The Holo-form of the Nucleotide Binding Domain of the KdpFABC Complex from Escherichia coli Reveals a New Binding Mode*§

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P-type ATPases are ubiquitously abundant enzymes involved in active transport of charged residues across biological membranes. The KdpB subunit of the prokaryotic Kdp-ATPase (KdpFABC complex) shares characteristic regions of homology with class II–IV P-type ATPases and has been shown previously to be misgrouped as a class IA P-type ATPase. Here, we present the NMR structure of the AMP-PNP-bound nucleotide binding domain KdpBN of the Escherichia coli Kdp-ATPase at high resolution. The aromatic moity of the nucleotide is clipped into the binding pocket by Phe377 and Lys395 via a π–π stacking and a cation–π interaction, respectively. Charged residues at the outer rim of the binding pocket (Arg317, Arg382, Asp399, and Glu448) stabilize and direct the triphosphate group via electrostatic attraction and repulsion toward the phosphorylation domain. The nucleotide binding mode was corroborated by the replacement of critical residues. The conservative mutation F377Y produced a high residual nucleotide binding capacity, whereas replacement by alanine resulted in low nucleotide binding capacities and a considerable loss of ATPase activity. Similarly, mutation K395A resulted in loss of ATPase activity and nucleotide binding affinity, even though the protein was properly folded. We present a schematic model of the nucleotide binding mode that allows for both high selectivity and a low nucleotide binding constant, necessary for the fast and effective turnover rate realized in the reaction cycle of the Kdp-ATPase.

Ion transport is a vital prerequisite for cellular life, because ions play a crucial role in many biochemical processes, either as cofactors for enzyme function, as substrates for sym- and antiport with other substances, or maintenance of cellular pH or turgor. To maintain ion gradients across their membranes, cells employ highly sophisticated transport systems. Primary transport systems are driven by ATP to achieve transport against concentration gradients. A classic representative is the family of P-type ATPases, which play a fundamental role in the transport of heavy metal, alkali, and earth-alkali ions. Because of their enormous importance, eukaryotic P-type ATPases, such as the Ca2+-ATPase and the Na+-K+-ATPase, have been studied intensively in recent years. A major breakthrough was achieved by Toyoshima et al. (1–3) with the elucidation of the Ca2+-ATPase structure in different reaction cycle intermediates. In addition, the structure of the nucleotide binding domain of the Na+-K+-ATPase was solved by x-ray crystallography (4). However, distantly related members of the P-type ATPase group are less well examined, and in particular, the important question of the evolutionary relationship of the different P-type ATPases remains uncertain.

An unusual prokaryotic P-type ATPase, the KdpFABC complex, serves as a good model to address several questions for both mechanistic and evolutionary aspects. In Escherichia coli, the KdpFABC complex serves as a highly specific potassium transport system, which is only synthesized when the cells’ need for K+ can no longer be satisfied by the constitutive K+ transport systems (5). This P-type ATPase differs from most others by the fact that catalytic activity (KdpB) and ion transport (KdpA) are associated with two different subunits (6–8). For communication between KdpB and KdpA, charged residues within transmembrane helix 5 of KdpB are essential (9). The catalytic subunit KdpB is a P-type ATPase with a unique membrane-bound region (seven transmembrane helices) and a small nucleotide binding domain (17 kDa), probably reflecting an elementary nucleotide binding mechanism. Recently, we solved the solution structure of the apo-form of the nucleotide binding domain of KdpB (KdpBN; Asn316–Gly451) by NMR spectroscopy (10). Based on nucleotide titration experiments and a small number of NOE distance restraints, we proposed a preliminary model of the complex where the nucleotide was docked into apoKdpBN (10). It has been concluded that KdpBN does not show major conformational changes upon ATP binding, in contrast to previous discussions in the literature (11). Rather, it is suggested that KdpBN undergoes a rigid body movement upon ATP binding and that it is connected to the phosphorylation domain via a flexible linker. Here, we report for the first time the atomic resolution structure of the nucleotide bound state of KdpBN based on a complete NOE data set, refining and extending the proposed model. To substantiate the role of certain residues, amino acids Phe377 and Lys395 as well as Ser396 and Asp399 were substituted and their influence on ATP binding in the KdpFABC complex as well as in the isolated KdpBN domain was studied. In contrast to the previously

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§ The on-line version of this article (available at http://www.jbc.org) contains supplementary Table S1 and Figures S1 and S2.

