Journal of Bioenergetics and Biomembranes, Vol. 33, No. 5, October 2001 (© 2001)

Role of Conserved TGDGVND-Loop in Mg^{2+} Binding, Phosphorylation, and Energy Transfer in Na,K-ATPase[1]

Peter Leth Jorgensen,[2,3] Jesper R. Jorgensen,[2] and Per Amstrup Pedersen[2]

In the P-domain, the 369-DKTGTLT and the 709-GDGVNDSPALKK segment are highly conserved during evolution of P-type E_1–E_2-ATPase pumps irrespective of their cation specificities. The focus of this article is on evaluation of the role of the amino acid residues in the P domain of the α subunit of Na,K-ATPase for the $E_1P[3Na] \rightarrow E_2P[2Na]$ conversion, the K^+-activated dephosphorylation, and the transmission of these changes to and from the cation binding sites. Mutations of residues in the TGDGVND loop show that Asp^{710} is essential, and Asn^{713} is important, for Mg^{2+} binding and formation of the high-energy $MgE_1P[3Na]$ intermediate. In contrast Asp^{710} and Asp^{713} do not contribute to Mg^{2+} binding in the E_2P–ouabain complex. Transition to E_2P thus involves a shift of Mg^{2+} coordination away from Asp^{710} and Asn^{713} and the two residues become more important for K^+-activated hydrolysis of the acyl phosphate bond at Asp^{369}. Transmission of structural changes between the P-domain and cation sites in the membrane domain is evaluated in light of the protein structure, and the information from proteolytic or metal-catalyzed cleavage and mutagenesis studies.

KEY WORDS: P-domain; Na,K-ATPase; K^+-activated; Asp.

INTRODUCTION

Renal [$\alpha 1 \beta 1 \gamma$] Na,K-ATPase consists of three subunits, α subunit with 1016 residues, β subunit with 302, and the γ subunit with 55 amino acids and it is the largest protein complex in the family of cation pump proteins (Jorgensen, 1962; Jorgensen and Andersen, 1988). The α subunit has about 30% amino acid sequence homology to Ca-ATPase of SR (994 residues) (Lingrel and Kuntzweiler, 1994). Their structural organization is similar and they show high homology in selective regions near sites for ATP binding, phosphorylation, Mg^{2+} binding, and in transmembrane segments (Axelsen and Palmgren, 1998). The structure at 2.6 Å resolution of SR Ca-ATPase appeared recently (Toyoshima *et al*., 2000). In this structure, each of two Ca^{2+} ions is coordinated by six oxygen groups of residues in transmembrane segments M4, M5, M6, and M8. The cytoplasmic protrusion is split in three domains with a single nucleotide binding N domain separated from the P domain comprising the phosphorylated 351-DKTGTLT and the C-terminal 701-TGDGVNDAPALKK segments, both highly conserved among P-type E_1–E_2-ATPase pumps of widely different cation specificities. The A domain, consisting of the second cytoplasmic loop and the N-terminus, is important for the conformational transitions and it may work as an anchor for the N domain. This is the first high-resolution structure of any member of the family of cation pumps and it forms an important provisional model for the α subunit of Na,K-ATPase. The available high-resolution structure is a model of the $E_1[2Ca]$ form, but information can also be inferred about the conformational transitions that are required for transfer of the energy from ATP hydrolysis to cation translocation sites in membrane-spanning segments of the α subunit. Conformational sensitive proteolytic splits can be localized in the sequences of Na,K-ATPase (Jorgensen and Andersen, 1988; Jorgensen, 1975; Jorgensen and Collins,

[1] Key to abbreviations: L23, loop between transmembrane segments 2 and 3 in α-subunit of Na,K-ATPase; M4, transmembrane segment 4 of 10 segments in α subunit of Na,K-ATPase; S4, cytoplasmic extension of M4.

[2] Biomembrane Research Center, August Krogh Institute, Copenhagen University, 2100 Copenhagen OE, Denmark.

[3] To whom all correspondence should be mailed. email: pljorgensen@aki.ku.dk

0145-479X/01/1000-0367$19.50/0

1986) and Ca-ATPase (Jorgensen and Andersen, 1988; Andersen *et al*., 1986; Møller *et al*., 1996). Structural changes have also been localized in site-directed mutagenesis experiments (Daly *et al*., 1996; Vilsen, 1997; Sorensen and Andersen, 2000) and by metal-catalyzed cleavage (Goldshleger and Karlish, 1999; Patchornik *et al*., 2000) of the protein.

In contrast to Ca-ATPase, the Na,K-pump protein alternates between two well defined cation complexes $E_1P[3Na]$ and $E_2[2K]$ to transport three Na^+ ions out and two K^+ ions into the cell in coupling with ATP hydrolysis, according to the reaction cycle in Fig. 1. Translocation of Na^+ is coupled to the transfer of γ-phosphate from ATP to an acyl bond at Asp^{369} of the α subunit and transition from the high-energy intermediate $E_1P[3Na]$ to the low-energy $E_2P[2Na]$ phosphoform is followed by exchange of Na^+ for K^+ and phosphoenzyme hydrolysis (Jorgensen and Andersen, 1988). Interactions between the phosphorylated segment 369-DKTGTLT and the neighboring 708-TGDGVNDSPALKK β-strand–loop–α-helix and the transfer of energy to the cation sites must be resolved to understand the molecular mechanism of these essential reactions. Until recently, it was not clear whether the TGDGVND loop would be involved in nucleotide interactions (Ohta *et al*., 1986; Ovchinnikov *et al*., 1987; Lane *et al*., 1993) or in Mg^{2+} binding (Girardet *et al*., 1993; Jorgensen *et al*., 1997). After solving problems related to expression in yeast of mutations to charged residues in this loop (Jorgensen and Pedersen, 2001), it has been possible to clarify their role for high-affinity binding of ATP and their contributions to Mg^{2+} binding and transfer of γ-phosphate to Asp^{369} in the E_1P form (Pedersen *et al*., 2000). Mutagenesis data show that the transition to E_2P involves a shift of Mg^{2+} coordination and that the TGDGVND loop becomes more important for hydrolysis of the acyl phosphate bond at Asp^{369}. Asp^{710} and Asn^{713} are essential for stabilization of the transition state and for the K^+-stimulated phosphoenzyme hydrolysis (Pedersen *et al*., 2000).

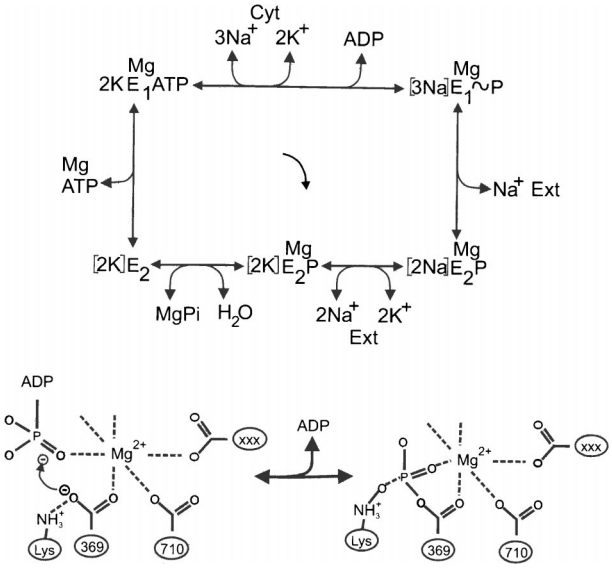

Fig. 1. (Upper) Minimum scheme of E_1–E_2 reaction cycle of the Na,K pump with ping-pong sequential cation translocation. The phosphoforms show specificity for Na^+ and dephosphoforms bind K^+, Rb^+, or $T1^+$ tightly. [Na^+] or [K^+] within brackets are tightly bound and prevented from exchanging with medium cations (Jorgensen and Andersen, 1988). (Lower) Model of the role of Asp^{710} in coordination of Mg^{2+} and transfer of the γ-phosphate from ATP to a covalent acyl-phosphate intermediate at Asp^{369}. Modified from a previous model (Ridder and Dijkstra, 1999) with suggestions concerning the nucleophilic attack on the phosphate group.

