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Review

Structure–function relationships of Na^+, K^+, ATP, or Mg^{2+} binding and energy transduction in Na,K-ATPase

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Abstract

The focus of this article is on progress in establishing structure–function relationships through site-directed mutagenesis and direct binding assay of Tl^+, Rb^+, K^+, Na^+, Mg^{2+} or free ATP at equilibrium in Na,K-ATPase. Direct binding may identify residues coordinating cations in the E_2[2K] or E_1P[3Na] forms of the ping-pong reaction sequence and allow estimates of their contributions to the change of Gibbs free energy of binding. This is required to understand the molecular basis for the pronounced Na/K selectivity at the cytoplasmic and extracellular surfaces. Intramembrane Glu^{327} in transmembrane segment M4, Glu^{779} in M5, Asp^{804} and Asp^{808} in M6 are essential for tight binding of K^+ and Na^+. Asn^{324} and Glu^{327} in M4, Thr^{774}, Asn^{776}, and Glu^{779} in 771-YTLTSNIPEITP of M5 contribute to Na^+/K^+ selectivity. Free ATP binding identifies Arg^{544} as essential for high affinity binding of ATP or ADP. In the 708-TGDGVND segment, mutations of Asp^{710} or Asn^{713} do not interfere with free ATP binding. Asp^{710} is essential and Asn^{713} is important for coordination of Mg^{2+} in the E_1P[3Na] complex, but they do not contribute to Mg^{2+} binding in the E_2P-ouabain complex. Transition to the E_2P form involves a shift of Mg^{2+} coordination away from Asp^{710} and Asn^{713} and the two residues become more important for hydrolysis of the acyl phosphate bond at Asp^{369}.

Keywords: Na,K-ATPase; Mutagenesis; Na^+ binding; K^+ binding; Tl^+ binding; Mg^{2+} binding; ATP binding; Cation binding site; Energy transduction

1. Introduction

Na,K-ATPase transforms chemical energy in ATP to osmotic work and maintains electrochemical Na^+ and K^+ gradients across cell membranes. At rest, Na,K-ATPase converts 20–30% of the current ATP production in mammals to active Na,K transport in kidney, central nervous system, and other cells of the body where Na,K gradients are required for maintaining membrane potential and cell volume. Renal [$\alpha 1\beta 1\gamma$] Na,K-ATPase consists of three subunits, α subunit with 1016 residues, β subunit with 302, and γ subunit with 55 amino acids, and it is the largest protein complex in the family of cation pump proteins [1,2]. The α subunit has about 30% amino acid sequence homology to Ca-ATPase of sarcoplasmic reticulum (SR) (994 residues) [3]. 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 [1]. The structure at 2.6 Å resolution of SR Ca-ATPase appeared recently [4]. This is the first

Abbreviations: M4, M5, M6, transmembrane segments 4, 5, and 6 in α subunit of Na,K-ATPase; Tris, tris(hydroxymethyl)-aminomethane

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high resolution structure of any member of the family of cation pumps. Its general architecture is therefore an adequate provisional model for the α subunit of Na,K-ATPase. In this structure, each of two Ca^{2+} ions are 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 Asp^{351} and the C-terminal 702-GDGVND segment. 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.

The Na,K-pump couples ATP hydrolysis to the active transport of three Na^+ ions out of and two K^+ ions into the cell in a ping-pong sequence, while alternating between two cation complexes $E_1P[3Na]$ and $E_2[2K]$ [1]. In the model of the reaction cycle (Fig. 1) relatively large E_1–E_2 conformational changes in the α subunit mediate long-range interactions between the ATP site and the cation sites in the membrane domain [1,5,6]. In the initial step, binding of ATP with low apparent affinity ($K_m \approx 0.2$–0.4 mM) to the $E_2[2K]$ conformation accelerates the $E_2[2K]$–$E_1(2K)$ transition with release of K^+ at the cytoplasmic surface. ATP is bound with high affinity in the E_1 conformation ($K_D \approx 30$–100 nM) [1,7] and 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 [8]. The next energy transducing steps are the Na-dependent transfer of γ phosphate from ATP to an acyl bond at Asp^{369} of the α subunit and isomerization between the occluded $E_1P[3Na]$–$E_2P[2Na]$ phospho forms in coupling with reorientation of cation sites and release of Na^+ ions at the extracellular surface.

Na,K-ATPase is unique among cation pumps in its ability to bind either one molecule of free ATP or two K^+ ions per α1β1 unit with high affinity in the absence of other ligands. High affinity binding of Rb^+ or Tl^+ can be assayed in purified renal Na,K-ATPase or in recombinant yeast enzyme at equilibrium with dissociation constants $K_D = 7$–9 μM [9–11]. Free ATP binds in ethylenediaminetetraacetic acid (EDTA)-containing buffer with high affinity ($K_D = 30$–100 nM) in the absence of other ligands

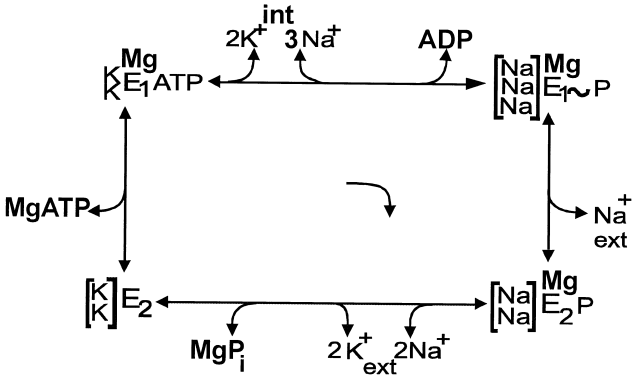

Fig. 1. Minimum scheme of E_1–E_2 reaction cycle of the Na,K-pump with ping-pong sequential cation translocation. The phospho forms show specificity for Na^+ and dephospho forms bind K^+, Rb^+, or Tl^+ tightly. [Na^+] or [K^+] within brackets are tightly bound and prevented from exchanging with medium cations [1].

[7]. The dissociation constants are equilibrium constants that can be related directly to the Gibbs free energy change for ligand binding to the protein [12]. Na,K-ATPase also exhibits ATP-K^+ antagonism because the two high affinity ligands stabilize alternative conformations, $E_2[2K]$ with tightly bound K^+ ions or E_1ATP with a preference for binding of Na^+ (cf. Fig. 1). The ATP and cation binding assays can therefore be exploited in estimation of the poise of the equilibrium between the E_1 and E_2 conformations of the protein and in determination of the affinities for K^+ [11,13]. Affinities for Na^+ or Mg^{2+} binding can be assayed in the $E_1P[3Na]$ form using oligomycin to stabilize the phosphorylated complex [11,14]. The E_2P form can be stabilized in the ouabain complex for estimates of the affinities for binding of Mg^{2+} or inorganic phosphate.

It is the purpose of this article to focus on progress in establishing structure–function relationships through the use of direct assay of cations and ATP binding at equilibrium in mutations of Na,K-ATPase. The important aspect is the possibility of determining the contribution of individual residues to the Gibbs free energy of binding of ligands in the partial reactions of this important reaction cycle. Direct binding assays can identify residues contributing to cation binding in the $E_2[2K]$ and $E_1P[3Na]$ forms of the ping-pong reaction sequence. This may answer the question whether the pump has separate sites with specificity for binding of Na^+ or K^+ or if the

same set of cation coordinating residues alternate between an E_1 form with high affinity for Na^+ and an E_2 form with high affinity for K^+. This is required for understanding the molecular basis for the pronounced Na/K selectivity at the cytoplasmic and extracellular surfaces. Studies of the binding of Mg^{2+} ions in the phosphorylated E_1P or E_2P conformations also provide a key to understanding the reactions involved in the formation of the E_1P[3Na] complex and in the Na^+ transport step coupled to transition form E_1P to the E_2P form of the protein.