The atomic coordinates and structure factors (codes 2A00 and 2A29) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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5 The abbreviations used are: NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; WaterLOGSY, water-lysigand observed via gradient spectroscopy; HSQC, heteronuclear single quantum correlation; AMP-PNP, adenosine 5’-(β,γ-imido)triphosphate tetra-lithium salt hydrate; CCH, three dimensional 13C-HSQC-NOESY-13C-HSQC; CNH, three dimensional 13C-HSQC-NOESY-1H-HSQC.
formulated notion that KdpB belongs to type I P-type ATPases (12, 13), this study corroborates that KdpB is more closely related to alkali- and earth-alkali-transporting P-type ATPases, sharing most similarities with the proton pump (type III).

MATERIALS AND METHODS

Growth Conditions, Media, and Supplements—E. coli cells were routinely grown in Luria Bertani medium (14) supplemented with the appropriate antibiotic. For the synthesis of recombinant KdpBN proteins, cells were grown in K115 minimal medium, as described previously (15). Strains carrying mutations in the kdp operon were grown in KML or K0 minimal medium with different potassium concentrations, according to Ref. 15. Supplements were added in the following concentrations: ampicillin, 100 μg ml⁻¹; thiamine, 1 μg ml⁻¹; isopropyl-β-D-thiogalactopyranoside, 1 mM.

Generation of KdpB Mutants—The kdpFABC genes expressed from the pSMC10His vector (5) result in a KdpFABC complex, with KdpC carrying a C-terminal decahistidinyl epitope. The primer pairs KdpBK395A/BamHI_rev and KdpBD399A/BamHI_rev (see supplemental Table S1) were used to create constructs carrying the point mutations described above. The resulting PCR fragments were ligated into BamHI sites into appropriately opened pSMC10His, in which a silent BamHI site was previously introduced. Using primer KdpBN2_for in combination with one of the mutagenesis primers KdpBF377A, KdpBF377Y, KdpBD399A, or KdpBS384T (see supplemental Table S1), PCR products were obtained carrying point mutations at positions Phe377 and Ser384, respectively. These DNA fragments were used to create constructs carrying point mutations at positions Phe377 and Ser384, respectively. These DNA fragments were used to create constructs carrying the point mutations described above. The resulting PCR products were ligated via Clal/BamHI into appropriately opened pSMC10His, in which a silent BamHI site was previously introduced. Using primer KdpBN2_for in combination with one of the mutagenesis primers KdpBF377A, KdpBF377Y, KdpBD399A, or KdpBS384T (see supplemental Table S1), PCR products were obtained carrying point mutations at positions Phe377 and Ser384, respectively. These DNA fragments were used to create constructs carrying the point mutations described above. The resulting PCR products were ligated via Clal/BamHI into appropriately opened pSMC10His. Modified KdpFABC complexes were synthesized using TKW3205 cells (5). This strain was routinely used for the expression of kdpFABC constructs, because it lacks the chromosomally encoded kdpFABC, atp, trkA, and trkD genes. Expression of the kdpFABC genes is under the control of the endogenous kdpD and kdpE gene products (6). Because not all kdpB mutants constructed enabled growth on low potassium concentrations, K⁺ concentrations in the millimolar range were used for induction, although the KdpD/E system triggers kdpFABC expression best below 1 mM KCl. Therefore, in these mutants, the expression of kdpFABC differed compared with wild type, which in consequence resulted in low yields of purified KdpFABC complexes. To generate modified KdpB nucleotide binding domains (KdpBN), the primer pair KdpBNfor and KdpBNrev was used to amplify the corresponding DNA fragments from the different pSMC10His derivatives carrying the point mutations described above. The resulting PCR fragments were ligated into NdeI/Xhol into appropriately digested pET16b vector (Novagen). Modified KdpBN proteins were synthesized using BL21(DE3) cells carrying pLYs5 (Novagen). All constructs were checked by DNA sequencing (Department of Botany, University of Osnabrück, Osnabrück, Germany).