The focus of this article is on evaluation of the role of the amino acid residues in the P domain for the catalytic process and in the structural changes accompanying $E_1P[3Na] \rightarrow E_2P[2Na]$ conversion and dephosphorylation to the $E_2[2K]$ form. Transmission of these changes to and from the cation binding sites is discussed as well. The results of mutagenesis studies will be evaluated in light of the three-dimensional structure model (Toyoshima *et al*., 2000) and the available information from proteolytic cleavage (Jorgensen and Andersen, 1988), mutagenesis studies (Daly *et al*., 1996; Vilsen, 1997; Sorensen, and Andersen, 2000, and metal-catalyzed cleavage (Patchornik *et al*., 2000) to identify and locate structural transitions in the protein accompanying the cation translocation processes.

Structure of P and N Domain

The structure of the P domain of Ca-ATPase, SERCA1 (1EUL) (Toyoshima *et al*., 2000) is an adequate model of the P domain of Na,K-ATPase, since there is extensive homology between the important catalytic segments in the P domain of the two proteins. Analysis of the quality of the 1EUL structure model shows only minor deviations from stereochemical ideality for the sequences in the P domain. Procheck analysis (Laskowski *et al*., 1993) indicates that 97% of the residues are in allowed regions of the Ramachandran plot. Most outlier residues in the plot are located in extracellular loops and in connections between domains.

The P domain in the central part of the cytoplasmic headpiece is organized in a Rossman fold comprising two parts (Toyoshima *et al*., 2000), (Figs. 2 and 3). The N-terminal part consists of 30 residues and treads from M4 via helix-1 and a β-strand to the phosphorylated residue, Asp^{351}. It continues via the conserved loop 352-KTGTV and the TTN link to the N domain. The C-terminal part

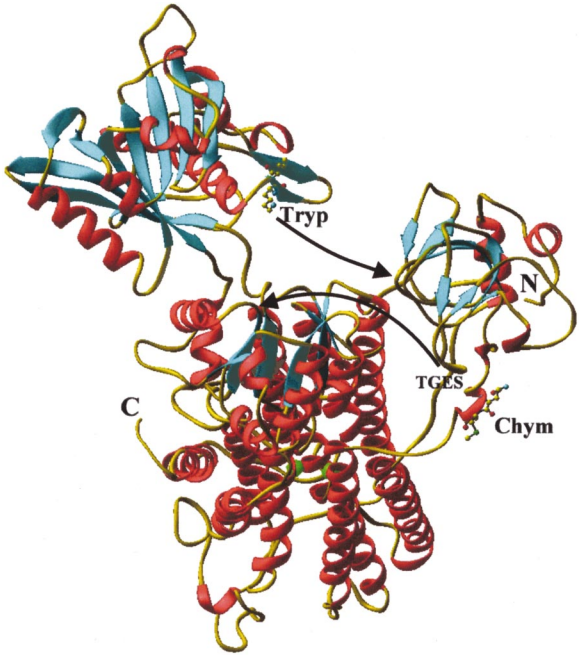

Fig. 2. Ribbons model of the overall structure of 1EUL Ca-ATPase in the E_1[2Ca(form (Toyoshima *et al*., 2000). In the N-domain, (Tryp) indicates the position in the sequence of Ca-ATPase of residues homologous in position to the peptide bond (Arg^{438}) that is cleaved by trypsin in the E_2[2K] conformation of α subunit of Na,K-ATPase. In the A domain, (Chym) indicates the homologous position of the peptide bond (Leu^{266}) that is cleaved by chymotrypsin in the E_1[3Na] form of α subunit of Na,K-ATPase. An arrow from 181-TGES points at the 625-TGD loop in P domain to indicate the movement of the A domain accompanying transition from E_1 to E_2 forms of the protein. An arrow from (Tryp) indicates the movement of the N domain to interact with the A domain in the E_2[2K] conformation of Na,K-ATPase (see text).

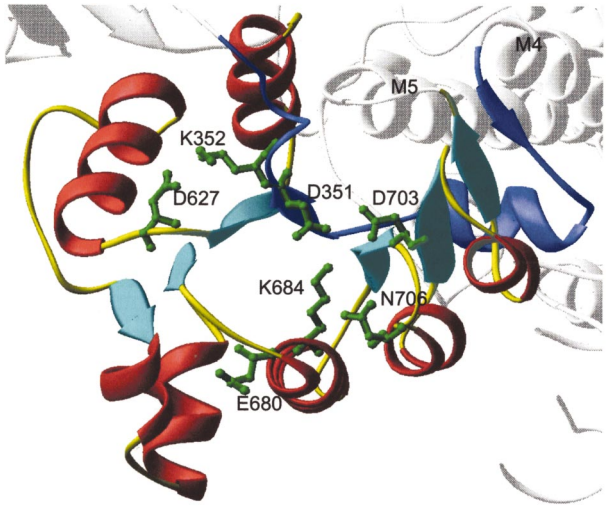

Fig. 3. Ribbons model of detailed structure of the P domain of Ca-ATPase in the E_1[2Ca] form (Toyoshima *et al*., 2000). The C-terminal component is colored according to secondary structure. The function of numbered residues in green color is discussed in the text.

(132 residues) begins at the 661-DPPR hinge from the N domain and connects to the 60 Å-long helical rod of S5 and M5. It forms six parallel β-strands with seven associated helixes. The fourth of these parallel β-strands continues as the 701-TGDGVNDAPALKK loop–α-helix and it is adjacent to the β-strand containing the phosphorylation site. The oxygens of the carboxylate group of Asp^{703} (Asp^{710} in Na,K-ATPase) are positioned less than 3 Å from the oxygens of the carboxylate group of the phosphorylated residue Asp^{351}(Fig. 3).

As illustrated in Fig. 2, the nucleotide binding N domain is inserted as a subdomain of the P domain with the N-terminal connection extending from the phosphorylation site with the 352-KTGTV sequence and the C-terminal 661-DPPR hinge. This structure is characteristic of the Rossman fold, originally described in the structures of nucleotide binding domains of dehydrogenases (Rossman and Liljas, 1974) and also in dehalogenases (Hisano *et al*., 1996). In these structures, the conserved residues in the loops connecting the β-strands and α-helixes of the P domain, the 625-TGDNK, 351-DKTGT, 684-KSK, and 701-TGDGVND sequences in Ca-ATPase contribute to catalysis in the active site.

The sequence of the N domain in Ca-ATPase, Gln^{360}–Arg^{604} (Toyoshima *et al*., 2000), correspond to Arg^{383}–Arg^{589} in α subunit of Na,K-ATPase. Both sequences terminate with the invariant 586-DPPR (Axelsen and Palmgren, 1998) motif connecting to the P domain. Other homologies in alignments suggest that Phe^{475} (McIntosh *et al*., 1999a) in Na,K-ATPase is important for binding of the adenine moiety. The receptor for 8-azido-ATP, Lys^{480} (Tran *et al*., 1994; Scheiner-Bobis and Schreiber, 1999), the site of FITC labeling, Lys^{501} (Karlish, 1980; Wang and Farley, 1992), and Arg^{544} (Jacobsen *et al*., 2001) may also be part of the ATP binding domain. Other parts of the sequences differ considerably and the overall amino acid sequence homology between the two N domains is limited to 18%.

