. This classification was mainly based on their substrate specificity and domain sizes. A common characteristic of P-type ATPases is the coupling of ATP hydrolysis and ion transport in a central subunit (many P-type ATPases contain additional regulatory subunits, for instance the γ-subunit of the Na^+,K^+-ATPase). However, the Kdp-ATPase (KdpFABC complex), a prokaryotic member of the P-type ATPases, appears to be an exception to this rule. Subunit KdpB (72 kDa) mediates ATP hydrolysis and shares the key motifs of P-type ATPases [Altendorf and Epstein, 1996; Altendorf et al., 1998; Bramkamp and Altendorf, 2004], while subunit KdpA (59 kDa), which shows homologies to other potassium-transporting proteins like KcsA, KtrB, and HKT, is required for potassium binding and transport [Bertrand et al., 2004; Dorus et al., 2001; Durell et al., 2000; van der Laan et al., 2002]. KdpC (21 kDa) might be involved in the assembly of the complex [Gassel and Altendorf, 2001], and the small hydrophobic peptide, KdpF (3 kDa), was shown to stabilize the complex at least in vitro [Gassel et al., 1999]. Besides several heavy metal-transporting ATPases, the KdpFABC complex of *Escherichia coli* is so far the best studied bacterial P-type ATPase. This complex, synthesized under potassium starvation or high osmolality, is an emergency system that transports potassium ions with a remarkably high affinity (K_m = 2 μ*M*), but moderate rates (v_{max} = 150 μmol g^{-1} min^{-1}) [Rhoads et al., 1976]. The expression of the *kdpFABC* operon is under the control of the KdpD/KdpE two-component system [Jung and Altendorf, 2002; Polarek et al., 1992; Walderhaug et al., 1992].

The understanding of the structure and function of the KdpFABC complex has developed rapidly in recent years. Whereas previously the focus in Kdp research was on the individual subunits KdpA, KdpC, and KdpF, which have no counterpart in other P-type ATPases, more recently, the central subunit KdpB came into focus, leading to a comprehensive understanding of nucleotide binding and domain organization in this central subunit of the Kdp complex. Investigations of the KdpB subunit were facilitated by the fact that KdpB, as well as the other P-type ATPases, has a modular design with a membrane integral part consisting of seven transmembrane helices (TM), a smaller cytoplasmic domain located between TM2 and TM3, which corresponds to the actuator (A-) domain, and a larger cytoplasmic loop between TM4 and TM5 (fig. 1). This latter H4H5 loop is composed of two separate modules, the phopshorylation (P-) domain and the nucleotide binding (N-) domain. The P- and N-domains are linked by a flexible hinge region that allows delivery of the γ phosphate group of the bound ATP to the P-domain. Furthermore, this review also summarizes the recent achievements in understanding of how KdpB facilitates ion transport in its own, unique way.

Biochemical Characteristics of KdpB

KdpB is the central catalytic subunit of the KdpFABC complex. Earlier studies revealed that the conserved P-type ATPase motif DKTGT is present in the KdpB protein. Site-directed mutagenesis within this motif identified aspartate 307 in KdpB as the site of phosphorylation [Puppe et al., 1992]. Enzymatic characterization of ATP hydrolysis and inhibitor studies revealed similarities between KdpB and other P-type ATPases [Siebers and Altendorf, 1988]. Formation of an acyl phosphate was demonstrated and the classical inhibitors of P-type ATPases (FITC, *o*-vanadate) were shown to inhibit ATP hydrolysis of KdpFABC [Siebers and Altendorf, 1988; Siebers et al., 1992]. Based on hydrophobicity plots and sequence comparison analyses, a KdpB model containing six TMs was proposed [Siebers and Altendorf, 1988]. Computer-based modeling on the structure of the sarcoplasmic reticulum Ca^{2+}-ATPase [Toyoshima et al., 2000] revealed that KdpB might contain seven instead of six TM domains. These findings do not necessarily contradict the hydrophobicity analysis, since the C-terminal part of the enzyme (where the additional helix was introduced) is mainly hydrophobic.

Previous classifications of the P-type ATPases grouped the Kdp-ATPase close to the heavy metal-transporting ATPases. However, the KdpB subunit shows profound differences to this class of ATPases. Most notably, sequence comparisons revealed that the KGSVD motif in the large cytoplasmic loop of KdpB is homologous to the KGAPE motif of other P-type ATPases and might therefore constitute the binding site for FITC. KdpB shares this motif with members of the type II-V classes of P-type ATPases. For other P-type ATPases, like Na^+,K^+-ATPase [Karlish, 1980] and Ca^{2+}-ATPase [Pick, 1981], it was shown that incubation with stoichiometric amounts of FITC leads to complete inhibition of ATP hydrolysis. The conserved lysine residues (K501 and K515) in the Na^+,K^+-pump and Ca^{2+}-ATPase, respectively, were found to be the modified residues [Farley et al., 1984; Pick and Bassilian, 1981]. These lysine residues belong to a conserved KGAPE motif that is found in many P-type

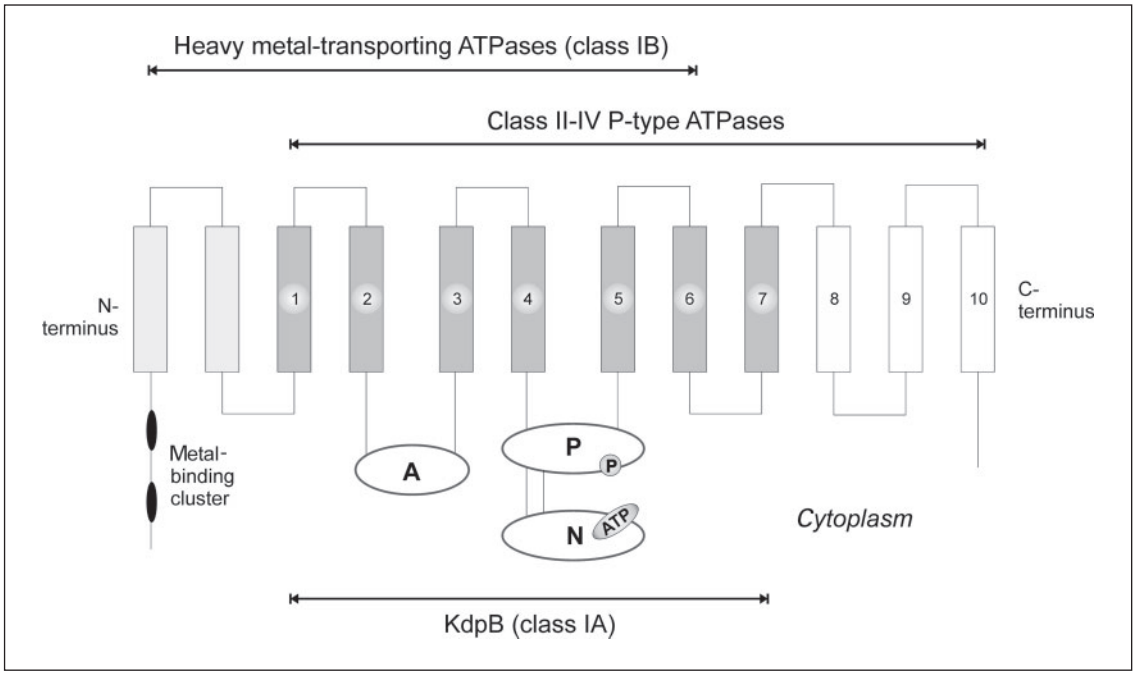


Fig. 1. Schematic topology of P-type ATPases depicting the modular design. The proposed topology for KdpB (class IA) reaches from TM1 to TM7. Heavy metal-transporting ATPases (class IB) have two additional transmembrane helices at the N-terminus and six in common with the other P-type ATPases. Class II-IV P-type ATPases have 10 transmembrane helices. Common to all are the large cytoplasmic loops connecting TM2 and TM3 (actuator or A-domain) and TM4 and TM5 (phosphorylation and nucleotide binding or P- and N-domains). The site of phosphorylation is characterized by the DKTGT motif (illustrated by a circled P). The N-domain comprises a conserved phenylalanine and the KGXXD/E motif responsible for nucleotide binding (illustrated by ATP) (modified according to figure 1 of Haupt et al. [2004a]).