2. Selection of residues for mutation and direct binding assay

Early mutagenesis screening of Ca-ATPase of SR, based on transient expression in COS cells, identified 40–50 essential residues as important for ligand binding and conformational transitions [3]. The oxygen-containing side chains Glu^{309}, Glu^{771}, Asn^{796}, Thr^{799}, and Asp^{800} in putative transmembrane segments were pinpointed as essential for Ca^{2+} binding [3,15,16]. Additional residues in M5 and M6 were shown to be important for the affinity of Ca^{2+} binding [3]. The predictions from mutagenesis work concerning cation-coordinating residues have been in remarkably good agreement with the cation site structure in the first high resolution structure of Ca-ATPase at 2.6 Å resolution [4]. The entry of Ca^{2+} from the cytoplasm appears to be guided via a hydrophilic pathway to direct interaction with oxygen groups of the coordinating residues pointing towards the cytoplasm, in particular Glu^{309} and Asp^{800}. There are no vestibules or gates, but the center parts of M4 and M6 are unwound loops with rows of exposed oxygen atoms, from M4 Pro^{312}–Glu^{309} and from M6, Gly^{801}–Asp^{800}. The side chains of Glu^{309}, Asn^{768}, Glu^{771}, Asn^{796}, Thr^{799}, Asp^{800}, and Glu^{908} plus a number of main chain carbonyls (Val^{304}, Ala^{305}, Ile^{307}) contribute six coordinating groups for each of two Ca^{2+} ions. At the exit there is a ring of oxygens with water molecules for rehydration, but apparently not a narrow water-filled access channel or ion well [4].

Homologous counterparts to these side chains in the α subunit of Na,K-ATPase are Glu^{327} (M4), Asn^{776}, Glu^{779} (M5), Asp^{804}, Thr^{807}, Asp^{808} (M6)

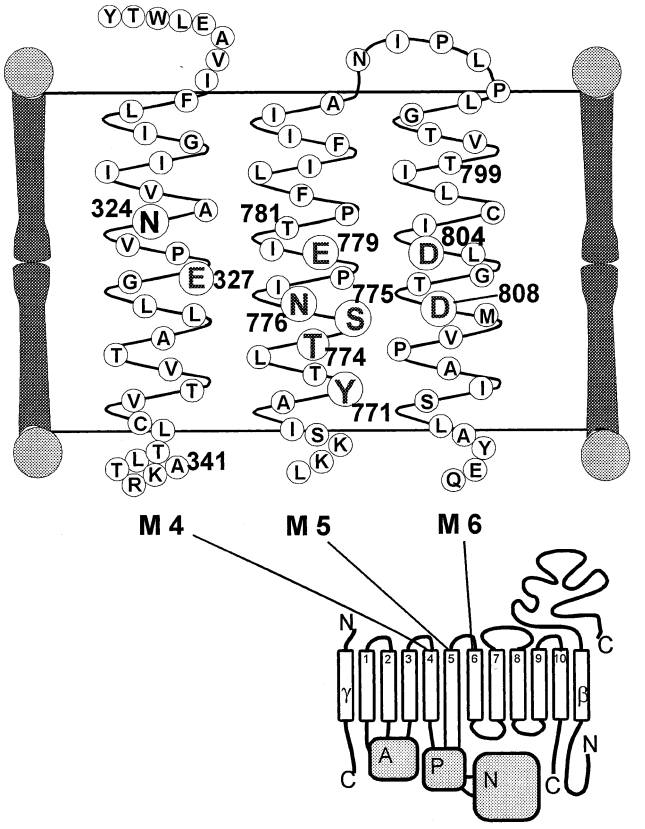

Fig. 2. Position of residues of importance for binding of Tl^+, K^+, or Na^+ in a helical representation of residues in M4, M5, and M6. With the indicated bilayer width (30 Å) and a pitch of 5.4 Å and 3.6 residues/turn, each intramembrane segment comprises 20 amino acid residues. The drawing is based on a topological model of renal [α1β1γ] Na,K-ATPase with 10 transmembrane segments in the α subunit. The cytoplasmic protrusion of the α subunit is split in three domains as in Ca-ATPase [4]. The N domain with a single nucleotide binding site separated from the P domain comprising the phosphorylated Asp^{351} and the C-terminal 702-GDGVND segment. 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.

(Fig. 2) and counterparts to the side chains exposing main chain carbonyls in M4 are Val^{322}, Ala^{323}, and Val^{325}. Mutagenesis studies in Na,K-ATPase have been delayed due to problems with expression of mutant protein. After transfection of the rodent ouabain-resistant α1 isoform of Na,K-ATPase in HeLa [17] or COS cells [18], mutations exhibiting active Na,K transport were selected by growth in ouabain medium. An alternative to this has been expression in baculovirus-infected cells [19], *Xenopus* oocytes

[20], or yeast [21,22]. Involvement in cation binding of several of the pertinent oxygen groups has earlier been questioned. After expression of ouabain-resistant mutations in HeLa [17,23] or COS cells [18,24] and assays of Na,K-ATPase activity and Na^+-dependent phosphorylation it is concluded that Glu^{327} is not essential for active transport [36] and it is questioned whether Glu^{327}, Glu^{779}, and Asp^{808} are cation-coordinating residues [17,23,25]. Only Asp^{804} and Ser^{775} appear to be essential for enzyme function [26], although studies of K^+–ouabain antagonism show that substitutions of both Asp^{808} and Asp^{804} disrupted K^+ interactions with Na,K-ATPase [27]. Major alterations are also observed after analysis of cation or membrane potential dependence of pump current of mutations of Glu^{327} and Glu^{779} in transfected cells [28,29]. Moderate yields of expression and the presence of endogenous Na,K-ATPase activity prevent direct binding assays.

Yeast cells are devoid of endogenous Na,K-ATPase and high yield expression of porcine Na,K-ATPase allows assay of high affinity binding of free ATP and cations at equilibrium [11,30]. Effects of mutations on high affinity binding of ATP are important for identification of residues involved in ATP binding and energy transduction. High affinity binding of Rb^+ or Tl^+ and assay of K^+–ATP antagonism forms the basis for estimating the free energy contributions of intramembrane residues to high affinity K^+ binding in the E_2[2K] conformation of Na,K-ATPase [30]. Using the Na^+-dependent phosphorylation from ATP as a dead end reaction in the presence of oligomycin allows monitoring of affinities for Na^+ ion or free Mg^{2+} ions in the E_1P[3Na] complex [11,14]. In the selection of additional residues for mutation, emphasis is placed on identification of side chains contributing to Na^+/K^+ specificity, e.g. the sequence 771-YTLTSNIPEITP in M5 of the α subunit [11], which is unique to Na,K-ATPase (Fig. 2).

3. High yield expression of recombinant Na,K-ATPase for assay of ligand binding at equilibrium

The combined effect of engineering the parameters of the expression system is to increase the density of Na,K pumps to $32\,500 \pm 3000$ sites per cell or 54 ± 5 μg Na,K pump protein per gram of yeast cells [22]. The concept behind the host/vector system for Na,K-ATPase expression is to separate the phase of yeast cell growth with low basal expression from the expression phase where the expression level of Na,K-ATPase in the yeast cell membranes increases to a maximum. Precise alteration of the α:β gene dose requires that the α and β cDNAs be present on the same plasmid and be expressed under control of identical promoters. The expression system allows for a high gene copy number, and a high transcriptional activity due to the strong galactose-inducible promoter. Transcriptional activity is increased by engineering the yeast host strain to express elevated concentrations of the GAL4 transactivating protein causing a 10-fold increase in the expression level. With respect to biosynthesis and translocation to cell membranes this system does not distinguish active Na,K-ATPase from lethal mutants. The recombinant enzyme is fully active, with one site for binding of ATP and ouabain per α subunit, and a range of molecular activities close to those of the native Na,K-ATPase of pig kidney. The relatively high activity, corresponding to 10–60 pmol ouabain binding sites per mg protein [22], and the lack of endogenous background allows for analyses not previously achieved for recombinant Na,K-ATPase, such as tight binding of K^+ (Rb^+ or Tl^+) ions [31] and high affinity [^{3}H]ATP binding [30] at equilibrium. Determination of cation stoichiometries requires precise definition of Na,K-ATPase concentration. In the yeast membranes, one αβ unit corresponds to one binding site for ouabain and all data of cation or ATP binding are expressed relative to the ouabain binding capacity [31].