Binding of ATP in Na,K-ATPase and Ca-ATPase

In accordance with the differences in amino acid sequence of the N-domain, the ATP binding properties of Na,K-ATPase are quite different from those of Ca-ATPase. Ca-ATPase binds MgATP with moderate affinity (K_d = 5.6 μM) and binding of free ATP cannot be assayed (Andersen *et al*., 1982). Na,K-ATPase is unique among cation pumps in its ability to bind free ATP with high affinity (K_d = 30–100 nM) in Mg^{2+} -free, EDTA-containing buffer, while the apparent affinity of the K^+

bound form is very low ($K_m \approx 0.2$–0.4 mM) (Jorgensen and Andersen, 1988; Norby and Jensen, 1988). The increase in binding energy of ATP associated with the $E_2[2K]$–$E_1(2K)$ conformational transition constitutes the driving force for transport of K^+ across the membrane (Lauger, 1991).

In the N domain, Arg^{544} is located near the interface to the P domain. This residue is essential for nucleotide binding, since substitution of Arg^{544} for Gln in the α subunit abolishes high-affinity binding of free ATP and Na,K-ATPase activity. From the double mutation with Asp^{369}, the contribution of the basic groups of Arg^{544} to the change in free energy of ATP binding is estimated to be close to 6 kJ/mol ($\Delta\Delta G_b$) (Jacobsen *et al*., 2001). Large changes in affinity for ADP after substitutions with Lys or Gln excludes the possibility that the arginine residue interacts exclusively with the γ-phosphate of ATP as observed for Arg^{182} in the β subunit of F_1-ATPase of *E. coli* (Nadanaciva *et al*., 1999). The data rather suggests that Arg^{544} in Na,K-ATPase has a role in stabilizing the α- and β-phosphates of ATP and ADP. These interactions could be important for aligning the γ-phosphate for interaction with the carboxylate group of Asp^{369}.

Catalytic Functions of P Domain

Consequences of Mutations of Asp^{369} and Asp^{710} in the P Domain for Binding of ATP

In Na,K-ATPase, binding of free ATP in absence of Mg^{2+} elicits strong electrostatic repulsion between the γ-phosphate and the negative charges at the surface of the P domain. Equilibrium binding shows that removal of the charge of the carboxylate groups in the Asp^{369} Ala mutation increases the affinity for ATP 30- to 40-fold (Pedersen *et al*., 1996). Substitution of Asp^{710} for Ala causes a more moderate two- to threefold increase of the ATP affinity (Pedersen *et al*., 2000). The assays of free $[^3H]$ ATP binding at equilibrium in Fig. 4 show that the dissociation constant for wild-type Na,K-ATPase of $K_d = 39$ nM is reduced to $K_d = 13$ nM in Asp^{710} Ala and in the Asp^{369} Ala mutation the K_d is only 1.8 nM. From these data, estimates of the free energy required to overcome the electrostatic interaction between the γ-phosphate of ATP and the carboxylate groups amounts to 2 kJ/mol for Asp^{710} and 7.9 kJ/mol for Asp^{369} (Pedersen *et al*., 1996, 2000). Double mutant cycle analysis provides evidence for interaction between the two negatively charged groups (Pedersen *et al*., 2000). The two negative groups contribute to stabilizing E_1 forms of the protein, since substitution of Asp^{369} or Asp^{710} for Ala causes a large two- to fourfold increase of K_c, the conformational constant for the E_1–E_2 conformational equilibrium

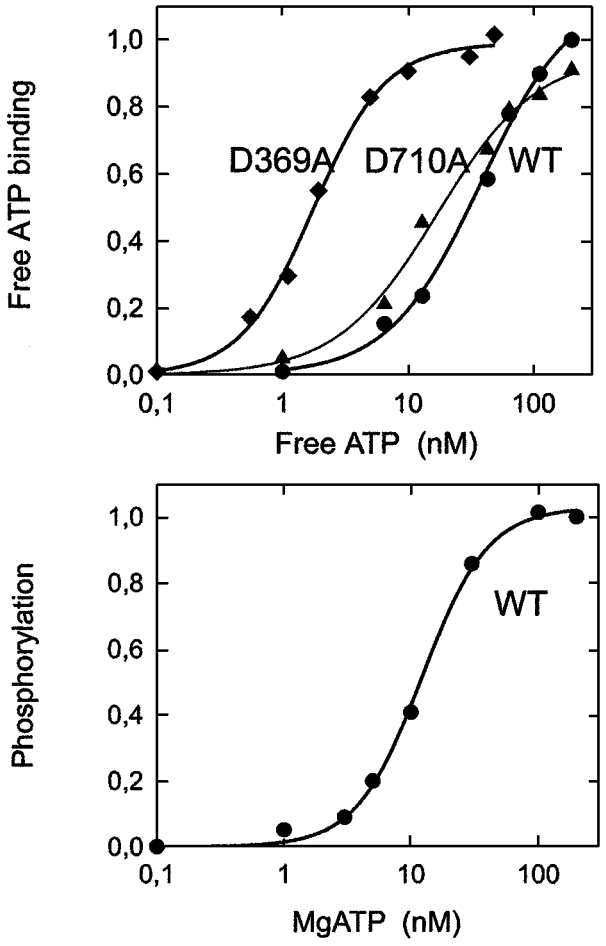

Fig. 4. (Upper) High-affinity free ATP binding of wild-type Na,K-ATPase and the Asp^{369} Ala or Asp^{710} Ala mutations. Yeast membranes were incubated on ice with 5 mM MOPS-Tris pH 7.2, 10 mM EDTA-Tris, protease inhibitors, $[^3H]$ATP (Amersham, specific activity 36Ci/mmol) to a final concentration of 6 to 21 nM plus cold Tris-ATP to final total ATP concentrations of 6 to 200 nM. Specific binding is estimated as that in 10 mM NaCl minus that in 10 mM KCl. Bound and unbound $[^3H]$ATP were separated by centrifugation at 265,000 $\times$ *g* for 30 min at 4°C. The supernatant was discarded and the pellet was resuspended for measurement of bound $[^3H]$ATP by scintillation counting and determination of protein content (Pedersen *et al*., 2000). Estimation of K_d from the equation: ATP binding $= a\,[ATP]^n/(c^n + [ATP]^n)$ in which *a* is the maximum ATP binding, *n* is the Hill coefficient, and *c* is the dissociation constant. K_d for wild type (•) is 38 ± 5 nM, for Asp^{710} Ala (▲) 13 ± 2 nM, and for Asp^{369} Ala (◆) 1.8 ± 0.1 nM. (Lower) Na^+-dependent phosphorylation as a function of ATP in presence of 3 mM $MgCl_2$ and oligomycin to stabilize the E_1P form. Aliquots of 200 to 300 μg TDS-treated wild-type yeast membranes were incubated for 10 min at 20°C in Eppendorf tubes containing 190 μl of 3 mM $MgCl_2$, 0.2 mM EDTA, 30 μM oligomycin, 10 mM NaCl or KCl, 20 mM TES-Tris, pH 7.5, and protease inhibitors (Pedersen *et al*., 2000). Phosphorylation was started by adding 10 ml $[\gamma^{32}$-P]ATP and the phosphoenzyme was isolated and counted as described before (Pedersen *et al*., 2000). The lines were fitted and $[ATP]_{1/2}$ values were estimated from the equation: phosphorylation $= a^*[ATP]^n/(c^n + [ATP]^n)$ in which *a* is the maximum phosphorylation level, *c* is $K_{0.5(ATP)}$, and *n* is the Hill coefficient. The $[ATP]_{1/2}$ value for wild type was 12.2 ± 0.7 nM (•)

corresponding to a shift toward the E_2 form (Pedersen *et al*., 1996, 2000). Substitution of Asp^{369} for the hydrophobic Ala allows a spontaneous transition from the E_1 to the E_2 form to support high-affinity binding of [^{3}H]ouabain in absence of Mg^{2+} (Pedersen *et al*., 1996; Ohtsubo *et al*., 1990). The position of the free charges of the carboxylate groups of Asp^{369} and Asp^{710} on the surface of the P domain may be such that they contribute to prevent the E_1–E_2 transition in the absence of phosphate and magnesium. Removal of the charge and hydrophobic substitution favors transmission of the conformational transition originating in the phosphorylated segment through the transmembrane segment to the ouabain site at the extracellular surface.