ATPases. Previous studies demonstrated that the ATPase activity of the KdpFABC complex is inhibited by FITC, however the exact binding site remained unclear [Siebers and Altendorf, 1988]. Finally, CNBr cleavage of FITC-labeled KdpB demonstrated that the ^{395}KGSVD motif constitutes the FITC-binding site in KdpB. Protection assays using adenine nucleotides revealed that the FITC-binding site is located in the nucleotide-binding domain of KdpB. Furthermore, it was shown that the KdpFABC complex hydrolyses pNPP [Bramkamp et al., 2004]. Remarkably, ATPase as well as pNPPase activity were inhibited by FITC, which is in contrast to the larger eukaryotic P-type ATPases, where ATP but not pNPP hydrolysis is inhibited by FITC [Bramkamp et al., 2004]. Unlike other P-type ATPases, pNPP hydrolysis of the Kdp-ATPase is not stimulated by the transported ion. This observation might be due to the lack of a counter ion, which usually stimulates pNPP hydrolysis in other P-type ATPases (e.g. the K^+-stimulated pNPPase activity in the Na^+,K^+-ATPase). The existence of a FITC-binding motif in KdpB is a major difference between the Kdp-ATPase and heavy metal-transporting ATPases.

The Membrane Integral Domain of KdpB

In comparison to other P-type ATPases, large differences exist in the way ion binding is accomplished by the KdpFABC complex. The ion-binding sites of related P-type ATPases were shown to be composed of residues of TM4–6 and TM8 of the large subunit [Inesi et al., 2004; Jørgensen et al., 1998; Rice and MacLennan, 1996; Toyoshima et al., 2000]. However, in case of the Kdp-ATPase, KdpA contains the ion-binding site and is responsible for the translocation of the ion across the membrane. Consequently, a mechanism should exist for the coupling of ATP hydrolysis via KdpB and ion binding and translocation via KdpA. Surprisingly, computer analyses and se-

quence comparison with other P-type ATPases revealed that KdpB has two highly conserved charged residues (D583 and K586) in its putative TM5. It has been shown recently that these residues are involved in the coupling of ATP hydrolysis and ion transport [Bramkamp and Altendorf, 2005]. Complementation assays revealed that replacing the charges at D583 and K586 reduced the ability to promote bacterial growth on low potassium. Strikingly, the effect was more pronounced if D583 was replaced suggesting a major role for this residue in the function of KdpB. It was shown that removal of the negative charge at position 583 in KdpB leads to uncoupling of ATP hydrolysis and ion transport. Two plausible explanations for this result are possible: (i) The mutant enzyme complex is disturbed in the intra-subunit communication of KdpB. (ii) The function of KdpB is not affected, but the hydrolysis of ATP is no longer coupled to ion transport. 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 [Toyoshima and Mizutani, 2004; Toyoshima and Nomura, 2002]. This necessary inter-subunit communication could be disturbed by the removal of the charges at position 583 and 586 within KdpB. Future studies will focus on the interaction between KdpB and KdpA.

The Soluble Domains of KdpB

The cytoplasmic parts of KdpB display a modular design, which allows separate synthesis and characterization of these domains without the difficulty of handling a hydrophobic membrane protein. The catalytic domain of KdpB is located between the TM4 and TM5, similar as in the well-characterized eukaryotic Ca^{2+}-ATPases and Na^+,K^+-ATPases. As mentioned above, the H4H5 loop is composed of the P- and N-domain. The P-domain comprises a classical Rossman-fold which was identified in a variety of enzymes. For that reason, Aravind and coworkers proposed a new superfamily of enzymes: the HAD superfamily (named after the L-2-haloacid dehalogenase) [Aravind and Koonin, 1998]. This family of hydrolases includes several phosphatases (including phosphoserine phosphatase, phosphomannomutase, phosphoglycolate phosphatase, and sucrose-phosphate synthase), dehalogenases, epioxide hydrolases, response regulator proteins, as well as the P-type ATPases [Ridder and Dijkstra, 1999].

Biochemical studies on the hydrolytic activity of KdpB were mainly performed using the catalytic domain (e.g. ATP hydrolyzing) encompassing the region between helices TM4 and TM5. The constructs, the H4H5 loop (methionine 282 to glutamine 567) and the nucleotide-binding domain KdpBN (asparagine 316 to glycine 451) were separately synthesized [Bramkamp and Altendorf, 2004]. FITC-labeling experiments demonstrated that the soluble modules of KdpB were properly folded, which was substantiated by CD spectroscopy and nucleotide-binding assays [Bramkamp et al., 2004]. TNP-ATP, TNP-ADP, and TNP-AMP were used to test nucleotide binding to the H4H5 loop and the KdpBN domain. Using titration experiments (TNP-ATP displacement by ATP, ADP or AMP), the existence of a single nucleotide-binding site in H4H5, which is located in KdpBN, was demonstrated [Bramkamp and Altendorf, 2004].

The Structure of the Nucleotide-Binding Domain KdpBN

The question whether KdpB is more closely related to the heavy metal (type I) or alkali and earth alkali type II and III transporting P-type ATPases was approached by the elucidation of the three-dimensional structure of KdpBN. Previous attempts to arrive at a model of the whole subunit, based on the structural data from the SERCA pump [Toyoshima et al., 2000], provided only a fragmentary picture of the structure [unpubl. data]. Nevertheless, the existence of seven TMs could be substantiated and the actuator domain, i.e. the H2H3 loop, showed a rather good alignment. More difficulties were encountered for the catalytic H4H5 loop. The phosphorylation domain, comprising the invariant DKTGT motif, proved to align reasonably well and it can be assumed that it contains the characteristic Rossman fold, as it is the case for the Ca^{2+}-ATPase. In contrast, no alignment was possible with KdpBN. Although this domain contains the KGXXE/D motif and a conserved phenylalanine residue approximately 25 amino acids upstream in the sequence (see above), it is much smaller than any other known N-domain from type II-V P-type ATPases.