The ouabain selection methodology as applied to eukaryotic cell lines like HeLa [17] and COS cells [18] is based on the expression of ouabain-resistant pumps and does not allow characterization of the interesting mutants with total blocks of the reaction cycle. The eukaryotic cell lines and *Xenopus* oocytes [20] also express endogenous Na,K-ATPase of almost the same magnitude as the transfected activity. Recently, systems for expression of Na,K-ATPase at relatively high concentrations, 10–100 pmol/mg protein, have been developed for baculovirus-infected insect cells (Sf9 and High Five) based on isolation of plasma membranes devoid of endogenous Na,K-

ATPase on sucrose gradients [32] with separation from inactive denatured protein.

4. Assay of direct cation binding in Na,K-ATPase

4.1. High affinity binding of K^+ analogues, Rb^+ or Tl^+, in Na,K-ATPase

Assays of direct cation binding of Rb^+ or Tl^+ with high affinity (K_D 7–9 μM) have been developed for the purified membrane-bound Na,K-ATPase from kidney. After equilibration, separation between bound and free cations ($^{86}Rb^+$ or $^{204}Tl^+$) can be achieved either by passage over Dowex columns [9,33] or by sedimentation of membranes by centrifugation [10,34]. Tight binding in the cation binding cavity prevents the cations from exchanging with medium cations during the passage over Dowex columns, in both the membrane-bound and soluble monomeric αβ units [35]. The term 'tight binding' is preferred to 'occlusion' [9], since the ions interact directly with the coordinating residues at the membrane surface and the high resolution structure of Ca-ATPase [4] is devoid of gates or vestibules. A cation site concentration of 100–200 nM corresponding to a concentration of Na,K-ATPase molecules (ouabain binding sites) of 50–100 nM is required to measure the affinity (K_D) and the capacity for cation binding. To ensure equal access to both membrane surfaces, membranes are pretreated by incubation with tris(hydroxymethyl)-aminomethane (Tris) salt of dodecyl sulfate in concentrations that do not interfere with catalysis [22]. Due to a background of unspecific binding sites for Rb^+ or Tl^+ in yeast membranes, ouabain is used as inhibitor to define specific and background binding [13,31]. Hyperbolic curves fit the data of binding of two Rb^+ [10] or Tl^+ [34] ions per α subunit or ouabain binding (Fig. 3) like in purified renal Na,K-ATPase. The two Tl^+ binding sites per α subunit are therefore equivalent and independent. At low temperature, the rate of release of Rb^+ or Tl^+ from renal Na,K-ATPase is negligible [35] and the $K_{0.5(Tl+)}$ for Tl^+ occlusion is an equilibrium constant which is directly related to the Gibbs free energy change for cation binding to the protein [11,31]. As estimated from the equilibrium constant for wild type of 7 μM and the maximum binding of 1.9 ± 0.3 Tl^+ ions per ouabain binding site, the change in Gibbs free energy for binding of Tl^+ in Na,K-ATPase is estimated to be $\Delta G_b = -28.8$ kJ/mol at 4°C for each of two ions [11] using the equation:

$$\Delta G_{b(Tl^+)} = -RT \ln (Tl^+/\text{ouabain binding}/K_{0.5(Tl^+)})_{WT} \quad (1)$$

To estimate the consequences of amino acid substitutions, changes in both affinity and capacity of Tl^+ binding should be taken into account. The changes in Gibbs free energy of binding ($\Delta\Delta G_b$) are therefore estimated from the ratios of maximum binding to $K_{0.5(Tl^+)}$ using a modification of Ferhst's equation [12]:

$$\Delta\Delta G_b = -RT \ln [(\text{maximum binding}/K_{0.5})_{mut}/(\text{maximum binding}/K_{0.5})_{wt}] \quad (2)$$

In several cases, e.g. after substitution of Glu^{779} or Asn^{776} (Fig. 3), the curves of Tl^+ binding are sigmoid as an indication that the cation sites are not independent. In these cases the $K_{0.5(Tl^+)}$ values may represent the site with the highest affinity and the contribution of the side chain to the change of the Gibbs free energy of binding ($\Delta\Delta G_b$) thus represents a minimum value.

4.2. K^+ displacement of ATP binding

The K^+–ATP antagonism reflects the alternative stabilization of the E_1 form by high affinity ATP binding and of the E_2[2K] form by high affinity occlusion of K^+ ions. At a low concentration of ATP (13 nM) the $K_{1/2} = 45$–60 μM for K^+ displacement of ATP from wild type Na,K-ATPase (Fig. 4). This value is close to the expected binding constant for K^+ at equilibrium, since the affinities for Rb^+ or Tl^+ are 5–10-fold higher than for K^+ [34]. Tables 1 and 2 show a comparison of changes of Gibbs free energies estimated from Tl^+ binding studies with those based on $K_{1/2}$ values for K^+ displacement of ATP. It is seen that the data reflect a close correlation between the consequences of the amino acid substitutions for high affinity Tl^+ ion binding and their effects on the K^+–nucleotide antagonism.