The strong electrostatic interaction with the negative charges of Asp^{369} and Asp^{710} shows that the γ-phosphate of the tightly bound ATP approaches the surface of the P domain in Na,K-ATPase. The gap between the N- and P-domains must, therefore, be closed in the ATP-E_1[3Na] form of the α subunit of Na,K-ATPase, even in the absence of Mg^{2+}. In the crystal structure of Ca-ATPase in the E_1[2Ca] form (Toyoshima *et al*., 2000), the N domain is separated from the P domain by a distance of 20–25 Å. Accordingly, Ca-ATPase of SR does not bind free ATP, but only MgATP with moderate affinity (Andersen *et al*., 1982). In Ca-ATPase, mutations of Asp^{351} enhances the affinity for MgATP binding up to 156-fold in the absence of Ca^{2+} and even more in the presence of Ca^{2+}, in agreement with strong electrostatic effects between the γ-phosphate and Asp^{351} in the wild-type pump. In line with this, the role of the neighboring Lys^{352} (Fig 3), appears to create a salt linkage with the phosphate group. Removal of the positive charge of Lys^{352} causes a pronounced decrease in ATP affinity of at least 1000-fold (McIntosh *et al*., 1999).

Binding of MgATP in Na,K-ATPase

Coordination of Mg^{2+} in the negatively charged surface of the P domain is expected to reduce electrostatic repulsion of the phosphate groups and thus facilitate ATP binding. The affinity of MgATP cannot be determined in equilibrium conditions because the γ-phosphate of bound ATP is rapidly transferred to the Asp^{369} residue in the protein with a rate of 190 s^{-1} at 21°C (Froehlich *et al*., 1997) in the reaction:

$$\mathrm{MgATP + E \leftrightarrow MgEATP \rightarrow MgE_1P + ADP}$$

The phosphorylated intermediate can be trapped in an oligomycin–MgE_1P[3Na] complex to prevent transition to the E_2P conformation. It is seen from Fig. 4 that $K_{1/2}$ values as low as 12 nM ATP in medium with 3 mM Mg^{2+} can be obtained when the protein concentrations of both yeast and pig kidney wild-type Na,K-ATPase are kept at 3–5 nM, as estimated from the ouabain binding capacity (Jacobsen *et al*., 2001). The $K_{1/2}$ value of 12 nM ATP is at least threefold lower than the dissociation constant, $K_d = 38 \pm 5$ nM (Fig. 4), for binding of free ATP at equilibrium in absence of Mg^{2+}. This result suggests that Mg^{2+} increases the apparent affinity of wild-type Na,K-ATPase for ATP. Previous estimates of the apparent affinities for MgATP are in the micromolar range, but the assays are usually conducted at much higher concentrations (>1 μM) of Na,K-ATPase protein (Helmich-De Jong, 1985).

Assay of Mg^{2+} Affinities in the E_1P and E_2P Conformations

Mg^{2+} is essential for all phosphoryl transfer reactions and for the E_1P–E_2P conversion in Na,K-ATPase. The experience from Mg^{2+} binding studies is that the binding affinity and the coordination pattern depends strongly on the conformational state (Pedersen *et al*., 2000). In assays of Mg^{2+} binding it is therefore important to stabilize either the E_1P or the E_2P conformations of the protein. In pig kidney Na,K-ATPase, the E_1P–E_2P equilibrium is poised heavily in favor of the E_2P and oligomycin is required to stabilize the E_1P form (Jorgensen and Andersen, 1988). The high affinity of the MgE_2P complex for ouabain provides a convenient assay of Mg^{2+} binding in the E_2P. Trapping of the intermediate in the ouabain complex also allows comparison of Mg^{2+} binding of E_2P to that of the E_2 complex with vanadate, the presumed pentacovalent transition state analog.

Contribution of TGDGVND Loop to Binding of Mg^{2+} and Phosphorylation of E_1P. While high-affinity ATP binding at equilibrium is preserved or increased in mutations of residues in the 708-TGDGVND loop, the Na,K-ATPase activity is severely reduced in mutations of Thr^{708}, Asp^{710}, Asn^{713}, and Asp^{714}, both at high (3 mM) and low ATP (25 μM) concentrations in the assay (Pedersen *et al*., 2000). Mutations of Asp^{714} are expressed, but the protein is devoid of any catalytic activity. To determine apparent affinities for Mg^{2+} for activation of transfer of γ-phosphate to Asp^{369}, phosphorylation is determined at saturating concentration of Na^+, 150 mM NaCl minus phosphorylation in 150 mM KCl, in an Mg–EDTA buffer system. In plots of specific phosphorylation versus free Mg^{2+}, the apparent Mg^{2+} affinity for wild type is $[Mg^{2+}]_{1/2} = 24 \pm 5$ μM. For the Asp^{710}Asn mutation, a 27-fold reduction of Mg^{2+} affinity to $[Mg^{2+}]_{1/2} = 648 \pm 88$ μM is observed, while

phosphorylation is almost abolished for the Asp^{710}Ala mutation with $[Mg^{2+}]_{1/2} => 10$ mM. A moderate fourfold increase to $[Mg^{2+}]_{1/2} = 90 \pm 23$ μM is observed after removal of the carboxamide group of Asn^{713} (Pedersen *et al*., 2000). The reaction model in Fig. 1 and the structure model in Fig. 3 illustrate how coordination of Mg^{2+} to Asp^{710} can establish a link via the γ-phosphate to Asp^{369}. The role of Asp^{710} in this scheme is to stabilize Mg^{2+} in a position that facilitates nucleophilic attack of Asp^{369} to form the acyl phosphate intermediate (cf. Ridder and Dijkstra, 1999) and Mg^{2+} shields the negative charges of the phosphate moiety and the two carboxylates.

It can be difficult to distinguish effects of the mutation on Mg^{2+} binding from interference with the nucleophilic attack on the γ-phosphate bond and the covalent insertion at the carboxylate group of Asp^{369}. In view of the selective and pronounced effects of mutations on the affinities for Mg^{2+} of the E_1P form, the straightforward interpretation is that Asp^{710} and Asn^{713} contribute to coordination of Mg^{2+} during transfer of γ-phosphate to the MgE_1P[3Na] form with three occluded Na^+ ions. Contributions of Asp^{710} and Asp^{714} to coordination of Mg^{2+} are also proposed in attempts at modeling the active site of cation ATPases on the dehalogenase fold, although the haloacid–dehalogenase reaction does not involve Mg^{2+} (Ridder and Dijkstra, 1999). In the Mg^{2+}-requiring phosphoserine phosphatase, mutation of Asp^{179} (a homolog of Asp^{710} in Na,K-ATPase α subunit) in the sequence 178-GDGATD decreased Mg^{2+} affinity about tenfold, while mutation of Asp^{183} (a homolog of Asp^{714}) did not interfere with Mg^{2+} binding (Collet *et al*., 1999).