Sample Preparation—KdpFABC complexes were purified by affinity chromatography via a histidine tag at the C terminus of KdpC (9). 15N- and 13C-labeled KdpBN were synthesized and purified as described previously (10). Unlabeled KdpBN was purified as described by Bramkamp and Altenendorf (16).

ATPase Assay—ATPase activity of purified KdpFABC complex was determined using the microtitrerie plate assay of Henkel et al. (17) following the modifications described previously (18).

NMR Spectroscopy of KdpBN—The AMP-PNP-bound NMR experiments (22) and WaterLOGSY experiments (23) were performed on unlabeled samples of wild type and mutants F377A, F377Y, K395A, and D399A at protein concentrations of 0.5 mM and AMP-PNP concentrations of 5 mM. For the integration of the one-dimensional proton spectra of the saturation transfer difference and WaterLOGSY experiments, the exact relative sample concentration was determined by integrating over the δ-methyl signal of Leu355 at ~0.2 parts/million. However, there is still a residual background from protein resonances visible in the WaterLOGSY spectra; therefore, the signal of H1‘ was chosen for evaluation, as it does not overlap with protein signals. In a reference WaterLOGSY experiment, a 5 mM solution of AMP-PNP only was recorded under similar buffer conditions. This represents the nonbinding case for the specific ligand signal and thus the integral over the H1‘ signal was set to ~100%. All spectra were processed with X-WINNMR (Bruker Analytik GmbH, Karlsruhe, Germany) and evaluated with SPARKY software programs. Integration of the one-dimensional spectra was performed with X-WINNER.

 Backbone Dynamics—For R₁ and R₂, experiments, typically 10 relaxation delays and three duplicate points for error estimation were recorded using experiments published earlier (24). In the 15N dimension, 80 complex points were sampled with 48 transients and a recycle delay of 2 s between successive scans. For the heteronuclear NOE, two sets of experiments consisting of spectra with and without saturation of the triad amide protons were recorded. A 15-fold molar excess of AMP-PNP was used. All NMR experiments (22) and WaterLOGSY experiments (23) were performed on unlabeled samples of wild type and mutants F377A, F377Y, K395A, and D399A at protein concentrations of 0.5 mM and AMP-PNP concentrations of 5 mM. For the integration of the one-dimensional proton spectra of the saturation transfer difference and WaterLOGSY experiments, the exact relative sample concentration was determined by integrating over the δ-methyl signal of Leu355 at ~0.2 parts/million. However, there is still a residual background from protein resonances visible in the WaterLOGSY spectra; therefore, the signal of H1‘ was chosen for evaluation, as it does not overlap with protein signals. In a reference WaterLOGSY experiment, a 5 mM solution of AMP-PNP only was recorded under similar buffer conditions. This represents the non-binding case for the specific ligand signal and thus the integral over the H1‘ signal was set to ~100%. All spectra were processed with X-WINNMR (Bruker Analytik GmbH, Karlsruhe, Germany) and evaluated with SPARKY software programs. Integration of the one-dimensional spectra was performed with X-WINNER.

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Structural Consequences of Nucleotide Binding—In general, the chemical shifts of apo- and holoKdpBN show only minor differences. Shifts characteristic of secondary structure elements, C\(^\alpha\), H\(^\alpha\), and C\(^\beta\) show changes that do not influence the classification of the residues according to the chemical shift index protocol (33). The secondary structure and the average estimated solvent accessibility of both sets of 19 structures, as determined by the program PROCHECK (34), are shown in Fig. 2, left panel. The only significant modification occurs to the C-terminal end of helix a1, which is extended by three residues upon nucleotide binding. The superimposition of the minimized averaged structures of the apo- and holoenzyme, shown in Fig. 2, right panel, exhibits a root mean square deviation of 1.26 Å over ordered backbone residues.