The K^+–ATP antagonism is also used to monitor

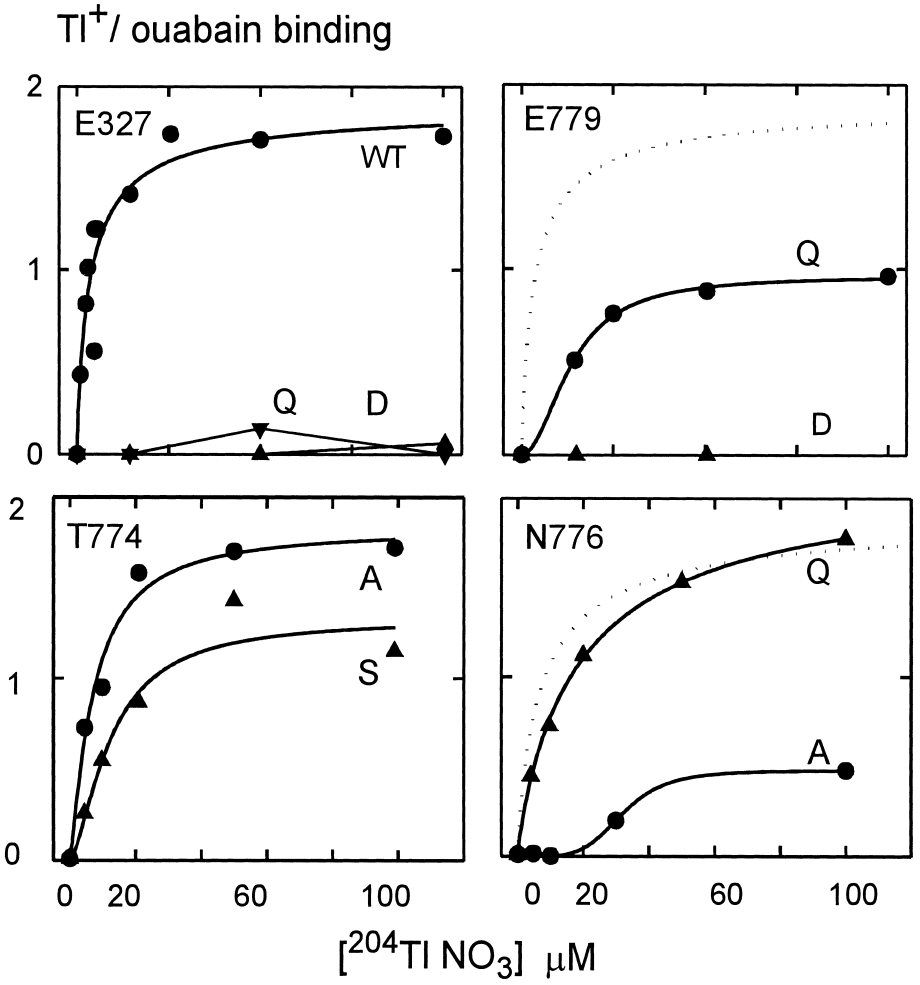

Fig. 3. High affinity $^{204}Tl^+$ binding after mutations of Glu^{327} in M4 or Glu^{779}, Thr^{774}, or Asn^{776} in M5. (Upper left) High affinity $^{204}Tl^+$ in wild type (WT) (●) with maximum capacity 1.9 ± 0.3 Tl^+ ions per ouabain binding site and $K_{1/2} = 7.3 \pm 3.3$ μM. Tl^+ ion binding is abolished after conservative mutations $Glu^{327}Gln$ (▼) or $Glu^{327}Asp$ (▲). (Upper right) In mutation $Glu^{779}Gln$ (●) binding is reduced to 0.96 ± 0.03 Tl^+ ions per ouabain site and $K_{1/2} = 27 \pm 1$ μM, while Tl^+ ion binding is abolished in $Glu^{779}Asp$ (▲). (Lower left) In the mutation $Thr^{774}Ala$ (●) binding capacity is unchanged 1.9 ± 0.3 Tl^+ ion per ouabain site and $K_{1/2}$ 7 ± 2 μM. In $Thr^{774}Ser$ (▲) binding capacity is reduced to 1.3 ± 0.2 Tl^+ ions per ouabain site and $K_{1/2}$ 12 ± 4 μM. (Lower right) In the mutation $Asn^{776}Gln$ (▲) binding capacity is unchanged at 2.2 ± 0.1 Tl^+ ions per ouabain site and $K_{1/2}$ is increased to 20 ± 3 μM. After $Asn^{776}Ala$ (●) binding capacity is reduced to 0.5 ± 0.1 Tl^+ ions per ouabain site and $K_{1/2}$ is increased to 32 ± 4 μM. Data points are average values of two separate experiments with double determinations at each concentration of cation. Assays were as described in detail before [11]. Using Sigmaplot 6.0 (Jandel Scientific) lines were fitted by non-linear least squares regression analysis to the Hill/Michaelis–Menten equations: occluded Tl^+/ouabain binding ratio $= a \times [TlNO_3]^n/(c^n + [TlNO_3]^n)$ in which a is the maximum Tl^+ occlusion/ouabain binding ratio, c is $K_{0.5(Tl+)}$ and n is the Hill coefficient. For the Michaelis–Menten equation $n = 1$.

the consequences of mutations for the E_1–E_2 conformational equilibrium. The $K_{1/2}$ values for displacement by KCl of [^{3}H]ATP reflect both the binding of K^+ to the E_1 form (K_p) with relatively low affinity, and the conformational equilibrium (K_c) between the E_1 and E_2 forms of the protein [13,30]. Mathematical analysis of the equilibrium binding of ATP to Na,K-ATPase with respect to K^+ concentration leads to the following equation for the $K_{1/2}$ value for K^+ inhibition of ATP binding:

$$K_{1/2} = K_p\,([ATP]/K_{ATP} + 1)/K_c \quad (3)$$

Given the experimentally determined $K_{1/2}$ values for K^+ and the affinity for ATP, K_c can be calculated for the mutations and the wild type on the assumption that K_p is unaltered by the mutations. A 3–4-

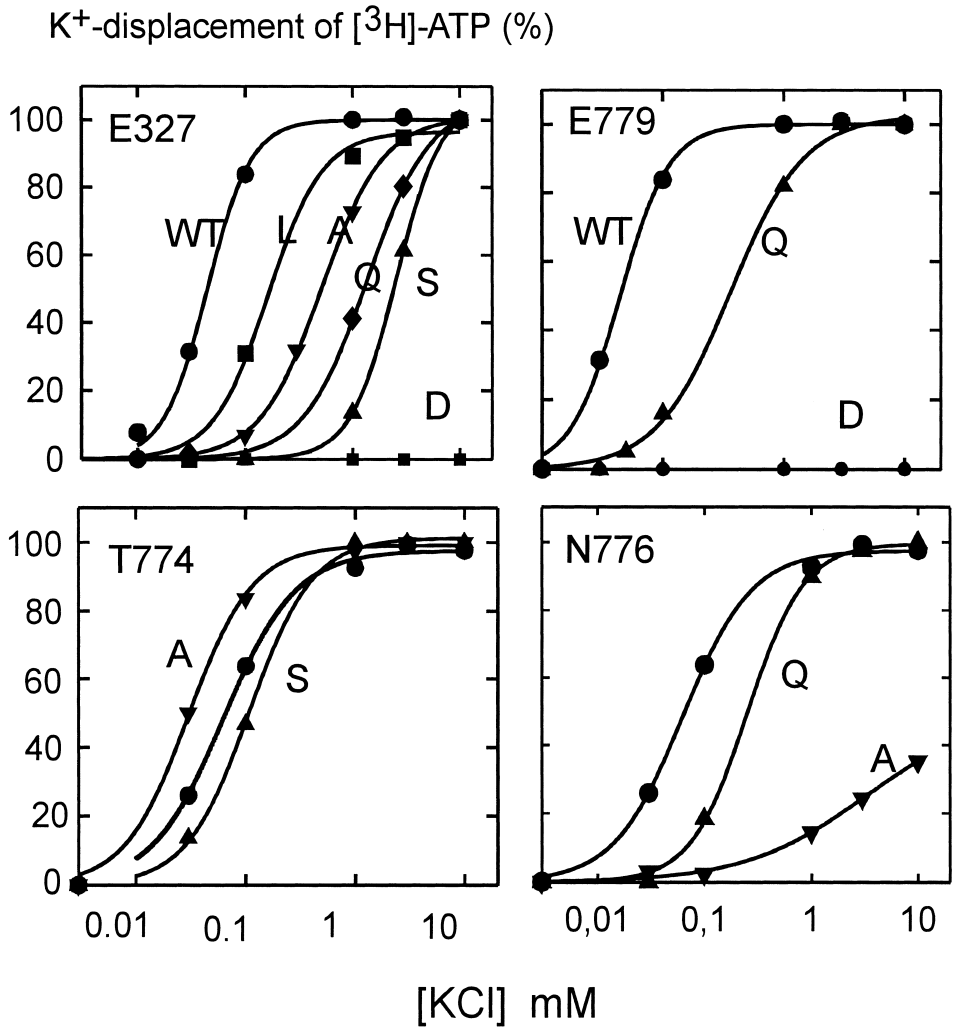

Fig. 4. Consequences for K^+ displacement of ATP binding of mutations of Glu^{327} in M4, Glu^{779}, Thr^{774}, or Asn^{776} in M5. (Upper left) $K_{0.5(K^+)}$ for wild type (●) 45 μM KCl, for E327L (■) 164 μM, for E327A (▼) 520 μM, for E327Q (♦) 1.4 mM, for E327S (▲) 2.6 mM, and for E327D (■) >10 mM KCl. (Upper right) $K_{0.5(K^+)}$ for wild type (●) 45 μM KCl, for E779Q (▲) 357 μM, and for E327D (●) >10 mM KCl. (Lower left) $K_{0.5(K^+)}$ for wild type (●) 63 μM KCl, for T774A (▼) 31 μM, and for T774S (▲) 109 μM KCl. (Lower right) $K_{0.5(K^+)}$ for wild type (●) 63 μM KCl, for N776Q (▲) 254 μM, and for N776A (▼) >10 mM KCl. Membranes were incubated with 13 nM [^{3}H]ATP in the presence of 0–10 mM KCl. Choline chloride was added to maintain constant ionic strength. The procedure was as described before [11]. Data points are average values of two separate experiments. The lines were fitted and $K_{0.5(K^+)}$ values were estimated by non-linear least square regression analysis using the equation: Displaced ATP = $a \times [KCl]^n / (c^n + [KCl]^n)$ in which a is the maximum ATP displacement, c is $K_{0.5(K^+)}$ and n is the Hill coefficient.

fold increase of K_c with a shift of conformational equilibrium towards the E_2 form is seen after substitution of the transiently phosphorylated Asp^{369} to Ala [30].