Asp^{710} and Asp^{713} in TGDGVND Loop Do Not Coordinate Mg^{2+} in E_2P. The high affinity of the MgE_2P complex for ouabain provides a sensitive assay of Mg^{2+} binding in the E_2P form. A correction is required for the reduction of ouabain binding affinity in mutations of Asp^{710} and Asp^{713}. As shown below, this reduction is mainly due to interference with binding of vanadate or phosphate, while the affinity for binding of Mg^{2+} is unaffected or increased in both mutations. The concentrations of ouabain in the assays are adjusted accordingly and the binding constants for Mg^{2+}, phosphate, and vanadate can be estimated from the equations:

$$K_{Mg} = [Mg]_{1/2} \frac{K_d + [O]}{K_d}$$

or

$$K_{VO_4^{3-}} = [VO_4^{3-}]_{1/2} \frac{K_d + [O]}{K_d}$$

where the vanadate concentrations required to give half-maximal ouabain binding $[VO_4^{3-}]_{1/2}$ can be replaced by $[PO_4^{3-}]_{1/2}$ to estimate the binding constant for phosphate.

The single mutations cause moderate increases of the affinity for Mg^{2+} in the E_2P or E_2 vanadate complexes with ouabain (Table I). Double mutations of Asp^{710} and Asp^{369} reveal interactions of moderate strength to prevent binding of Mg^{2+} in E_2P (Pedersen *et al*., 2000). It can thus be an important part of the E_1P to E_2P conversion to shift Mg^{2+} coordination away from the TGDGVND loop prior to hydrolysis of the aspartyl phosphate bond at Asp^{369}. Based on ^{60}Co binding studies, it has previously been proposed that one Mg^{2+} ion is occluded in the E_1P[3Na] form and that Mg^{2+} is released in the K^+-dependent dephosphorylation reaction (Richards, 1988; Hansen and Skou, 1973).

Table I. Consequences of Mutations of Asp^{710} and Asn^{713} for Binding of Mg^{2+} and Phosphate or Vanadate in Ouabain Complexes[a]

	Ouabain K_d (nM)	$K_{Mg^{2+}}$ (μM)	K_{pi} (mM)
A. Phosphate medium			
WT	3.9 ± 0.4 ($n = 5$)	22.8	2.74 ± 0.07
D710A	37 ± 4 ($n = 2$)	15.4	1.08 ± 0.12
N713A	43 ± 8 ($n = 4$)	5.6	>10
B. Vanadate medium			
WT	1.9 ± 0.2	0.63	1.4 ± 0.7
D710A	111 ± 7	0.16	>1000
N713A	7 ± 1	2.5	27 ± 3

[a] Ouabain binding was determined as before (Pedersen *et al*., 2000) and the mean values of the dissociation constant (K_d) are given with the number of experiment in parenthesis. Ouabain binding was assayed as a function of Mg^{2+}, phosphate, or vanadate concentrations and $K_{Mg^{2+}}$, K_{pi}, or K_{VAN} values were estimated from $K_{1/2}$ values using the equations in the text (Pedersen *et al*., 2000).

Coordination of Mg^{2+} in E_2P—A Bridge between P Domain and A Domain

The mutagenesis studies have not yet identified residues ligating Mg^{2+} in the E_2P conformation, but alternative ligating loops are proposed from analysis of the high-resolution structure of Ca-ATPase. Transition to the E_2 form may involve a 90° turn of the A domain to approach the P domain, so that the 181-TGES loop can meet the 625-TGD motif in the P-domain, (Fig. 2) (Toyoshima *et al*., 2000). The effects of mutations in the TGDGVND loop on binding of Mg^{2+} in E_1P and E_2P forms are complimentary to the results of metal-catalyzed cleavage. In the ATP–Fe^{2+} complex, Fe^{2+} substitutes for Mg^{2+} in activating phosphorylation and Fe^{2+}–ascorbate–H_2O_2 cleavage is sensitive to E_1–E_2 conformational transitions in Na,K-ATPase (Goldshleger and Karlish, 1999; Patchornik *et al*., 2000). In the E_1P conformation, two major cleavages are detected at Val^{712} and nearby. In the E_2P conformation, a major cleavage occurs near 212-TGES in the A domain, whereas cleavages in the P domain are less prominent (Patchornik *et al*., 2000). The results suggest that a shift of Mg^{2+} coordination to the TGES segment in the A domain can form a bridge to stabilize interactions between the P and A domains in the E_2 forms of the protein. In the E_2[2K] form, cleavage from Fe^{2+} bound in the protein suggests that residues within 212-TGES in the A domain and 367-CSDK, 658-MVTGD, and 712-VNDSPALKK in the P domain of Na,K-ATPase remain in proximity (Goldshleger and Karlish, 1999). The structure analysis suggests that that there are at least two binding sites for Fe^{2+}, one that does not change in E_1 and E_2 forms and one that exists only in E_2 forms (Goldshleger and Karlish, 1999; Patchronik *et al*., 2000). This may affect the conclusions concerning interactions between loops that are sensitive to cleavage.

TGDGVND Loop Forms a Part of the Active Site of the K^+ Phosphatase Activity in E_2P

In the E_2P form, the mutagenesis data show that Asp^{710} and Asn^{713} are more important for the interactions with vanadate and phosphate and for the communication of the conformational transition to the cation sites in M4, M5, and M6. The mutations have dramatic and very specific effects, as removal of the carboxylate group of Asp^{710} eliminates vanadate binding and removal of the carboxamide of Asn^{713} eliminates the phosphate dependence of ouabain binding (Table I) (Pedersen *et al*., 2000). The TGDGVND loop is, therefore, required for stabilization of the transition state and for the phosphorylation reaction, suggesting that the loop is important for transmission of the K^+ stimulation via M5 to hydrolysis of the acyl-phospate bond in the forward reaction. Asp^{710} may contribute directly to coordination of pentacovalent vanadate in the transition state (Cantley *et al*., 1978). Removal of the Asp^{710} also interferes with transmission of the structural change to the cation sites and ouabain binding domain at the extracellular surface. The Asp^{710} Ala mutation causes a threefold reduction of the affinity for binding of $T1^+$ (Pedersen *et al*., 2000). In the forward reaction, this corresponds to a role of Asp^{710} in transmission of the K^+ stimulation of phosphoenzyme hydrolysis from M5 to the P domain.

Accordingly, alanine substitution of each of the residues Asp^{369}, Asp^{710}, or Asn^{713} eliminates the K^+-stimulated *p*-nitrophenylphosphate hydrolysis. The observation that the Asp^{369} Ala mutation eliminates the K^+ phosphatase activity suggests that transient phosphorylation of Asp^{369} is part of the phosphatase reaction. Elimination of the K^+ phosphatase activity in the Asp^{710} Ala or Asn^{713} Ala mutations falls in line with the observation that these mutations eliminate vanadate binding or phosphate interactions in the ouabain binding assay. Together, the observations show that this triad of residues Asp^{369}, Asp^{710}, and Asn^{713} in the P domain form the active catalytic site of the K^+ phosphatase activity.