A more thorough examination of the chemical shift data helps in understanding the binding mechanism. In supplemental Fig. S1, the difference of the chemical shifts of holo- to apoKdpBN of selected nuclei is plotted against the sequence. C\(^\alpha\), H\(^\alpha\), and C\(^\beta\) show noticeable changes that do not influence the classification of the residues according to the chemical shift index protocol (33). The secondary structure and the average estimated solvent accessibility of both sets of 19 structures, as determined by the program PROCHECK (34), are shown in Fig. 2, left panel. The only significant modification occurs to the C-terminal end of helix a1, which is extended by three residues upon nucleotide binding. The superimposition of the minimized averaged structures of the apo- and holoenzyme, shown in Fig. 2, right panel, exhibits a root mean square deviation of 1.26 Å over ordered backbone residues.

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Corresponding residue in strand β3. Ala379 and Arg382 are both located at the edges of the turn, their side chains pointing toward the binding pocket. The positively charged side chain of arginine stabilizes the triphosphate group of the nucleotide. The side chain of Arg317 points toward the binding pocket and forms a hydrogen bond to the triphosphate group opposite to that formed by Arg382.
Chemical shifts of methyl groups are sensitive probes for structural alterations and electrostatic interactions caused by the ligand. Interestingly, the methyl carbons are hardly affected at all in contrast to the methyl protons close to aromatic systems. Significant changes in chemical shift were observed for residues in the loop region between helices H9251 and H9252 (see supplemental Fig. S1), as well as for Leu431 situated on strand H9252; its side chain is located in proximity to the purine ring. Both methyl groups come as close as 3–4 Å to the aromatic system, explaining the strong upfield shift.

Characterization of the Binding Pocket

The core of the binding pocket is dominated by neutral residues. Only one negatively charged residue, Asp344, is situated at the center and forms a hydrogen bond to the amino group of the nucleotide (N6). The only positively charged residue within the core is Lys395, forming a cation-π-stacking interaction to the six-membered part of the purine ring of AMP-PNP. Two positively charged residues, Arg317 and Arg382, are situated diametrically opposed to each other on the outer rim of the binding pocket (Fig. 3a). Both form hydrogen bonds to the triphosphate group of the nucleotide and orient it toward the phosphorylation domain. Somewhat further away (6–8 Å) are two negatively charged residues, Glu348 and Asp399. They are also diametrically opposed to each other, and their axis forms an angle of ≈45° with respect to the axis defined by Arg317 and Arg382 (Fig. 3b). These residues may provide stabilization by electrostatic repulsion within a certain range of flexibility. Furthermore, electrostatic interactions between the protein and the ligand are established by Ser317 and N1 of AMP-PNP. Most residues in the binding pocket are, however, hydrophobic. The most important interaction is established by Phe377, which exhibits a π-π stacking with the nucleotide, whereas Leu431 approaches the five-membered ring of AMP-PNP from the other side.

Backbone Dynamics

To further characterize structural changes upon binding of AMP-PNP, the backbone dynamics of apo- and holo-KdpBN were investigated on a fast (pico- to nanosecond) and a slow (millisecond) time scale, using the amide nitrogen as probes. Model-free analysis (35, 36) of the R1, R2 and heteronuclear NOE data show that internal motions on the fast time scale in the secondary structure elements of KdpBN are highly restricted, with averaged squared order parameters of 0.90 ± 0.01 for the apoprotein and 0.91 ± 0.01 for the holo-form, respectively. Regions with higher internal mobility are found in the loops connecting helix α2 and the 310 helix (Val367–Leu370), between helices α3 and α4 (Asn408–Phe412), and at the C terminus (Ile448–Gly451); for the latter residues, virtually unrestricted motion is
Nucleotide Bound State of KdpBN