4.3. Assay of Na^+ binding

Direct binding of three $^{22}Na^+$ ions per αβ unit can be assayed in purified renal Na,K-ATPase [36], but the relatively high $K_D = 0.5$ mM Na^+ and low specific activity of the isotope prevent direct binding measurements in recombinant Na,K-ATPase. In the phosphorylation assay, oligomycin prevents E_1P–E_2P transition and allows monitoring of the formation of the $MgE_1P[3Na]$ complex as a dead end reaction. The phosphorylation data are expressed relative to parallel determinations of the ouabain binding capacity for each membrane preparation to cancel

Table 1
Summary of the effects of mutations of intramembrane glutamate and aspartate residues in α subunit of Na,K-ATPase on alterations in Gibbs free energy ($\Delta\Delta G_b$) of Tl^+, K^+, or Na^+ ion binding at 4°C

Allele	Alterations in Gibbs free energy $\Delta\Delta G_b$ (kJ/mol)		
	Tl^+	K^+	Na^+
M4			
E327D	> +8	> +11	+3.0
E327Q	> +8	+7.9	+4.0
E327S		+9.3	+2.9
E327A		+5.6	+2.7
E327L		+3.0	+2.5
M5			
E779Q	+4.3	+4.9	+6.8
E779D	> +8	> +10	> +10
M6			
D804A	> +8	> +12	
D804E	> +8	+4.8	> +12
D804N	> +8	> +12	+3.9
D808A	> +8	> +12	+3.2
D808E	> +8	> +12	
D808N	> +8	> +12	> +12

Calculations as described in the text.

variations among different fermentations and preparations of individual mutant proteins (Fig. 5). The $K_{0.5(Na^+)}$ for Na^+ dependence of phosphorylation for wild type is close to the K_d for $^{22}Na^+$ ion binding at equilibrium to the oligomycin complex of purified pig kidney Na,K-ATPase [32]. The Na^+ dependence of transfer of γ phosphate from ATP to a covalent acyl bond with Asp^{369} in the α subunit may therefore reflect the apparent affinity of the complex for Na^+ [37]. For wild type, the $K_{0.5(Na^+)}$ value of 0.62 mM (Fig. 5) reflects a change in Gibbs free energy of binding (ΔG_b) of −17 kJ/mol per Na^+ ion if three ions bind with the same affinity. After the substitutions several sigmoid-shaped curves are observed (Fig. 5), and the estimated values of $\Delta\Delta G_{b(Na^+)}$ represent approximations to minimum values for the contribution of the amino acid residues to the free energy of Na^+ binding.

5. Contribution of intramembrane glutamate and aspartate residues to binding of Rb^+, Tl^+, K^+, or Na^+

The first set of data showed that binding at 100 μM Rb^+ ions is eliminated in mutations of Glu^{327} to Asp or Gln (M4), of Asp^{804} and Asp^{808} to Asn or Glu (M6), and of Glu^{779} to Asp (M5), while 33 ± 10% of the Rb^+ binding level is preserved in Glu^{779}Gln [31]. Since Rb^+ is the only ligand required for stabilization of the E_2[2Rb] conformation, the severe effects of conservative substitutions of Glu^{327}, Asp^{804}, and Asp^{808} on Rb^+ occlusion suggest that these carboxylic acids contribute to tight binding of Rb^+ ions in the E_2[2Rb] complex. Although mutation of Glu^{779} to a residue with the shorter side chain Asp completely abolishes Rb^+ binding, an appreciable amount of binding is conserved when the carboxylate group of Glu^{779} is replaced with the carboxamide of Gln. It is important to decide if the mutation Glu^{779}Gln only reduces the apparent affinity of the enzyme for Rb^+ ions, or if the capacity for the ions is also affected. Due to the higher affinity of Tl^+, binding of $^{204}Tl^+$ is developed as an alternative assay [31].

Conservative substitutions of the carboxylic acids Glu^{327}(Gln,Asp), Asp^{804}(Asn,Glu), Asp^{808}(Asn,Glu), and Glu^{779}(Asp) abolish high affinity binding of Tl^+. Substitution of Glu^{779} to Gln reduces the binding capacity to one Tl^+ ion per α1β1 unit with a threefold decrease of the apparent affinity for the ion

Table 2
Identification of residues of importance for Na^+/K^+ specificity in M4 and M5

Allele	Alterations in Gibbs free energy $\Delta\Delta G_b$ (kJ/mol)		
	Tl^+	K^+	Na^+
M4			
N324A	+1.3	+2.6	+4.0
M5			
T781A	+0.3	0	−0.3
T781S	+0.6	+0.3	+1.3
N776A	+6.7	+9.9	+6.8
N776Q	+1.9	+3.1	+8.0
S775A	+3.6	+7.7	+5.9
S775T	+6.3	+8.3	+4.1
T774A	+3.7	+1.7	+6.5
T774S	+3.3	+1.3	+3.8
T772A	+3.7	+2.7	+3.7
T772S	+3.3	+1.2	+3.3
Y771F	+6.7	+5.0	+6.6

Alterations in Gibbs free energy ($\Delta\Delta G_b$) of Tl^+ or Na^+ ion binding at 4°C of mutations of residues in M4, M5, M6, or M8 of α subunit of Na,K-ATPase. $\Delta\Delta G_b$ values were calculated from binding data using Eq. 2.