Energy Transfer: Coupling of E_1P to E_2P Conversion to Na^+ Translocation and K^+-Activated Phosphoenzyme Hydrolysis

Analysis of the mutations of residues in the 708-TGDVNDSPALKK segment identifies reactions that are catalyzed by these residues in formation of the high-energy E_1P form, transition to the low-energy E_2P form, and in the dephosphorylation reaction. The next step is to understand the transfer of energy, that is, the coupling of these scalar reactions to vectorial translocation of Na^+ ions out of the cell and binding of extracellular K^+ ions. The phosphoenzyme conversion in the P domain is coupled to adjustment of the cation sites from the E_1P[3Na] form with specificity for Na^+ over K^+ to the E_2P[2Na] form with specificity for K^+ over Na^+. In the cation sites in M4, M5, and M6, this transition involves a change of both the number of coordinating groups per ion and the distances between coordinating groups to adapt to the different diameters of Na^+ (1.9 Å) and K^+ (2.7 Å) (Pedersen *et al*., 1998). The structural change must be transmitted over long distances from the P domain to the cation sites. A reciprocal structural change is required to transmit the change accompanying exchange of K^+ ions for 2 Na^+ ions and activation of dephosphorylation of E_2P[2K] in the P domain.

Routes for Transmission of Long-Range Structural Transitions

There are two possible routes for these long-range transitions, direct transmission through M4 and M5 or an indirect route via the A domain. Considering the position of the P domain in direct connection via S5 to M5 and S4 to M4, one could expect that the structural changes would be transmitted directly to the cation coordinating residues in M4, M5, and M6 to alter their cation specificity. There is, however, strong evidence that the predominant route for transmission of the structural accompanying the E_1P–E_2P conversion is indirect via the A domain. The proteolytic cleavage experiments show that this transition is interrupted by chymotryptic cleavage (Jorgensen and Andersen, 1988). The split at Leu^{266} in the L23 loop breaks the connection from the A domain to S4 (Fig. 2 and 5), to stabilize the E_1P[3Na]form and prevent Na^+ translocation.

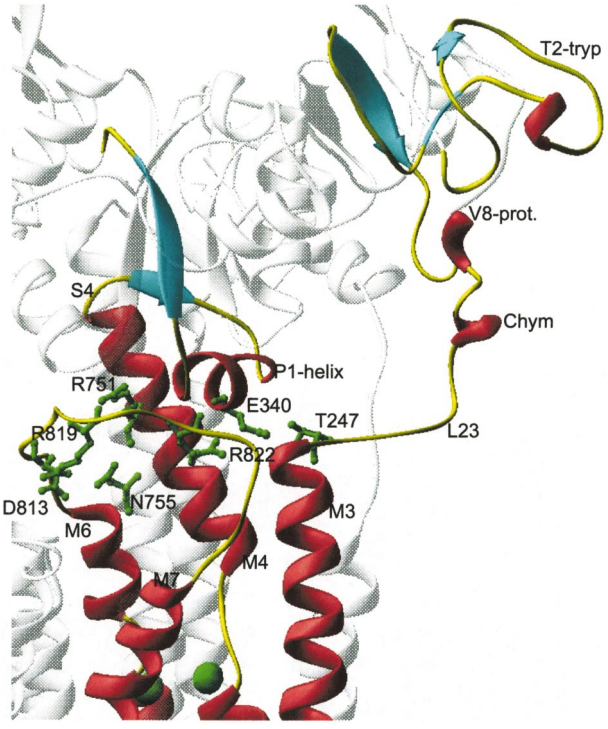

Fig. 5. Ribbons model of detailed structure of the connections from the A domain via L23 to M3, Pl helix, and L67 of Ca-ATPase in the E_1[2Ca] form (Toyoshima *et al*., 2000). Two Ca^{2+}-atoms in the cation sites are shown in green. (Chym) indicates the homologous position (Gln^{238}) of the peptide bond (Leu^{266}) that is cleaved by chymotrypsin in the E_1[3Na] form of α subunit of Na,K-ATPase. Peptide bonds that are cleaved in the E_1[2Ca] form of Ca-ATPase are shown as T2-tryp (Arg^{198}) (Andersen *et al*., 1986) and V8-prot. (Glu^{231}) (Møller *et al*., 1996). Numbered residues, engaged in hydrogen bond formation, are shown in green.

Model of Structures Linking A Domain and Cation Sites

The structure model in Fig. 5 details the architecture of the connections from the A domain via L23 to S4 and L67 in Ca-ATPase (Toyoshima *et al*., 2000). The position of the chymotryptic split at Leu^{266} in α subunit of Na,K-ATPase is marked in L23. It is seen that L23, near the cytoplasmic end of M3, is hydrogen bonded to S4 and L67 in the region where M3, M4, and L67 gather. The residues engaged in this triangular connection, Thr^{247} in L23, Glu^{340} in the P1 helix, and Arg^{822} in L67 are well conserved in Na,K-ATPase. Figure 3 also illustrates some of the conserved hydrogen bond connections between L67 and S5, i.e., between Asp^{813} in L67 and Asn^{755} in S5 and between the main chain carbonyl of Arg^{819} and Arg^{751} in S5. Additional hydrogen bonds and van der Waal interactions between L67 and M5 are described in the structure of Ca-ATPase (Toyoshima *et al*., 2000). Structural changes can also be transmitted from the N-terminal part of the A domain through the loop–helix connecting to M2. Both the transmembrane segment M2 and S2, the helix–loop connecting to the A domain, are in close contact with M4 and its extension S4 (Toyoshima *et al*., 2000).

Monitoring Interactions between P Domain and A Domain by Proteolysis

The interactions between P and A domains can be monitored by chymotryptic cleavage in Na,K (Jorgensen and Andersen, 1988) and tryptic or V8-protease cleavage in Ca-ATPase (Andersen *et al*., 1986; Møller *et al*., 1996). In Na,K-ATPase, chymotrypsin cleaves at Leu^{266} (Cl) in the E_1[3Na] or E_1P[3Na] forms and this bond is protected in the E_2 forms. Trypsin cleaves the E_1 forms rapidly at Lys^{30} (T2) and 30-fold more slowly at Arg^{262} (T3) to produce the characteristic biphasic pattern of inactivation (Jorgensen and Andersen, 1988). The peptide bonds at Leu^{266} (C1) and Arg^{262} (T3) are located near the L23 loop connecting the A domain to M3. Figure 5 shows the location of the homologous positions in Ca-ATPase (Arg^{234} for T3 and Gln^{238} for C1) in the L23 loop connecting the A domain to M3. In the same L23 loop, the peptide bond at Arg^{198}, is exposed to trypsin (T2) (Andersen *et al*., 1986) and that at Glu^{231} is exposed to V8 protease (V8) (Møller *et al*., 1996) in the E_1[2Ca] bound form of Ca-ATPase. These bonds are also protected after removal of Ca^{2+} and stabilization of the E_2 conformation. In the E_2 form, the segment of L23 containing bonds 231–238 may be hidden in the interface between the P and A domains. As shown in Fig. 2 and Fig. 5, transition to the E_2 form may involve a large turn of the A domain, up to 90° to approach the P domain (Toyoshima

et al., 2000), thus protecting bonds in segment 231–238 to proteolysis.