Based on the structure, a detailed model of nucleotide binding for KdpBN was reported. In contrast to results published for the Na^+,K^+-ATPase N-domain [Hilge et al., 2003], no large-scale conformational changes could be observed upon nucleotide binding, implying that ATP-induced domain rearrangements are not necessary for the reaction cycle of the KdpFABC complex. In the light of

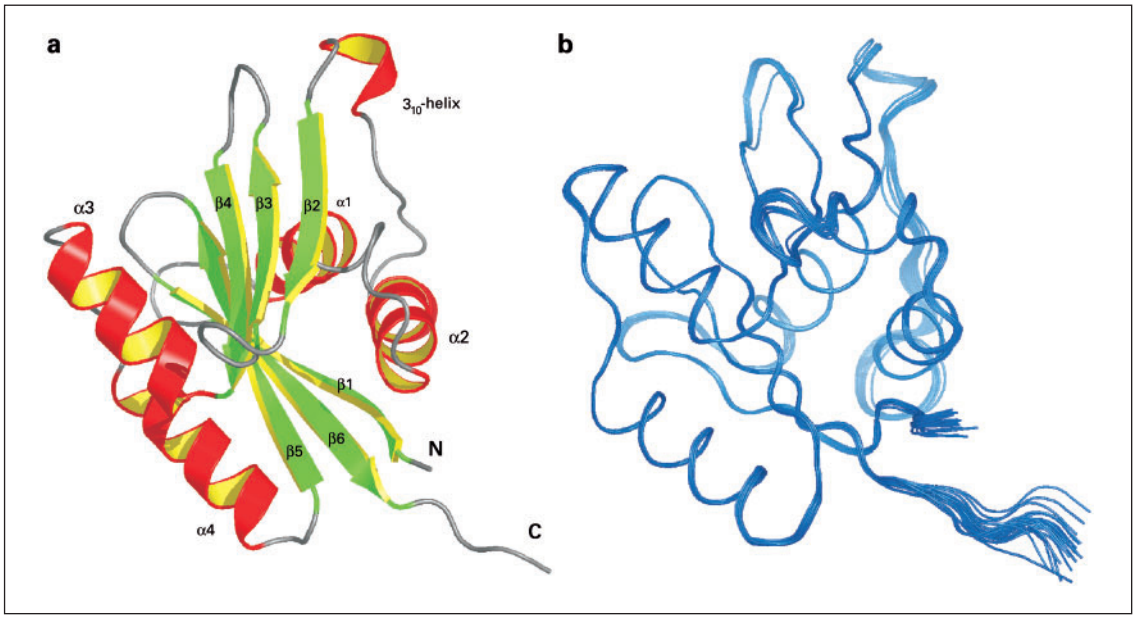


Fig. 2. The structure of KdpBN. **a** Secondary structure elements illustrating the high degree of ordered residues constituting the structure. **b** Definition of a final set of 20 structures which are superimposed over ordered residues. This figure and figures 4–6 were produced using PyMol [DeLano, 2003] (modified according to fig. 2 of Haupt et al. [2004a]).

these results, a revised picture for the conformational rearrangements that form the basis of the reaction cycle in this enzyme family was proposed [Haupt et al., 2004a].

Structure Description of KdpBN

The structure of KdpBN was solved using high-resolution, heteronuclear, multidimensional NMR spectroscopy [Haupt et al., 2004b]. KdpBN forms a curved, six-stranded, antiparallel β-sheet domain flanked by two pairs of α-helices, one on the concave side of the sheet and tightly packed to form the hydrophobic core of the domain (α1 and α2), the other more solvent exposed on the convex side (α3 and α4, see fig. 2). In other P-type ATPases the loop between helix α2 and the β2 strand contains an additional helix. In KdpBN this is replaced by a short 3_{10}-helix at the C-terminal end of the loop. Both, the C-terminus and the slightly shorter N-terminus show high flexibility.

NMR Titration and Nucleotide-Binding Studies

In order to identify the location of the nucleotide-binding site and to observe the effects of nucleotide binding on the structure of the domain, ^{15}N-labeled KdpBN was titrated with AMP-PNP, a stable, non-hydrolysable ATP analogue. TNP-ATP displacement experiments verified that AMP-PNP has equal binding properties as other nucleotides. The K_D values at pH 6 were all in the same range, i.e. 1.3, 1.7 and 2.2 m*M* for ATP, ADP and AMP-PNP, respectively [unpubl. results]. The superimposition of ^{15}N-HSQC spectra recorded on the free domain and in the presence of an increasing excess of AMP-PNP showed a gradual change of chemical shifts, representing a fast ligand exchange on the NMR time scale. Fitting of the curves showed that AMP-PNP binds weakly to the protein (K_D = 1.4 m*M*). Mapping of the normalized weighted chemical shift differences (Δ_{av}/Δ_{max}) upon the 3D structure of KdpBN defined the nucleotide-binding pocket (fig. 3a). K395, which had previously been determined by FITC (fluorescein 5-isothiocyanate) labeling to be critical for nucleotide binding [Bramkamp et al., 2004], shows a significant Δ_{av}/Δ_{max} value. It is located in the center of the antiparallel β-sheet (β4). However, the largest changes occur in the loop region between helix α1 and helix α2, which forms the base of the pocket, leaving an opening for the nucleotide close to the N- and C-termini. Though some cross-peaks in the ^{15}N-HSQC are shifted considerably, the consensus plots of secondary chemical shift index (CSI) for the native and the AMP-PNP-bound protein hardly vary, indicating that no change in secondary structure and thus in the tertiary structure