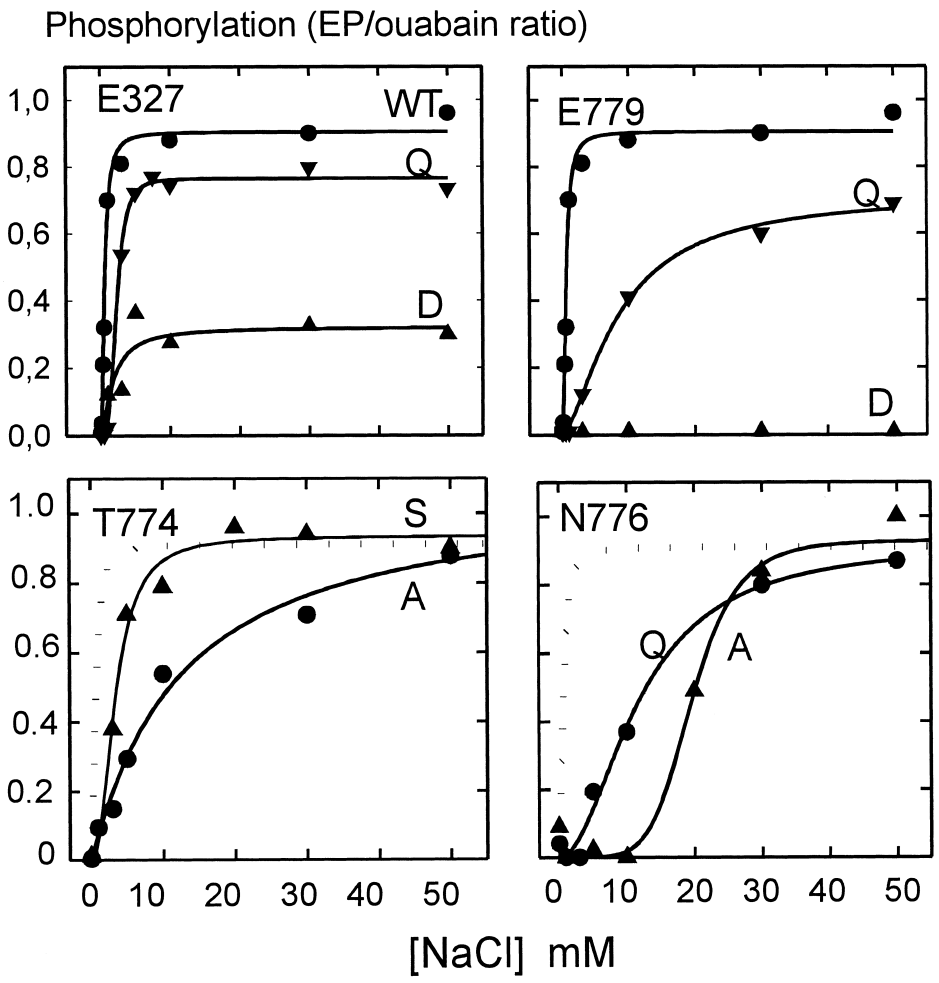

Fig. 5. Consequences for Na^+-dependent phosphorylation from ATP of Glu^{327} in M4, Glu^{779}, Thr^{774}, or Asn^{776} in M5. Phosphorylation for 4 s at 2 μM [γ-^{32}P]ATP, 3 mM $MgCl_2$, 30 μM oligomycin, 0–150 mM NaCl, 0–150 mM choline chloride, 20 mM TES–Tris, pH 7.5, and protease inhibitors as before [11]. The lines were fitted and maximum phosphorylation/ouabain binding ratios and $K_{0.5(Na^+)}$ values were estimated by non-linear least square regression analysis using the equation: Phosphorylation/ouabain ratio = $a \times [NaCl]^n/(c^n+[NaCl]^n)$ in which a is the maximum phosphorylation level, c is $K_{0.5(Na^+)}$ and n is the Hill coefficient. (Upper left) EP/ouabain and $K_{0.5(Na^+)}$ for wild type (●) 0.91 and 0.61 mM, for E327Q (▼) 0.76 and 2.4 mM, for E327D (▲) 0.32 and 1.8 mM NaCl. (Upper right) EP/ouabain and $K_{0.5(Na^+)}$ for E779Q (▲) 0.72 and 8.6 mM, and for E327D (▲) 0 and >50 mM NaCl. (Lower left) EP/ouabain and $K_{0.5(Na^+)}$ for T774A (●) 1.06 and 12.3 mM, and for T774S (▲) 0.93 and 3.4 mM NaCl. (Lower right) EP/ouabain and $K_{0.5(Na^+)}$ for N776Q (●) 0.92 and 12 mM, and for N776A (▲) 0.93 and 20 mM NaCl.

($K_{d(Tl^+)} = 24 \pm 8$ μM) (Fig. 3) [31]. These effects on occlusion are closely correlated to effects of the mutations on $K_{0.5(K^+)}$ for K^+ displacement of ATP binding (Table 1). The strong inhibition of binding after mutation of carboxylate-containing residues precludes measurements of binding affinities for cations from the Tl^+ binding data, since binding is abolished in the range 0–200 μM Tl^+ [31]. Assuming $K_{0.5(Tl^+)}$ values of ≥200 μM, the estimated change of the free energy of binding ($\Delta\Delta G_b$) is in excess of 8 kJ/mol. Data from K^+ displacement of ATP binding suggest that this value is higher than 11–12 kJ/mol (Table 1). These values approach the values for the changes of free energy of binding ($\Delta G_b = 14$–17 kJ/mol) between an ammonium group in a substrate and a carboxylate group in an enzyme [38].

Reduced affinities for Na^+ dependence of phosphorylation in the presence of oligomycin after substitution of carboxylate groups Glu^{327}(Gln,Asp), Asp^{804}(Asn,Glu), Asp^{808}(Asn,Glu), and Glu^{779}(Asp)

indicated that these residues also contribute to coordination of Na^+ in the E_1P[3Na] form. The contributions of Asp^{804}, Asp^{808}, and Glu^{779} were of the same magnitude as for Tl^+ or K^+ binding. For Glu^{327} of M4, the change in free energy of binding of Na^+ in E_1P[3Na] was 2–3-fold smaller than that for binding of Tl^+ and K^+ (Table 1). The conclusion from the binding data is therefore that each of the four carboxylate residues Glu^{327}, Glu^{779}, Asp^{804}, and Asp^{808} in transmembrane segments M4, M5, and M6 are essential for cation binding and that these groups alternately engage in binding of Na^+ or K^+ in the occluded E_1P[3Na] or E_2[2K] conformations according to the reaction cycle in Fig. 1. The data fit into a model in which amphiphilic helices of M4 and the M5–M6 helical loop (Fig. 2) are organized with the carboxylate side chains oriented towards a central cavity [11,31], as recently shown in the structure of the Ca-ATPase of SR [4].

5.1. Controversies concerning the role of Glu^{327} in M4

In HeLa [17,23] or COS cells [18,24] the mutations Glu^{327}Gln, Glu^{327}Leu, Glu^{779}Ala, and Asp^{808}Glu can be expressed with relatively high activity and moderate alterations of kinetic parameters in assays of Na,K-ATPase activity and Na^+-dependent phosphorylation. It is concluded that Glu^{327} is not essential for active transport [24] and it is questioned whether it is a cation-coordinating residue [25]. In contrast, after expression in ouabain-resistant NIH3T3 cells, K^+ competition of ouabain binding to mutations of Glu^{327} to Ala, Asp, Glu, and Leu displays severely altered interactions between these proteins and K^+ [39]. Electrophysiological analysis of mutations of Glu^{327} in transfected cells shows that loading of K^+ sites is seriously impaired in the range of 0–3 mM K^+, while 5–20 mM extracellular K^+ is sufficient to activate the Na,K pump current of the Glu^{327}Gln mutant in HeLa cells [29]. Also more recent data for the Glu^{329}Gln mutation [40] reveal large reductions of the apparent affinity for extracellular K^+ and a 30-fold enhancement of the rate of release of tightly bound K^+ to the cytoplasmic side of the membrane, while the changes of apparent Na^+ affinities are more moderate. The conclusions from these data [40] agree with those from the binding data in Figs. 3–5 and Table 1, that Glu^{327} is important for stabilization of the tightly bound K^+ and for cation binding from both the cytoplasmic and extracellular medium.

Much of this controversy concerning Glu^{327} in M4 may be ascribed to the differences in assay conditions and different choices of substituting residues. The pronounced effects in the yeast system of conservative substitutions of Gln or Asp for Glu^{327} on binding of Tl^+ and K^+ [31] are questioned because previous hydrophobic substitutions with hydrophobic residues Ala or Leu had only minor effects on $K_{0.5}$ values for Na^+/K^+ stimulation of ATPase activity [24]. One problem is that $K_{1/2}$ values are calculated for normalized curves for K^+ or Na^+ activation of ATP hydrolysis [17,23], rather than the k_{cat}/K_M ratio. This ratio is the proper specificity constant for the discrimination between two competing substrates in an enzymatic reaction [12]. Another problem is the comparison of the effects of hydrophobic Leu or Ala substitutions with those of conservative substitutions of Glu^{327} with Gln or Asp. The K^+ binding data in Fig. 4 and Table 1 show that Leu reduces K^+ affinity three-fold less than Ala, and 16-fold less than Ser. A probable explanation for this may be that Glu^{327} is free to rotate in its position as the +1 residue after a proline and with a neighboring Gly without side chain. The hydrophobic Leu and Ala substitutions may cause rotation of the side chain with exposure of main chain carbonyl(s) for cation binding. The mobility of the residue may be even more pronounced if Glu^{327} is positioned in an unwound loop between two M4 helices like in the high resolution structure of Ca-ATPase [4]. Hydrophobic substitution with Leu or Ala is therefore not relevant for judging the contribution of the carboxylate group of Glu^{327} to cation binding, while the conservative substitutions with Gln or Asp may cause fewer structural rearrangements.