As expected, the majority of residues showed no alteration in their dynamic behavior on a fast time scale upon ligand binding (see Fig. 4). Changes in the squared order parameter, defined as $\Delta(S^2) = S^2(\text{holo}) - S^2(\text{apo})$, are considered to be significant, if the uncertainty is less than half of the corresponding parameter value. A dramatic decrease in $S^2$ upon ligand binding is observed for Ser341. This increase in mobility, also reflected in the relaxation parameters ($R_2$ is reduced by a factor of 2 in the complex), is somewhat surprising, because Ser341 is located in a loop region in the apoprotein, whereas it is part of helix α1 in the complex. Phe377, located at the C-terminal end of strand β2, also experiences a higher degree of internal mobility in the holo-form, as indicated by the negative value of $\Delta(S^2)$. In the apo-form, the amide proton of Phe377 is hydrogen-bonded to A343C, an interaction that is absent in the complex as a consequence of a different orientation of Ala343 due to the elongation of helix α1. In contrast, Thr378 features a positive $\Delta(S^2)$ value. Thr378 is involved in two hydrogen bonds with Met383 (T378HN–M383M and M383HN–T378CH); surprisingly, a negative $\Delta(S^2)$ value is observed for Met383. The two regions of higher mobility in the apo-form appear more rigid in the complex.

On a slow (ms) time scale, 42 residues subject to chemical exchange, with an average exchange rate constant ($k_{\text{ex}}$) of $881 \pm 40 \text{ s}^{-1}$, were identified by apoKdpBN. In the complex, 63 residues were found to be affected by an exchange process, with an $k_{\text{ex}}$ of $414 \pm 36 \text{ s}^{-1}$. For residues subject to chemical exchange, the data were refitted assuming that all residues were affected by the same process, and the average values of $k_{\text{ex}}$ were used. For residues fitted to the general equation, averaged populations of the major populated state of 0.98 ± 0.01 and 0.96 ± 0.01 were obtained for the apo- and holo-form, respectively. In the absence of ligand, residues with exchange contributions cluster around the nucleotide binding site. In addition, exchange contributions are found for a number of residues in helix α4. For complexed KdpBN, chemical exchange affected a larger number of residues. In addition to residues in proximity to the binding site, the N-terminal winding of helix α2, virtually all residues in helix α4, and the C-terminal part of strand β5 showed significant exchange contributions. The differences in chemical shift for the apo- and holo-form, as obtained from the fitting procedure assuming a two-site exchange, suggest that the conformations adopted by KdpBN are not altered by ligand binding (see Fig. 5). A proposed mechanism by which ligand binding can be linked to dynamic changes distant from the binding site is presented in supplemental Fig. S2.

ATPase Activity of Different KdpB Mutants—To investigate the importance of individual residues for ATP hydrolysis, several KdpFABC mutants were constructed. The residues chosen for mutation were those of KdpBN, which showed strong alterations in their backbone 15N chemical shift upon nucleotide addition (10). In each case, mutant KdpFABC complexes were detergent-solubilized from the membrane, affinity-purified via a histidine tag at the C terminus of KdpC, and analyzed for subunit composition by SDS-PAGE. Mutations in the N-domain of KdpB did not destabilize the KdpFABC complex as observed for mutations within the transmembrane region of KdpB (9). The ATPase activities of KdpFABC complexes are usually stimulated with 1 mM KCl by a factor of 3–5, and this was also observed for these mutant protein complexes. Because the $K^+$ affinity of the wild-type KdpFABC complex is quite high (2 μM), $K^+$ impurities in the reaction buffer contribute to an already elevated basal activity of the protein, explaining the low stimulation factor for the wild type. In addition, the sensitivity of the ATPase activity to ortho-vanadate is comparable with wild type, indicating that all mutant protein complexes were capable of performing a full reaction cycle with E1–E2 transition. The results, summarized in Table 2, illustrate the important roles of amino acids Phe377 and Lys395. Although changes in other amino acids located within the nucleotide binding

![FIGURE 4. Plot of $\Delta S^2$ versus the residue number of KdpBN. Changes are considered to be significant if the error is less than half of the parameter value and are represented by filled circles.](image1)

![FIGURE 5. Correlation of the chemical shift differences obtained from the analysis of millisecond motions for KdpBN in the absence (x-axis) and presence (y-axis) of AMP-PNP; the regression coefficient of the correlation is 0.91. ppm, parts/million.](image2)