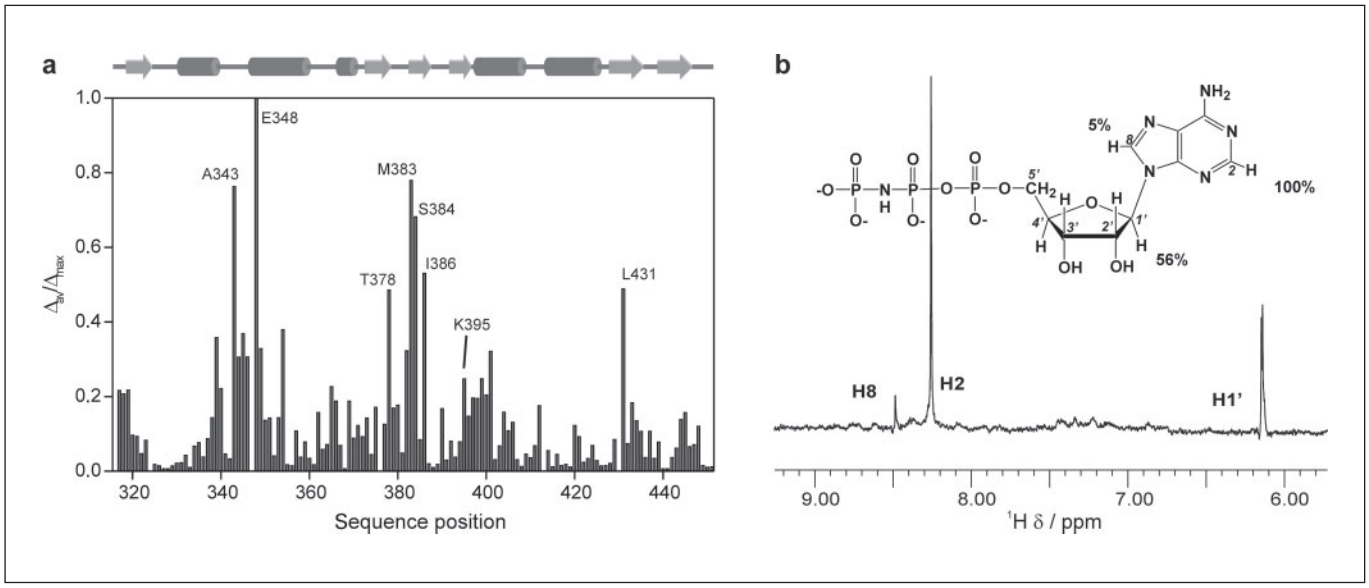


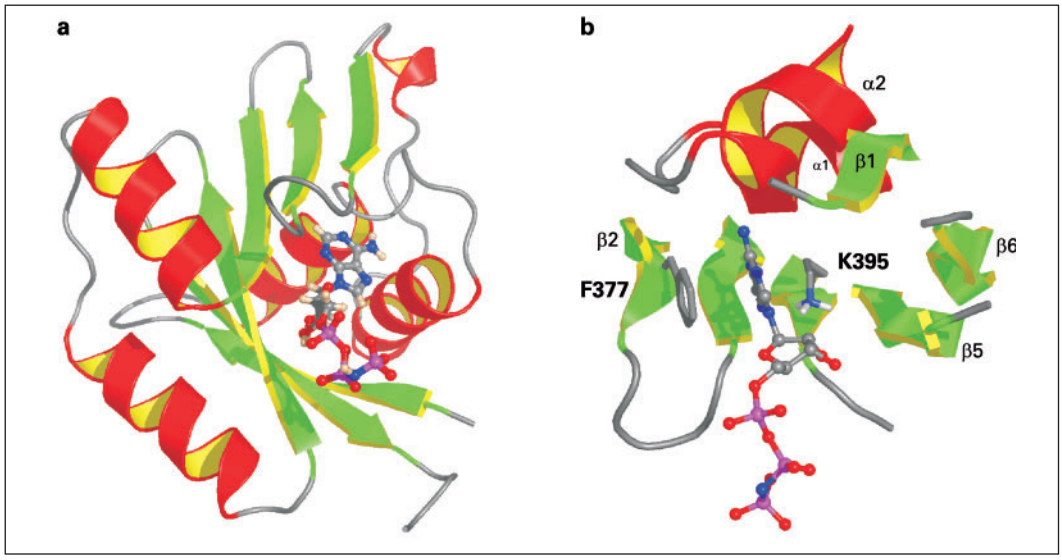
Fig. 3. Characterization of the nucleotide-binding properties. **a** Normalized weighted chemical shift differences (Δ_{av}/Δ_{max}) for KdpBN upon AMP-PNP binding per residue. The secondary structure elements are indicated schematically above. It can be seen that the changes are centered around the cavity forming the binding pocket, i.e. the loop between helices α1 and α2 and the strands β2, β3, and β4, carrying the residues F377 and K395, which are critical for nucleotide binding. **b** Saturation transfer difference (STD) experiment on nucleotide-bound KdpBN and the numbering scheme for AMP-PNP. H2 and H1′ are in close contact to the protein surface, resulting in a high transfer rate, whereas H8 points towards the opening of the cavity.

of the N-domain occurs upon nucleotide binding. Comparison of the side chain chemical shifts also revealed only minor differences [Haupt et al., 2004b]. As chemical shifts respond extremely sensitive to conformational alterations, substantial changes in the overall structure can also be ruled out. Additionally, a CNH-NOESY experiment was also performed on the holoenzyme. The NOE connectivities for critical residues in the core region and in the C- and N-terminal strands show an almost identical pattern, indicating that the local structure in this region has hardly changed.

Characteristics of the Nucleotide-Binding Pocket

Intermolecular NOE coupling restraints were applied for the calculation of the AMP-PNP-binding mode. NOE cross-peaks were observed from M383, K395, and G396 to H2 and H1′ of AMP-PNP (fig. 3b), whereas no NOE cross-peaks were observed for H8 of the nucleotide. In consequence, H8 must be at least 4 Å away from all other aliphatic protons of the protein. The nucleotide-binding mode was calculated using inter-molecular NOE data to position AMP-PNP into the structure of the free domain. F377 undergoes an aromatic π stacking with the purine ring system, whilst the ζ-amino group of K395 is drawn close to the purine from the other side, forming a cation-π interaction with the purine ring. The purine ring is sandwiched between the side chain residues of strands β3 and β5 and capped by helices α1 and α2 in such a way that H8 of AMP-PNP points out of the binding pocket. The triphosphate end group protrudes into the solvent via the opening close to the N- and C-termini (fig. 4).

The calculated binding mode is supported by saturation transfer difference (STD) NMR experiments (fig. 3b), which identify those protons in the ligand that are in close proximity to the protein surface [Mayer and Meyer, 2001; Meyer and Peters, 2003]. Proton H2 shows the most intense transfer of saturation (defined as 100% relative to a standard 1D experiment), thus it has to be closest to the protein surface. H8 exhibits an STD of only 5%, which might be a result of the neighboring aromatic phenyl ring, which cannot be observed in the NOESY spectrum. The H1′ proton of the sugar ring which has a transfer of 56% points towards the β3, β4 and β5 strands. The other protons of the sugar, H3′, H4′, and H5′, show no transfer of magnetization at all (the transfer for H2′ could not be observed, as its resonance overlaps with wa-



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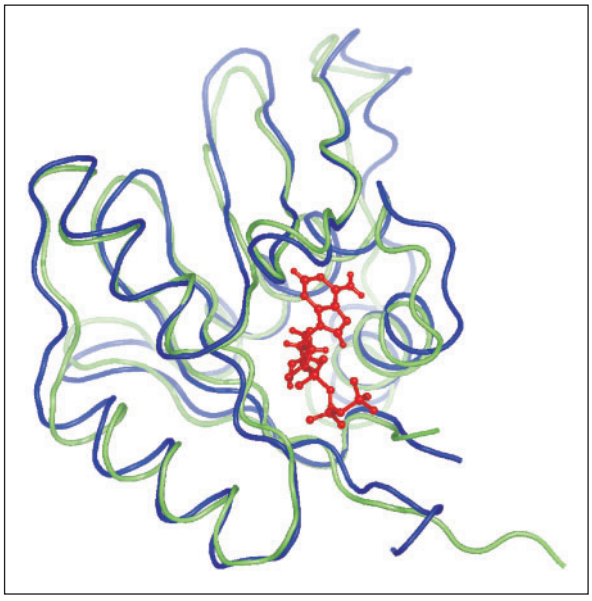

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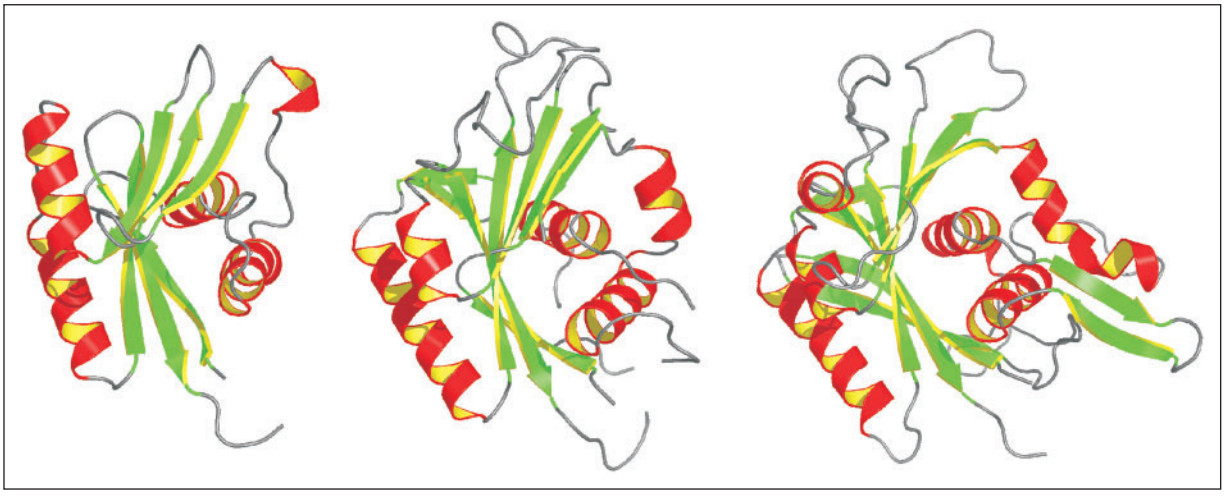