5.2. The role of Glu^{779} in M5

Substitutions of Glu^{779} caused partial disruption of occlusion. It is striking that the Glu^{779}Gln mutation reduced the capacity to one site for Tl^+ per α subunit with three-fold lower affinity than in wild type. This could suggest that Glu^{779} contributes to coordination of both tightly bound K^+ ions in the E_1[2K] form. In the Glu^{779}Asp mutation, the binding of Rb^+ and Tl^+

ions is abolished and this is in agreement with the data of K^+ displacement of bound ATP (Table 1). These changes in binding of Rb^+, Tl^+, and K^+ agree with changes in catalytic properties of the mutations of Glu^{779} [18,19]. Na,K-ATPase activity is also retained after substitutions of Glu^{779}, but with two major changes in catalytic properties. Firstly, for the Glu^{779}Ala mutation, the activity in the presence of Na^+ alone is increased to 40–50% of total Na,K-ATPase activity as compared to 2–3% in wild type as an indication of a major change of cation specificity. Secondly, a large, 4–8-fold decrease of the K_m for ATP of the overall Na,K-ATPase reaction may reflect a shift of the E_1–E_2 equilibrium due to interference with high affinity binding of K^+ [18,19].

Electrophysiological analysis shows that the membrane potential dependence of $K_{0.5}$ for extracellular K^+ activation of pump current is abolished in Glu^{779}Gln and Glu^{779}Ala, while it is preserved in the Glu^{779}Asp mutation. To explain this it has been proposed that the movement of Glu^{779} in the membrane electric field could be rate-limiting for ion transport and that the polar side chain of Glu^{779} may form a portion of the access channel [28]. This proposal does not fit easily with the observations of a reduced stoichiometry of Tl^+ occlusion in the Glu^{779}Gln mutation, the abolished high affinity occlusion in the Glu^{779}Asp mutation, and the consequences of these mutations for K^+ displacement of ATP binding [31]. These data rather suggest that Glu^{779} contributes coordinating groups to K^+ ions in the occlusion cavity per se or that it is important for stability of the cation binding cavity.

5.3. The role of Asp^{804} and Asp^{808} in M6

The deleterious effects of substitutions of Asp^{804} and Asp^{808} on Rb^+ and Tl^+ ion occlusion are in agreement with assays of the enzymatic properties. Analysis of K^+ displacement of ATP binding, Na^+-dependent phosphorylation [41] and K^+ competition with ouabain binding [27] have identified Asp^{804} and Asp^{808} as important residues for high affinity interaction with both Na^+ and K^+. Analysis of sequence homologies among the cation pump proteins supports the notion that these residues are important for occlusion of K^+ in Na,K-ATPase. The carboxylate at Asp^{808} is a conserved feature and a carboxylate at the position homologous to Asp^{804} is only observed in H,K-ATPase [42]. The homologous carboxylates at positions 820 and 824 in H,K-ATPase are also involved in binding of K^+ ions [43], while the Ca-ATPase of plasma membranes [44] and SR [45] or H-ATPase from yeast [46] or plants [47] have Asn or Ala at the position of Asp^{804} in the α subunit of Na,K-ATPase.

6. Cation selectivity

6.1. Contribution of residues in M4 and M5 to cation selectivity

Transitions between E_1 and E_2 forms of the protein are accompanied by large shifts of selectivity for Na^+ and K^+ ions, but little is known about the molecular basis for Na/K selectivity. In addition to the carboxylates, a number of oxygen-containing residues like Asn, Ser, Thr, and Tyr are important for cation binding, in particular for Na/K selectivity [11]. Sequences in M4 (324-NVPEG) and in M5 (771-YTLTSNIPEITP) are unique to Na,K-ATPase (Fig. 2). Mutagenesis screening with assays of direct binding of Tl^+, K^+, or Na^+ has identified groups of importance for Na^+/K^+ selectivity at the cytoplasmic and extracellular membrane surfaces. Binding assays of the mutation Gln^{324}Ala show that the carboxamide group contributes more to coordination of Na^+ than to Tl^+ or K^+ (Table 2). Mutagenesis screening of six oxygen-carrying residues of the specific M5 sequence Y^{771}TITSNIPEITP^{782} shows that the carboxamide group of Asn^{776} or the hydroxyl groups of Ser^{775} and Tyr^{771} contribute more than 6 kJ/mol to the free energy of $Tl^+(K^+)$ binding. Removal of these groups causes parallel changes of K^+ displacement of ATP binding. These oxygen-containing groups also contribute $\geq$6 kJ/mol to the free energy of Na^+ binding as an indication that Asn^{776}, Ser^{775}, and Tyr^{771} may alternately engage in binding of K^+ or Na^+ in the occluded E_1P[3Na] or E_2[2K] conformations [13]. An important differential effect is observed for Thr^{774} as its hydroxyl group contributes to binding of Na^+ ions, while it is not required for high affinity occlusion of K^+ ions (Table 2). Measurements of the changes in free energy of binding of Tl^+ or Na^+ for the substitution of Thr^{774} to Ser or

Ala allow separation of the contributions of the hydroxyl and the methyl groups to cation binding. The hydroxyl group in Thr^{774} contributes 2.7 kJ/mol to the free energy of Na^+ binding, but it prevents binding of Tl^+ with 1.8 kJ/mol and its net contribution to Na^+ binding is 4.5 kJ/mol larger than the contribution to interaction with Tl^+ [11] (Fig. 3 and Table 2). This specific effect of the Thr^{774}Ala mutation on Na^+ interactions suggests that the hydroxyl group in Thr^{774} is involved in the ability of the enzyme to discriminate between Na^+ and K^+ at the cytoplasmic surface.

Differential effects on binding of Na^+ or K^+ are also observed after changing the geometry of the carboxamide group of Asn^{776} (Asn^{776}Glu) or the hydroxyl of Ser^{775} (Ser^{775}Thr). The consequences of mutations of Asn^{776} have not been examined in Na,K-ATPase before. Analysis of the Asn^{776}Ala mutation shows that the carboxamide group contributes almost equally ($\Delta\Delta G_b = 6.7$ or 6.8 kJ/mol) to the free energy of Tl^+ or Na^+ binding [11]. The homologous counterpart in Ca-ATPase of SR, Asn^{768}, also contributes directly to Ca^{2+} coordination [4]. The displacement of the carboxamide group of Asn^{776} by insertion of a methyl group in the side chain (Asn^{776}Gln) has a larger cost for Na^+ binding ($\Delta\Delta G_b = 8.0$ kJ/mol) than for binding of Tl^+ ($\Delta\Delta G_b = 1.9$ kJ/mol) (Figs. 3–5 and Table 2). The increase of side chain volume and altered geometry of the carboxamide group of Asn^{776} is therefore more critical for binding of Na^+ than for binding of K^+.

Ser^{775} also contributes to binding of both K^+ and Na^+. Introduction of a methyl group in the Ser^{775}Thr mutation destabilizes Tl^+ binding with 6.3 kJ/mol ($\Delta\Delta G_b$) and Na^+ binding with 4.1 kJ/mol ($\Delta\Delta G_b$) indicating that K^+ binding is more sensitive to the change in side chain volume or hydrophobicity. The Ser^{775}Ala mutation shows that the hydroxyl group contributes more to Na^+ binding ($\Delta\Delta G_b = 5.9$ kJ/mol) than to binding of Tl^+ ($\Delta\Delta G_b = 3.6$ kJ/mol) (Table 2) [11]. This is at variance with an earlier report stating that this residue is specific for K^+ [26], but the binding data agree with a subsequent report suggesting an alteration in intrinsic Na^+ binding in the Ser^{775}Ala mutation [48]. The residues involved in binding of K^+ in the E_2[2K] form, Asn^{776}, Ser^{775}, Tyr^{771}, and Glu^{779}, align as a patch to form a polar surface that spans about 140° of the 360° helix [11]. In the E_1P[3Na] conformation, the addition of Thr^{774} to the group of coordinating residues means that the patch of mutation-sensitive residues extends by about 100° to involve two thirds of the circumference of the helix [11].