Identification of Residues of Importance for the Structural Change

The significance of these connections through the A domain is also apparent from the changes in catalytic properties after proteolytic cleavage or from site-directed mutagenesis of individual residues in L23 and S4. Tryptic cleavage at Lys^{30} in the N-terminus of the α subunit strongly reduces the rate of the E_1P to E_2P conversion, due to a large shift of conformational E_1–E_2 equilibrium toward E_1 forms with a three- to fourfold decrease of the K_c, the conformational equilibrium constant. This favors the E_1 form with high affinity for ATP and Na^+, while the turnover rate is reduced by 50% (Jorgensen and Andersen, 1988). Chymotryptic cleavage at Leu^{266} blocks transitions from E_1 to E_2 forms. The E_1P[3Na] phosphoform is stabilized and transition to the E_2P form is prevented (Jorgensen and Andersen, 1988). The chymotryptic cleaved protein was, in fact, used for the first demonstration of occlusion of three Na^+ ions in the E_1P[3Na] form of Na,K-ATPase (Glynn *et al*., 1984). In the cleaved protein, cation sites are not damaged, but binding of K^+ no longer reduces the affinity for free ATP binding.

In agreement with the consequences of proteolytic cleavage, mutations comprising deletion of the N-terminal 30 residues of the α subunit and the $Glu^{233} \rightarrow$ Lys substitution in L23 in the $\alpha 1$ subunit result in a shift in the steady-state E_1–E_2 conformational equilibrium toward E_1 form(s) (Daly *et al*., 1996). The combined effects of the N-terminal deletion and the Glu^{233} substitutions are synergistic, consistent with an interaction between the N-terminal region and the L23 loop in the A domain (Boxenbaum *et al*., 1998). In Ca-ATPase, about 20 residues are crucial for the E_1P to E_2P transformation of the phosphoenzyme. They are located in the portion of L23 that connects the A domain to transmembrane segment M3 (Sorensen and Andersen, 2000). Also, mutations of a number of residues in S4, the stalk segment that links the P domain to transmembrane segment M4, characteristically cause a shift of conformational equilibrium toward the E_1form (Zhang *et al*., 1955). In yeast plasma membrane H^+-ATPase, systematic alanine screening of residues in S4 identified mutations of 13 consecutive residues, from Ile^{-359} through Gly^{-371}, that are highly resistant to orthovanadate (Ambesi *et al*., 2000). Insensitivity to vanadate indicates that the rate of transition from E_1 to E_2 forms is reduced. The segment Ile^{359}–Gly^{371} in H-ATPase occupies three full turns of an α-helix that is homologous to the P1 helix in S4 of Ca-ATPase (Toyoshima *et al*., 2000). In the center of the P1-helix of Ca-ATPase, Glu^{340} is hydrogen bonded to Thr^{247} in L23 and to Arg^{822} in L67 and thus establishes a triangular connection between the loop from the A domain and the transmembrane segments (Fig 5).

Importance of the L67 Loop for the E_1–E_2 Structural Transition

The loop L67 runs along the bottom of domain P in a position to mediate interactions between the P domain and the transmembrane domain. The hydrogen bond of Arg^{822} in L67 to Glu^{340} in the P1 helix and from Glu^{340} to Thr^{247} will couple movements from the A domain to M4 and M5 (Toyoshima *et al*., 2000). Single or cluster mutations of aspartic residues in the cytoplasmic loop between transmembrane segments 6 and 7 causes a reduction of the affinity for Ca^{2+} in Ca-ATPase. This was interpreted in a model where these negative charges serve as an entrance to Ca^{2+} sites within the membrane (Menguy *et al*., 1998). Similarly, it was proposed that negatively charged residues in L6/7 recognize either Na^+ or K^+ ions or their competitive antagonists and thus also serve as an entrance port in Na, K-ATPase (Shainskaya *et al*., 2000). These hypotheses are untenable after appearance of the structure of Ca-ATPase, as the position of L67 in the structure is not in agreement with a function as entrance port to the cation sites (Toyoshima *et al*., 2000). The structure information rather suggests that changes of cation affinities after mutations of residues in L67 may be secondary to altered conformational equilibria.

Together with the structural information (Toyoshima *et al*., 2000), the results of proteolytic cleavage and the analysis of mutations in Na, K-ATPase, H-ATPase, and Ca-ATPase support the notion that the route depicted in Fig. 5, from the A domain via L23, P1-helix to S4-M4 and via L67 to M5 and M6 is of central importance for transmission of the structural change accompanying E_1p–E_2P conversion to the cation sites.

Long-Range Structural Transitions Accompanying K^+-Activated Dephosphorylation

Binding of K^+ to E_2P[2K] elicits a long-range structural change that can be transmitted from the cation sites in the membrane domain to the P domain to activate nucleophilic attack on the Asp^{369}-C–O–P bond by Asp^{710} and Asn^{713} in the GDGVND segment (Pedersen *et al*., 2000) and allow hydrolysis of the covalently bound phosphate at Asp^{369}. Mutation of Asp^{710} also affect high-affinity

binding of $T1^+$ in Na, K-ATPase as an indication that this residue is important for transmission of this structural change from K^+ binding sites in M4, M5, and M6 to the P domain. This structural change does not alter the exposure of peptide bonds to proteolytic cleavage. It is, therefore, not likely that it is transmitted via the connections with L23 to alter the interactions between the A and the P domains. Rather the K^+ activating signal is transmitted directly through M5 and S5 to the active site for the K^+ phosphatase in the P domain, consisting of the triad of Asp^{369}, Asp^{710}, and Asn^{713} (Pedersen *et al*., 2000). This route of transmission has been proposed after analysis of a series of mutations of residues in S5 of Ca-ATPase (Sorensen and Andersen, 2000). The mutations interfere with transmission of conformational changes from the cation binding sites in the membrane domain to the P domain controlling phosphorylation and dephosphorylation. Arg^{751} (Arg^{758} in Na, K-ATPase) in S5 is hydrogen bonded to L67 (Fig. 5) and this residue is important for the structural integrity of the enzyme. Through this and other connections in the network of hydrogen bonds and van der Walls interactions of L67, the structural change can also be transmitted from K^+, coordinating residues in M4 and M5 to the P domain without involving the A domain.

In this context, it can be important to consider the possibility that the rodlike M5–S5 helix in Ca-ATPase forms a more flexible structure in the α subunit of Na,K-ATPase. The unwound loop sequence 307-IPEGLP, dividing M4 in Ca-ATPase in two helixes, is remarkably similar to the 777-IPEITP sequence in M5 in α subunit of Na,K-ATPase, while M5 in Ca-ATPase is devoid of prolines. It is, therefore, possible that this segment of M5 is unwound in Na,K-ATPase. This would expose a row of negative charges of this segment in M5 that has been demonstrated to be of particular importance for Na^+/K^+ selectivity (Pedersen *et al*., 1998; Vilsen, 1995). A more flexible loop could facilitate transmission of a structural change accompanying high-affinity binding of K^+ ions via M5 to the P domain.

Interactions between A Domain and N Domain in the E_2 [2K] Form

In the E_2[2K] or E_2P[2Na] forms, trypsin cleaves at Arg^{438} in the N domain and subsequently at Lys^{30} in the N-terminus of the α subunit of Na,K-ATPase. The loss of Na,K-ATPase activity is linear and associated with cleavage at Arg^{438}. The time course of K^+-phosphatase loss has a characteristic delay, indicating that tryptic cleavage at Arg^{438} must precede cleavage at Lys^{30} (Jorgensen and Andersen, 1988). As shown in Fig. 2, Arg^{438} is exposed at the surface of the N domain close to a loop pointing toward the A domain. The cleavage pattern, therefore, suggests that there is direct interaction between the N and the A domains in the E_2[2K] conformation of Na,K-ATPase. In the E_2[2K] form, the N, P, and A domains may, therefore, be more tightly associated in the cytoplasmic headpiece as compared to the widely separated domains in the structure in Fig. 2.