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Fig. 4. The calculated model of AMP-PNP bound to KdpBN. **a** The aromatic moiety of the nucleotide is inserted into the cavity formed by the curved β-sheet and helices α1 and α2, whereas the hydrophilic triphosphate group protrudes into the solvent. **b** A zoom into the binding pocket, depicting all residues that are within a sphere of 10 Å around the nucleotide.

Fig. 5. Superimposition of the apo- and the holoenzyme. The overall structure does not change considerably upon nucleotide binding as already indicated by the chemical shift index evaluation [Haupt et al., 2004b]. Only the N- and C-terminal residues show a high degree of flexibility.

Fig. 6. Comparison of the structures of the nucleotide-binding domains of KdpB (15VJ) [Haupt et al., 2004a], the Na^+, K^+-ATPase (1Q3I) [Håkansson, 2003] and the Ca^{2+}-ATPase (1SU4) [Toyoshima et al., 2000]. KdpBN is the smallest one and represents the basic core motif of a six-stranded antiparallel β-sheet flanked by two α-helices on either side. The additional β-hairpins and α-helices as they are found in class II-IV P-type ATPases are likely to be secondary achievements acquired in the course of evolution (reprinted from Haupt et al. [2004a], with permission from Elsevier).

ter at 4.75 ppm). These data clearly show that only the aromatic head group is positioned within the binding pocket, whereas the hydrophilic sugar ring is not in close contact to the surface of the N-domain.

The Nucleotide-Binding Mode

The nucleotide-binding mode of KdpBN shows the aromatic ring of AMP-PNP to be clipped into the binding pocket by π interactions on both sides. F377 approaches the purine with a π-π stacking interaction. K395 stabilizes the complex from the other side via a cation-π interaction (fig. 4). Both interactions contribute with low binding energies of probably –2 to –5 kcal/mol [Boehr et al., 2002; Gallivan and Dougherty, 2000], which corresponds to the low observed K_D value of 1.4 m*M*. The nucleotide-binding mode of KdpBN thus differs from other ATP-binding domains. In the P-loop ATPases, for example, considerable binding energy is derived from the triphosphate group capping the N-terminus of a helix, in addition to hydrophobic interactions with a conserved phenylalanine or tyrosine residue on the surface of the protein, resulting in a binding constant in the μ*M* range. In contrast, the current study shows that only the aromatic purine system is essential for ATP binding in the P-type ATPase N-domains, whilst the triphosphate group projects into the solvent. This observation is important, as the γ-phosphate group has to be transferred to D307 in the adjacent P-domain. The superimposition of the apo- and the holoenzyme shown in figure 5 clarifies the low-scale conformational changes of the N-domain upon nucleotide binding.

Phylogenetic Classification of KdpB

Comparison of KdpBN to the other known N-domain structures at atomic resolution, i.e. the Na^+,K^+-ATPase crystal structure (1Q3I) [Håkansson, 2003] and the Ca^{2+}-ATPase crystal structure (1SU4) [Toyoshima et al., 2000] revealed that KdpBN represents a core N-domain motif (fig. 6). Pairwise structural alignments (using DALI [Holm and Sander, 1993]) yielded RMSDs of 2.7 Å for KdpBN versus 1Q3I and 2.8 Å versus 1SU4, which indicates good structural agreement in the core region. The structures of the other N-domains possess additional α-helices, an extended β-sheet and additional β-hairpins. Thus, it seems that KdpBN displays not only a minimal scheme for the nucleotide-binding domain, but also a rudimentary ATP-binding motif comprised of F377, K395, and G396. These residues are completely conserved among the type II-IV P-type ATPases. It might be speculated that additional interactions with ATP found in the Na^+,K^+-ATPase and Ca^{2+}-ATPases are secondary acquirements which resulted in higher ATP-binding affinities (E2 site ATP affinity of 30 μ*M* in case of the Ca^{2+}-ATPase [Moutin et al., 1994] compared to 80–120 μ*M* in case of KdpB). Therefore, it is proposed that F377 and K395 provide the basis for nucleotide binding, whereas G396 seems only to be conserved for steric reasons.

Interestingly, neither the KGXXE/D motif nor an equivalent for F377 can be found in the type IB (or heavy metal-transporting) ATPases. Therefore, it was concluded that nucleotide binding in type IB P-type ATPase differs somewhat from that of all other representatives, and that the Kdp-ATPase is misgrouped as a class I P-type ATPase [Axelsen and Palmgren, 1998; Lutsenko and Kaplan, 1995]. Thus, the evolutionary tree of P-type ATPases needs to be redrawn, since the Kdp-ATPase seems to share more similarities with the alkali and earth alkali-transporting (class II-IV) P-type ATPases, than with the heavy metal-transporting class I.

Implications on the Reaction Cycle of the KdpFABC Complex

Ion transport, driven by ATP hydrolysis, is the central feature of all P-type ATPases. Two intermediate states have been identified: the unphosphorylated E1 state with high-affinity ion-binding sites on the extracytoplasmic side of the membrane and the phosphorylated E2 state with low-affinity ion-binding sites on the cytoplasmic side. Their catalytic turnover was first explained for the Na^+,K^+-ATPase in the so-called Post-Albers scheme [Albers, 1967; Post et al., 1972] (fig. 7a). In this scheme, the enzyme has two additional intermediate states; the first where E1 is loosely bound to inorganic phosphate whilst three sodium ions are released to the extracytoplasm and the second where the dephosphorylated E2 state releases two potassium ions into the cytoplasm. A popular extension of the Post-Albers scheme was introduced by DeMeis and Vianna [1979] (fig. 7b) who suggested a spontaneous transition from the E2 to the E1 state prior to ion binding and spontaneous release of the two bound Ca^{2+} ions in the E2-P state without any change in conformation. Both reaction schemes suffer from the fact that the stabilization of transition states has not been taken into account. By now, high-resolution crystal structures of the Ca^{2+}-ATPase are available in both intermediate and transition state-like forms. These structures clearly demonstrated