**TABLE 2**

| ATPase activity of different KdpB mutants | 0 mM KCl | 1 mM KCl | Ortho-vanadate sensitivity |
|-----------------------------------------|---------|---------|---------------------------|
| Wild type                               | 1448    | 3164    | ND                        |
| F377A                                   | NA      | NA      | +                         |
| F377Y                                   | 953     | 2785    | +                         |
| S384A                                   | 272     | 1585    | +                         |
| S384T                                   | 294     | 1090    | +                         |
| K395A                                   | 130     | 468     | +                         |
| D399A                                   | 333     | 1258    | +                         |
Mutational effects on the ligand binding properties of Phe377 and Lys395. Displayed are the values for the wild-type enzyme (WT) and the mutants F377Y, F377A, and K395A. Both the WaterLOGSY experiment (a) and the saturation transfer difference experiment (b) show a nucleotide binding strength of 60–70% of the wild-type enzyme for the conservative mutant F377Y, whereas the alanine mutants F377A and K395A have almost completely lost their ability to bind nucleotide. ANP is the abbreviation for a solution of AMP-PNP at the same concentration and buffer condition as present in the protein samples.

Nucleotide Bound State of KdpBN

As strong binding ligands are indistinguishable from non-binding ligands in WaterLOGSY, saturation transfer difference experiments (22, 37) were also performed. This experiment shows positive signals for binding ligands, with intensities proportional to the distance of individual groups from the protein surface. The most intense signal in the complex of AMP-PNP and wild-type enzyme was standardized to 100%.

Ligand Binding Properties of Phe377 and Lys395. Mutants Examined by NMR Spectroscopy—The WaterLOGSY experiment (23) is an ideal tool for studying weak binding ligands. The non-binding case (i.e. AMP-PNP in buffer without protein) provides the maximum negative signal, which can be standardized to −100% (Fig. 6a). Binding ligands show increasingly positive signals, reaching a maximum at concentrations around binding constant. The ligand binds to the wild-type protein with an intensity of 64%. The conservative mutant F377Y has the highest intensity of +46%, which corresponds to 72% of wild-type binding capacity. F377A and K395A show only weak residual interaction with the ligand, i.e. −25% and −34%, respectively.

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This was observed for the aromatic proton H2, which points directly into the binding pocket and which is involved in a close network of NOE contacts to the surrounding protein. The second observable aromatic proton is H8. It points toward the mouth of the binding pocket and is far from the protein surface. Consequently, the observed signal is weak (5%). H1′, the only observable proton from the sugar moiety has an intensity of 56%; it is closely anchored to the strands β3 and β4 via multiple NOE contacts (Fig. 6b). H2′ resonates at the same frequency as water and is thus not detectable. The other protons, H3′, H4′, and H5′ do not show a detectable signal, but they also resonate close to the water frequency and hence may be suppressed by the water suppression scheme. The sugar proton signals may also be below the detection threshold of ~5%. The conservative F377Y mutant shows 61% residual signal intensity for the H2 proton compared with the wild type, and 2.6 and 33% for H8 and H1′, respectively. The net signal intensity of the F377A mutant to the ligand is already below 5% compared with the wild type, whereas the K395A mutant no longer shows any signals. These results confirm that the mutated residues are responsible for ligand binding, as the mutants no longer have the ability to bind nucleotide, although NMR shows them to be natively folded and stable over several days.