ACKNOWLEDGMENTS

Work in the authors laboratories is supported by the Danish Research Foundation and the Novo-Nordic and Carlsberg Foundations. We thank Kjell O. Hakansson for help with drawings in the Ribbons program.

REFERENCES

Ambesi, A., Miranda, M., Allen, K. E., and Slayman, C. W., (2000). *J. Biol. Chem*. **275**, 20545–20550.
Andersen, J. P., Vilsen, B., Collins, J. H., and Jørgensen, P. L. (1986). *J. Membr. Biol*. **93**, 85–92.
Andersen, J. P., Moller, J. V., and Jorgensen, P. L. (1982). *J. Biol. Chem*. **257**, 8300–8307.
Axelsen, K. B., and Palmgren, M. G. (1998). *J. Mol. Evol*. **46**, 84–101.
Boxenbaum, N., Daly, S. E., Javaid, Z. Z., Lane, L. K., and Blostein, R. (1998). *J. Biol. Chem*. **273**, 23086–23092.
Cantley, L. C., Jr., Cantley, L. G., and Josephson, L. (1978). *J. Biol. Chem*. **253**, 7361–7368.
Collet, J. F., Stroobant, V., and Schaftingen, E. V. (1999). *J. Biol. Chem*. **274**, 33985–33990.
Daly, S. E., Lane, L. K., and Blostein, R. (1996). *J. Biol. Chem*. **271**, 23683–23689.
Froehlich, J. P., Taniguchi, K., Fendler, K., Mahaney, J. E., Thomas, D. D., Albers, R. W. (1997). *Ann. N. Y. Acad. Sci*. **834**, 280–896.
Girardet, J. L., Bally, I., Arlaud, G., and Dupont, Y. (1993). *Eur. J. Biochem*. **217**, 225–231.
Glynn, I. M., Hara, Y., and Richards, D. E. (1984). *J. Physiol*. **351**, 531–547.
Goldshleger, R., and Karlish, S. J. D. (1999). *J. Biol. Chem*. **274**, 16213–16221.
Hansen, O., and Skou, J. C. (1973). *Biochim. Biophys. Acta* **233**, 122–132.
Helmich-De Jong, M. L., De Pont, J. J. H. H. M., Stekhoven, F. M. A. H., Swarts, H. G. P., and Bonting, S. L. (1985). *Fourth International Conference on Na,K-ATPase* (Company of Biologists, Cambridge), pp. 379–382.
Hisano, T., Hata, Y., Fujii, T., Liu, J. Q., Kurihara, T., Esaki, N., and Soda, K. (1996). *J. Biol. Chem*. **271**, 20322–20330.
Jacobsen, M. D., Pedersen, P. A., and Jorgensen, P. L. (2001). Submitted for publication.
Jorgensen, P. L. (1975). *Biochim. Biophys. Acta* **401**, 399–415.
Jorgensen, P. L. (1992). In *Molecular Aspects of Transport Proteins* (De Pont, J. J. H. H. M., ed.) Elsevier, New York, pp. 1–26.
Jorgensen, P. L., and Andersen, J. P. (1988). *J. Membr. Biol*. **103**, 95–120.
Jorgensen, P. L., and Collins, J. H. (1986). *Biochim. Biophys. Acta* **860**, 570–576.
Jorgensen, J. R., and Pedersen P. A. (2001). *Biochemistry* **40**, 7301–7308.
Jorgensen, P. L., Rasmussen, J. H., Nielsen, J. M., and Pedersen, P. A. (1997). *Ann. N. Y. Acad. Sci*. **834**, 161–174.
Karlish, S. J. D. (1980). *J. Bioenerg. Biomembr*. **12**, 111–136.

Lane, L. K., Feldmann, J. M., Flarsheim, C. E., and Rybczynski, C. L. (1993). *J. Biol. Chem*. **268**, 17930–17934.
Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993). *J. Appl. Cryst*. **26**, 283–291.
Lauger, P. (1991). *Electrogenic Ion Pumps*, Sunderland, Massachusetts.
Lingrel, J. B., and Kuntzweiler, T. A. (1994). *J. Biol. Chem*. **269**, 19659–19662.
Maruyama, K., and MacLennan, D. H. (1988). *Proc. Natl. Acad. Sci. USA* **85**, 3314–3318.
McIntosh, D. B., Woolley, D. G., MacLennan, D. H., Vilsen, B., and Andersen, J. P. (1999). *J. Biol. Chem*. **274**, 25227–25236.
Menguy, T., Corre, F., Bouneau, L., Deschamps, S., Moller, J. V., Champeil, P., le Maire, M., and Falson, P. (1998). *J. Biol. Chem*. **273**, 20134–20143.
Møller, J. V., Juul, B., and le Maire, M. (1996). *Biochim. Biophys. Acta* **1286**, 1–51.
Nadanaciva, S., Weber, J., and Senior, A. E. (1999). *Biochemistry* **38**, 7670–7677.
Norby, J. G., and Jensen J. (1988). *Methods. Enzymol*. **156**, 191–201.
Ohtsubo K., Noguchi, S., Takeda, K., Morohashi, M., and Kawamura, M. (1990). *Biochim. Biophys. Acta* **1021**, 157–160.
Ovchinnikov, YuA., Dzhandzhugazyan, K. N., Lutsenko, S. V., Mustayev, A. A., and Modyanov, N. N. (1987). *FEBS Lett.* **217**, 111–116.
Ohta, T., Nagano, K., and Yoshida, M. (1986). *Proc. Natl. Acad. Sci. USA* **83**, 2071–2075.
Patchornik, G., Goldshleger, R., and Karlish, S. J. D. (2000). *Proc. Natl. Acad. Sci. USA* **97**, 11954–11959.
Pedersen, P. A., Jorgensen J. R., and Jorgensen, P. L. (2000). *J. Biol. Chem*. **275**, 37588–37595.
Pedersen, P. A., Nielsen, J. M., Rasmussen, J. H., and Jørgensen, P. L. (1998). *Biochemistry* **37**, 17818–17827.
Pedersen, P. A., Rasmussen, J. H., and Jørgensen, P. L. (1996). *Biochemistry* **35**, 16085–16093.
Richards, D. E. (1988) *J. Physiol.* (*London*) **404**, 497–514.
Ridder, I. S., and Dijkstra, B. W. (1999). *Biochem. J*. **339**, 223–226.
Rossman, M. G., and Liljas, A. (1974) *J. Mol. Biol*. **85**, 177–181.
Scheiner-Bobis, G., and Schreiber, S. (1999). *Biochemistry* **38**, 9198–9208.
Shainskaya, A., Schneeberger, A., Apell, H. J., and Karlish, S. J. (2000). *J. Biol. Chem*. **275**, 2019–2028.
Sorensen, T. L., and Andersen, J. P. (2000). *J. Biol. Chem*. **275**, 28954–28961.
Toyoshima, C., Nakasako, M., Nomura, H., and Ogawa, H. (2000). *Nature* (*London*) **405**, 647–655.
Tran, C. M., Scheiner-Bobis, G., Schoner, W., and Farley, R. A. (1994). *Biochemistry* **33**, 4140–4147.
Vilsen, B. (1995). *Biochemistry* **34**, 1455–1463.
Vilsen B. (1997). *Biochemistry* **36**, 13312–13324.
Wang, K., and Farley, R. A. (1992). *J. Biol. Chem*. **267**, 3577–3580.
Zhang, Z., Sumbilla, C., Lewis, D., Summers, S., Klein, M. G., and Inesi, G. (1955). *J. Biol. Chem*. **270**, 16283–16290.