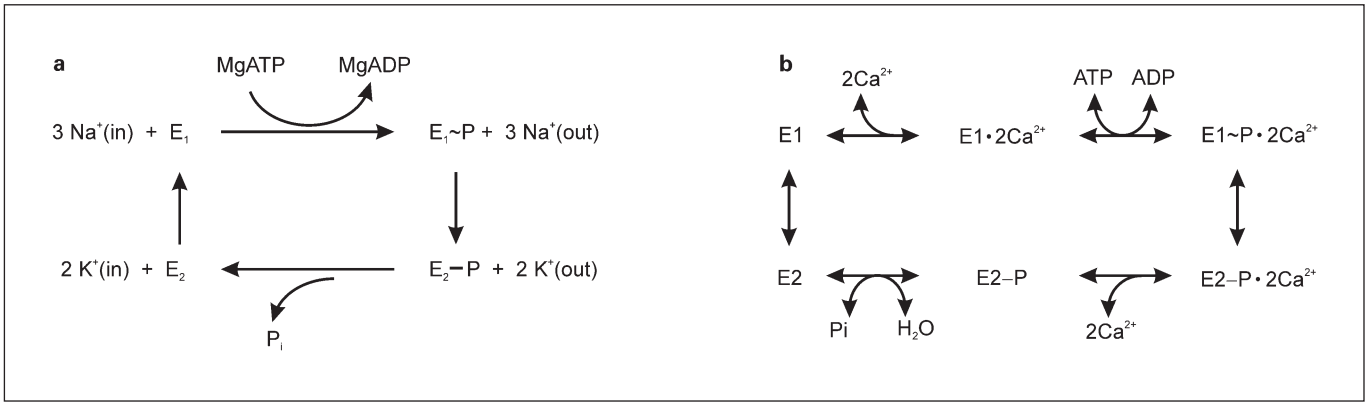

Fig. 7. Popular reaction cycles, approving the existence of the E1/E2 transition. **a** Original Post-Albers scheme [Albers, 1967; Post et al., 1972], defining the basic intermediates during the reaction cycle. **b** Thereof derived cycle of DeMeis and Vianna [1979], which suggests a spontaneous transition from E2 to E1 prior to ion binding and likewise from E1-P to E2-P prior to ion release.

the conformational changes, which occur in the transmembrane domain and the rearrangement of the cytoplasmic domains during the E1/E2 transition at different stages of the cycle [Lykke-Møller Sørensen et al., 2004; Toyoshima and Mizutani, 2004]. However, they do not explain the energy transduction process in this type of enzyme, therefore the mechanism of this rearrangement has still to be elucidated.

The modular design of P-type ATPases has convincingly been demonstrated by the work of Toyoshima et al. [2000]. A specific characteristic of the Ca^{2+}-ATPase structure in the presence of bound Ca^{2+} is that the N-domain is well separated from the phosphorylation site. In order to deliver ATP to this site, the N-domain must undergo a considerable rigid body movement of approximately 110°. A published NMR solution structure of the N-domain of the Na^+,K^+-ATPase from rabbit tried to explain this rigid body movement by structural changes in the secondary structure upon nucleotide binding [Hilge et al., 2003], which should induce rearrangements in the hinge region connecting the N-domain and the P-domain. The critical conformational change was centered on S583 in the C-terminal β-strand, which was reported to undergo a flip from phi/psi angles of 164°/57° in the free form to –79°/178° in the bound form, leading to a repositioning of the hinge region between the N- and P-domains. Although this might serve as a convenient explanation, the observed binding constant K_D of 5.1 m*M* is unlikely to deliver enough energy to disrupt a secondary structure element. Furthermore, the disruption of the β-sheet region was observed in the nucleotide-free form. This finding was later contradicted by the crystal structure of the N-domain of the Na^+,K^+-ATPase from rat solved by Håkansson [2003]. This structure of the apoenzyme was more similar to the structure of the holoenzyme of Hilge et al. [2003]. Thus it seems likely that the conformational change observed by Hilge et al. was due to an artifact in the calculation of the apoenzyme structure. Examination of the NMR data for both forms shows them to be more consistent with the crystal structure of Håkansson [2003] than with the published solution structure. Indeed, recalculation of the domain yields a structure consistent with the crystal structure after re-interpretation of just two ambiguous NOE contacts. This is in accord with our results of the study of the nucleotide-binding properties of the N-domain of the KdpFABC complex [Haupt et al., 2004a]. The absence of an ATP-induced conformational change supports the idea that the N-domain is flexibly linked to the P-domain and that it engages and disengages the P-domain at various stages of the reaction cycle through thermal motion. The very open structure displayed by the Ca^{2+}-ATPase in the Ca^{2+}-bound form most likely represents one extreme of the N-domain's flexible range, stabilized by crystal contacts, and therefore need not to represent the range covered during the reaction cycle. In contrast, it is more likely that the ATPases do not necessarily undergo such severe rigid body motions at each turn of the reaction cycle as this would represent a very time and energy-consuming process which would prevent the fast turnover rate of the cycle. It can be hypothesized that the actual rigid body movements of the cytoplasmic domains during the reaction cycle are only