DISCUSSION

A central question arising from the modular design of P-type ATPases has always been how nucleotide binding is realized in the clearly separated N-domain. In the very well studied case of P-loop ATPases, ATP is bound via a hydrophobic π–π stacking to a conserved phenylalanine or tyrosine residue on the surface of the enzyme, and additionally, the triphosphate group of ATP caps the N terminus of an α-helix, leaving it embedded in a close network of hydrogen bonds. Evidently, this cannot be the case for P-type ATPases. It had been found earlier that a highly conserved lysine residue in the KGXX(D/E) motif and a phenylalanine 20–25 amino acids upstream in the sequence are indispensable for nucleotide binding (11, 38). However, both crystal structures of the Ca2+-ATPase with AMP-PCP bound (1, 39) could not explain the necessity of the lysine residue. The modeling of the N-domain of KdpB with AMP-PNP bound, which was based on intermolecular distance restraints, suggested that the positively charged N′ end group is involved in a cation–π interaction with the aromatic part of the nucleotide residue. The α-carbon for the holo-form based on a completely new set of NMR data presented here confirms and extends this previous model. In the apo-form, the acidic side chain of Asp344 points directly into the binding pocket and which is involved in a close network of NOE contacts to the surrounding protein. The second observable aromatic proton is H8. It points toward the mouth of the binding pocket and is far from the protein surface. Consequently, the observed signal is weak (5%). H1′, the only observable proton from the sugar moiety has an intensity of 56%; it is closely anchored to the strands β3 and β4 via multiple NOE contacts (Fig. 6b). H2′ resonates at the same frequency as water and is thus not detectable. The other protons, H3′, H4′, and H5′ do not show a detectable signal, but they also resonate close to the water frequency and hence may be suppressed by the water suppression scheme. The sugar proton signals may also be below the detection threshold of ~5%. The conservative F377Y mutant shows 61% residual signal intensity for the H2 proton compared with the wild type, and 2.6 and 33% for H8 and H1′, respectively. The net signal intensity of the F377A mutant to the ligand is already below 5% compared with the wild type, whereas the K395A mutant no longer shows any signals. These results confirm that the mutated residues are responsible for ligand binding, as the mutants no longer have the ability to bind nucleotide, although NMR shows them to be natively folded and stable over several days.

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ing constant (1.4 mM for the isolated N-domain). The mobility on a slow
time scale observed for the loop connecting helices $\alpha_1$ and $\alpha_2$ suggests
that a motion of this stretch is necessary to provide access to the ligand
binding site. Furthermore, our data show that this motion is present in
both the apo- and holo-form of the protein; thus these slow motions
likely represent an inherent property and do not require energy associ-
ated with the ligand binding event.

To compensate for this low affinity binding site described above,
charged residues were placed strategically on the outer rim of the bind-
ing pocket, which directed the triphosphate end group via electrostatic
attraction and repulsion toward the solvent, thereby making it point
toward the phosphorylation domain. The nucleotide itself is forced to
adopt an almost linear form, accessorially facilitating the release of the
$\gamma$-phosphate to the neighboring P-domain. This peculiar binding mode
where the nucleotide is “clipped” into the binding pocket by Phe$^{377}$,
Lys$^{395}$, and Asp$^{344}$ in the core and stabilized and directed by Arg$^{317}$ and
Arg$^{382}$ on the surface, is depicted schematically in Fig. 8.

The Ca$^{2+}$-ATPase showed a similar arrangement of positive charges
around the outer rim of the nucleotide binding pocket; Arg<sup>489</sup> and Arg<sup>560</sup> both participate in the orientation of the β-phosphate (1). Mutation of Arg<sup>560</sup> in the Ca<sup>2+</sup>-ATPase reduced the apparent ATP affinity >100-fold (42) and showed a drastically reduced steady state level of phosphoenzyme production (5, 43), obviously because of a hydrogen bond formed between Arg<sup>298</sup> and the P-domain (1). Despite these similarities, there is one major difference concerning the apparent nucleotide binding mode. The salt bridge formed by Lys<sup>395</sup> and Asp<sup>442</sup> in apoKdpBN is opened in holoKdpBN in favor of newly established contacts toward the nucleotide; Asp<sup>442</sup> makes a hydrogen bond to the amino group of AMP-PNP and Lys<sup>395</sup> is drawn toward the purine ring. This salt bridge finds its counterpart in the Ca<sup>2+</sup>-ATPase, Arg<sup>560</sup> both participate in the orientation of the nucleotide phosphate (1). Muta-

tistical properties of the N-domain are a result of fine-

cycle. It can be speculated that the different geometries of the nucleotide

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