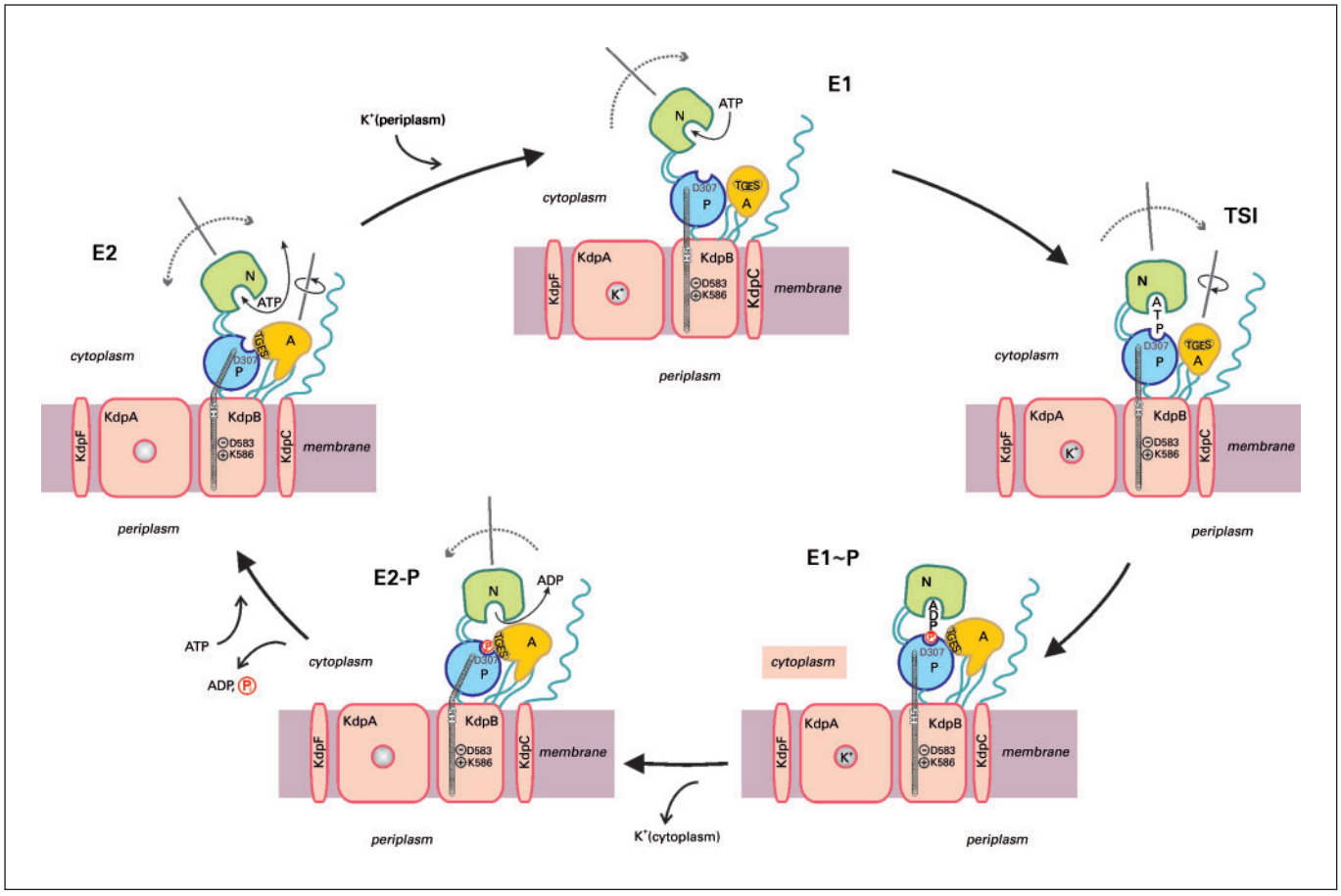

Fig. 8. The proposed reaction cycle for the KdpFABC complex [Haupt et al., 2004a]. The E2 state has high ion affinity on the extracellular side of the membrane. ATP, due to its low binding affinity, is in rapid exchange with the flexibly hinged N-domain (movement indicated by arrows). K^+ binding triggers the start of the reaction cycle, leading to the E1 state. The phosphorylation site (D307) is activated and the N-domain engages; the nucleotide is now bound between the N- and P-domains (TSI). Phosphorylation takes place, the A-domain engages via the TGES motif (E1~P state) followed by conformational changes in the transmembrane domain leading to the E2-P state with low ion-binding affinity on the cytoplasmic side of the membrane (release of K^+). D307 is dephosphorylated by the A-domain (E2 state). Periplasmic K^+ binds, the A-domain disengages and KdpB returns to the E1 state (reprinted from Haupt et al. [2004a], with permission from Elsevier).

at a scale of minimal vibrations, just large enough to allow an effective exchange of ADP by ATP and docking of the A-domain to the P-domain in order to hydrolyze the phosphorylated aspartate residue and re-establish activity. The data obtained for KdpBN support the view that the cytoplasmic domains of P-type ATPases show only minor internal conformational changes upon ligand binding, but are free to undergo considerable rigid body movement with respect to each other.

The concept of a flexible N-domain has important implications for the reaction cycle (fig. 8). It is commonly accepted that binding of the transported ion is a key step leading to a high ATP affinity conformation (E1 state). A flexibly linked N-domain implies that this increase in ATP affinity is the result of activation of the phosphorylation site rather than any effect on the N-domain itself, i.e. the high ATP affinity is the product of the affinities of the N- and P-domain-binding sites in the combined site formed by engagement of the N-domain. For the reaction cycle this means that as long as there are no ions to be transported, the N-domain could swing back and forth with cytoplasmic ATP being bound and released, presumably with a dissociation constant K_D that is similar to that of the isolated N-domain. Under physiological conditions this is not a likely scenario, thus the ATPases are either activated by reversible phosphorylation of a

regulatory domain (class II-IV) or as in the case of the KdpFABC complex, only synthesized when needed and rapidly degraded when no longer required. In this context it is worth mentioning that for the KdpC subunit a regulatory role has been proposed [Gassel and Altendorf, 2001]. Introduction of a charge, i.e. ion binding, induces the rearrangement of the transmembrane helices, here symbolized by stretching of TM5, leading to the E1 state. For the non-physiological case that no ATP is present, the N-domain is still free to swing back and forth, leading presumably to the open state as observed for the SERCA pump in the E1 state (1SU4). Binding of the ion activates at the same time the phosphorylation site in the P-domain. If the N-domain is now approaching the P-domain with an ATP bound, it is drawn towards it. The presence of two binding sites for the nucleotide increases the affinity for ATP drastically, the apparent K_D at pH 7.8 rises from 0.77 m*M* for the isolated N-domain to 100 μ*M* for the intact KdpB protein [Bramkamp and Altendorf, 2004]. This conclusion is consistent with the observation that ATPase activity is coupled to ion binding and not vice versa, not only in the Kdp-ATPase but also in all other P-type ATPases. The existence of this short-lived transition state is included in the figure as transition state I (TSI). A recently proposed mechanism based on considerations of transition state stabilization by Scarborough [2003] corroborates this assumption.

Nucleotide binding and subsequent activation of the γ-phosphate group leads to the E1~P state. The formation of the phosphoenzyme causes again a conformational change of the transmembrane region which lowers the ion affinity towards the release side (E2-P state). This intermediate has been stabilized and crystallized for the Ca^{2+}-ATPase in the presence of decavanadate and thapsigargin, but in absence of Ca^{2+} ions (1KJU) [Xu et al., 2002]. In this structure the N-domain is still in close contact with the P- and A-domain, but this might again represent a crystallization artifact and only represents one possible conformation, as the connecting link between the N- and the P-domain is cleaved and the N-domain is free to disengage. Aspartate 307 can now freely be accessed and dephosphorylated by the A-domain leading to the E2 state. This is immediately followed by ion binding on the uptake side of the membrane, returning the system to the E1 state and reactivating the phosphorylation site so that the cycle can start again.

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References

Albers RW: Biochemical aspects of active transport. Annu Rev Biochem 1967; 36:727–756.

Altendorf K, Epstein W: The Kdp-ATPase of *Escherichia coli*; in Lee AG (ed): Biomembranes (ATPases). London, JAI Press Inc, 1996, vol 5, pp 403–420.

Altendorf K, Gassel M, Puppe W, Möllenkamp T, Zeeck A, Boddien C, Fendler K, Bamberg E, Dröse S: Structure and function of the Kdp-ATPase of *Escherichia coli*. Acta Physiol Scand Suppl 1998;163:137–146.

Aravind L, Koonin EV: The HD domain defines a new superfamily of metal-dependent phosphohydrolases. Trends Biochem Sci 1998;23:469–472.

Axelsen KB, Palmgren MG: Evolution of substrate specificities in the P-type ATPase superfamily. J Mol Evol 1998;46:84–101.

Bertrand J, Altendorf K, Bramkamp M: Amino acid substitutions in putative selectivity filter regions III and IV in KdpA alter ion selectivity of the KdpFABC complex from *Escherichia coli*. J Bacteriol 2004;186:5519–5522.

Boehr DD, Farley AR, Wright GD, Cox JR: Analysis of the π-π stacking interactions between the aminoglycoside antibiotic kinase APH(3′)-IIIa and its nucleotide ligands. Chem Biol 2002;9:1209–1217.

Bramkamp M, Altendorf K: Functional modules of the KdpB subunit of the KdpFABC complex from *Escherichia coli*. Biochemistry 2004;43:12289–12296.

Bramkamp M, Altendorf K: 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. Biochemistry 2005;44:8260–8266.

Bramkamp M, Gassel M, Altendorf K: FITC binding site and *p*-nitrophenyl phosphatase activity of the Kdp-ATPase of *Escherichia coli*. Biochemistry 2004;43:4559–4567.

DeLano WL: The PyMol Molecular Graphics System 0.98beta32 edit. DeLano Scientific LLC, San Carlos, CA, USA. http://www.pymol.org

DeMeis L, Vianna AL: Energy interconversion by the Ca^{2+}-dependent ATPase of the sarcoplasmic reticulum. Annu Rev Biochem 1979;48:275–292.

Dorus S, Mimura H, Epstein W: Substrate-binding clusters of the K^+-transporting Kdp ATPase of *Escherichia coli* investigated by amber suppression scanning mutagenesis. J Biol Chem 2001;276:9590–9598.

Durell SR, Bakker EP, Guy HR: Does the KdpA subunit from the high affinity K^+-translocationg P-type Kdp-ATPase have a structure similar to that of K^+ channels? Biophys J 2000;78:188–199.

Farley RA, Tran CM, Carilli CT, Hawke D, Shively JE: The amino acid sequence of a fluorescein-labeled peptide from the active site of (Na,K)-ATPase. J Biol Chem 1984;259:9532–9535.

Gallivan JP, Dougherty DA: A computational study of cation-π interactions vs. salt bridges in aqueous media: implications for protein engineering. J Am Chem Soc 2000;122:870–874.

Gassel M, Altendorf K: Analysis of KdpC of the K^+-transporting KdpFABC complex of *Escherichia coli*. Eur J Biochem 2001;268:1772–1781.

Gassel M, Möllenkamp T, Puppe W, Altendorf K: The KdpF subunit is part of the K^+-translocating Kdp complex of *Escherichia coli* and is responsible for stabilization of the complex in vitro. J Biol Chem 1999;274:37901–37907.

Håkansson KO: The crystallographic structure of Na,K-ATPase N-domain at 2.6 Å resolution. J Mol Biol 2003;332:1175–1182.

Haupt M, Bramkamp M, Coles M, Altendorf K, Kessler H: Inter-domain motions of the N-domain of the KdpFABC complex, a P-type ATPase, are not driven by ATP-induced conformational changes. J Mol Biol 2004a;342:1547–1558.

Haupt M, Coles M, Truffault V, Bramkamp M, Altendorf K, Kessler H: ^1H, ^{13}C and ^{15}N resonance assignment of the nucleotide binding domain of KdpB from *Escherichia coli*. J Biomol NMR 2004b;29:437–438.

Hilge M, Siegal G, Vuister GW, Guntert P, Gloor SM, Abrahams JP: ATP-induced conformational changes of the nucleotide-binding domain of Na,K-ATPase. Nat Struct Biol 2003;10:468–474.

Holm L, Sander C: Protein structure comparison by alignment of distance matrices. J Mol Biol 1993;233:123–138.

Inesi G, Ma H, Lewis D, Xu C: Ca^{2+} occlusion and gating function of Glu309 in the ADP-fluoroaluminate analog of the Ca^{2+}-ATPase phosphoenzyme intermediate. J Biol Chem 2004;279:31629–31637.

Jørgensen PL, Nielsen JM, Rasmussen JH, Pedersen PA: Structure-function relationships of E1–E2 transitions and cation binding in Na,K-pump protein. Biochim Biophys Acta 1998;1365:65–70.

Jung K, Altendorf K: Towards an understanding of the molecular mechanisms of stimulus perception and signal transduction by the KdpD/KdpE system of *Escherichia coli*. J Mol Microbiol Biotechnol 2002;4:223–228.

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

Lutsenko S, Kaplan JH: Organization of P-type ATPases – Significance of structural diversity. Biochemistry 1995;34:15607–15613.

Lykke-Møller Sørensen T, Møller JV, Nissen P: Phosphor transfer and calcium ion occlusion in the calcium pump. Science 2004;304:1672–1675.

Mayer M, Meyer B: Group epitope mapping by saturation transfer difference NMR to identify segments of a ligand in direct contact with a protein receptor. J Am Chem Soc 2001;123:6108–6117.

Meyer B, Peters T: NMR spectroscopy techniques for screening and identifying ligand binding to protein receptors. Angew Chem Int Ed Engl 2003;42:864–890.

Moutin MJ, Cuillel M, Rapin C, Miras R, Anger M, Lompre AM, Dupont Y: Measurements of ATP binding on the large cytoplasmic loop of the sarcoplasmic reticulum Ca^{2+}-ATPase overexpressed in *Escherichia coli.* J Biol Chem 1994;269:11147–11154.

Pick U: Interaction of fluorescein isothiocyanate with nucleotide-binding sites of the Ca-ATPase from sarcoplasmic reticulum. Eur J Biochem 1981;121:187–195.

Pick U, Bassilian S: Modification of the ATP binding site of the Ca^{2+} ATPase from sarcoplasmic reticulum by fluorescein isothiocyanate. FEBS Lett 1981;123:127–130.

Polarek JW, Williams G, Epstein W: The products of the *kdpDE* operon are required for expression of the Kdp ATPase of *Escherichia coli*. J Bacteriol 1992;174:2145–2151.

Post RL, Hegyvary C, Kume S: Activation by adenosine triphosphate in the phosphorylation kinetics of sodium and potassium ion transport adenosine triphosphatase. J Biol Chem 1972;247:6530–6540.

Puppe W, Siebers A, Altendorf K: The phosphorylation site of the Kdp-ATPase of *Escherichia coli*: site-directed mutagenesis of the aspartic acid residues 300 and 307 of the KdpB subunit. Mol Microbiol 1992;6:3511–3520.

Rhoads DB, Waters FB, Epstein W: Cation transport in *Escherichia coli*. VIII. Potassium transport mutants. J Gen Physiol 1976;67:325–341.

Rice WJ, MacLennan DH: Scanning mutagenesis reveals a similar pattern of mutation sensitivity in transmembrane sequences M4, M5, and M6, but not in M8, of the Ca^{2+}-ATPase of sarcoplasmic reticulum (SERCA1a). J Biol Chem 1996;271:31412–31419.

Ridder IS, Dijkstra BW: Identification of the Mg^{2+}-binding site in the P-type ATPase and phosphatase members of the HAD (haloacid dehalogenase) superfamily by structural similarity to the response regulator protein CheY. Biochem J 1999;339:223–226.

Scarborough GA: Why we must move on from the E1E2 model for the reaction cycle of the P-type ATPases. J Bioenerg Biomembr 2003;35:193–201.

Siebers A, Altendorf K: The K^+-translocating KdpATPase from *Escherichia coli*. Purification, enzymatic properties and production of complex- and subunit-specific antisera. Eur J Biochem 1988;178:131–140.

Siebers A, Kollmann R, Dirkes G, Altendorf K: Rapid, high yield purification and characterization of the K^+-translocating Kdp-ATPase from *Escherichia coli*. J Biol Chem 1992;267:12717–12721.

Toyoshima C, Mizutani T: Crystal structure of the calcium pump with a bound ATP analogue. Nature 2004;430:529–535.

Toyoshima C, Nakasako M, Nomura H, Ogawa H: Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 Å resolution. Nature 2000;405:647–655.

Toyoshima C, Nomura H: Structural changes in the calcium pump accompanying the dissociation of calcium. Nature 2002;418:605–611.

Van der Laan M, Gassel M, Altendorf K: Characterization of amino acid substitutions in KdpA, the K^+-binding and -translocating subunit of the KdpFABC complex of *Escherichia coli*. J Bacteriol 2002;184:5491–5494.

Walderhaug MO, Polarek JW, Voelkner P, Daniel JM, Hesse JE, Altendorf K, Epstein W: KdpD and KdpE, proteins that control expression of the *kdpFABC* operon, are members of the two-component sensor-effector class of regulators. J Bacteriol 1992;174:2152–2159.

Xu C, Rice WJ, He W, Stokes DL: A structural model for the catalytic cycle of Ca^{2+}-ATPase. J Mol Biol 2002;316:201–211.