6.2. Energy requirements for Na/K selectivity

It is important to estimate if the free energy contributions of residues in M4 and M5 are relevant for the Na^+/K^+ selectivity at the cytoplasmic and extracellular surfaces of intact cells. The apparent affinities for Na^+ and K^+ at the cytoplasmic surface have been estimated to be 0.6 mM and 10 mM [8]. This affinity ratio corresponds to a 16-fold preference for Na^+ over K^+ at the cytoplasmic surface and this requires that Na^+ binding is stabilized by 6.4 kJ/mol more than binding of K^+[13]. The specific contribution of Thr^{774} to binding of Na^+ and contributions in the range of 4–7 kJ/mol from Thr^{776}, Ser^{775}, and Tyr^{771} in the cytoplasmic part of M5 may therefore be relevant for the selectivity for Na^+ at the cytoplasmic surface. At the extracellular surface the affinities for Na^+ and K^+ amount to 600 mM and 0.2 mM [8]. The extracellular K/Na affinity ratio of 3000-fold requires that K^+ binding is 18.4 kJ/mol more stable than binding of Na^+. Glu^{327} contributes 4–8 kJ/mol more to binding of Tl^+ or K^+ than to binding of Na^+, but the contribution of several residues appears to be required.

Our data agree with the notion that adjustment of the site from specificity for K^+ over Na^+, E_2[2K], to one with specificity for Na^+ over K^+ E_1P[3Na] is accompanied by 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). The adaptation of the residues contributing to cation binding in the cytoplasmic part of M5 may be achieved by twisting or tilting of the intramembrane segment. In a proposed model for energy transfer the structural change accompanying conformational E_1–E_2 transitions is transmitted to M4 and M5 via connections to the P domain containing the phosphorylated Asp^{351} and the C-terminal 702-GDGVND segment [49].

7. Role of intramembrane proline residues in M4, M5, and M6

As illustrated in Fig. 2, M5 and M6 assume the structure of a hairpin loop with a short extracellular loop (NIPLPLG) with two proline residues. This hairpin remains membrane-associated after extensive proteolytic cleavage of the membrane-bound Na,K-ATPase [50], but only when cation sites are occupied by K^+ during proteolysis. Upon removal of the tightly bound cations, the hairpin spontaneously leaves the membrane and it can be collected in the supernatant after sedimentation of the membrane residual [51]. These observations are in harmony with the previous findings that Na,K-ATPase is most thermostable in KCl medium [1] and that K^+ ion occlusion prevents inactivation of soluble Na,K-ATPase in non-ionic detergent ($C_{12}E_8$) [52].

The carboxylate groups are positioned in the vicinity of proline residues in the presumptive transmembrane segments, M4 (NVPE^{327}G), M5 (TS^{775}NIPE^{779}ITP), and M6 (ID^{804}LGTD^{808}MVP). The presence of proline in a helix of the sequence 774-TSNIPEITP of M5 may create a kink with positioning of these hydrophobic residues at the concave curvature, while Pro^{778} and its +1 (Glu^{779}), −3 (Ser^{775}), and −4 (Thr^{774}) polar neighbors are exposed on the convex side [53]. Another consequence is that the amide nitrogen of Pro^{778} cannot form the normal backbone hydrogen bond with the −4 neighbor, Thr^{774}, and it may sterically prevent the −3 neighbor, Ser^{775}, from making a hydrogen bond with its +1 neighbor. These effects of proline on the structure may serve to expose both side chain and main chain oxygens for coordination of cations. With this in mind it is interesting that the substitution of Pro^{778} to Ala causes a 5–6-fold increase in $K_{0.5(K^+)}$ without altering $K_{0.5(Na^+)}$ [54]. The replacement of Pro^{778} with Ala allows formation of hydrogen bonds with the carbonyl groups of Ser^{775} or Thr^{774} and this may interfere with the role of the carbonyl in coordinating K^+ ions. An important example of this is the 304-VAAIPEGL sequence in M4 of Ca-ATPase [3,4]. In the high resolution structure, the main chain carbonyl groups of the −4 (Val^{304}) and the −3 (Ala^{305}) neighbors to Pro^{309} contribute to coordination of one Ca^{2+} ion [4]. In Na,K-ATPase, the homologous α subunit sequence in M4 is 322-VANVPEGL and it is probable that the −3 (Ala^{323}) and −4 (Val^{321}) neighbors to Pro^{326} may also expose carbonyl groups to coordination of Na^+ or K^+. Similarly, carbonyls could be exposed in M6 where the essential Asp^{808} and Thr^{807} are −3 and −4 neighbors to Pro^{811} in the sequence 803-IDLGTDMVP.

The high resolution structure shows that in M4 of Ca-ATPase the sequence around Glu^{309} is unwound to form a loop with rows of exposed oxygen atoms that are important for entry of Ca^{2+} ions into the binding site [4]. With respect to distribution of intramembrane proline residues there are important differences between Ca-ATPase and the α subunit of Na,K-ATPase. The unwound loop sequence 307-IPEGLP dividing M4 in Ca-ATPase into two helices is remarkably similar to the 777-IPEITP sequence in M5 in the 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 [11] and this loop could be important for guiding the entry of Na^+ from the cytoplasm to the cation binding cavity.

8. Lysine substitutions of cation site residues of Na,K-ATPase in the salt gland of *Artemia salina*

After identification of Asn^{324} and Asn^{776} as cation-coordinating residues in the α subunit of Na,K-ATPase it was realized that a KK isoform of the α subunit with lysine substitutions at these two positions is expressed in the chloride cells of the salt gland of the brine shrimp *Artemia salina* [55,56]. In the intestine and other tissues of the shrimp, the normal α subunit NN isoform of Na,K-ATPase is expressed with Asn at the two positions. The protonated ε amino groups of Lys may provide an internal monovalent cation with properties like the NH_4^+ ion. It is known that NH_4^+ ions can replace K^+ at the transport sites of Na,K-ATPase with about the same affinity as for K^+ ions [8]. Examples of a functional lysine substitution are found in bacterial pyruvate kinases that do not require activation by external K^+. Here, substitution of Glu^{117} to Lys in the form of a protonated ε amino group serves as an internal monovalent cation [57].

Table 3
Single and double lysine substitutions of Asn^{324} in M4 and Asn^{776} in M5

Allele	Alterations in Gibbs free energy $\Delta\Delta G_b$ (kJ/mol)		
	Tl^+	K^+	Na^+
M4-M5			
N324K	+5.5	+4.0	+5.0
N776K	+8.0	+4.0	+7.0
N776K/N324K	+3.5	+4.0	+7.0

Alterations in Gibbs free energy ($\Delta\Delta G_b$) of Tl^+ or Na^+ ion binding at 4°C of mutations of residues in M4, M5, M6, or M8 of the α subunit of Na,K-ATPase.

To clarify the role of the Lys substitutions in the KK isoform of *A. salina*, the consequences of single and double mutations in the α subunit of pig kidney Na,K-ATPase were examined. The single replacement of Asn^{324} to lysine causes a large depression of high affinity Tl^+ occlusion with 11-fold reduction of the maximum binding/$K_{0.5(Tl^+)}$ ratio (Table 3). Substitution of Asn^{766} to lysine completely eliminates high affinity Tl^+ binding, but introduction of Asn^{324}Lys as a second mutation increases Tl^+ binding to one Tl^+ ion occluded per ouabain binding site with 2.5-fold higher $K_{0.5(Tl^+)}$ than in wild type (Table 3). Double mutant cycle energy analysis [11] of the binding data shows that the single substitution of Asn^{324} or Asn^{776} to lysine reduces the free energy of binding with 5.7 kJ/mol or 15.8 kJ/mol, while the interaction between the two lysine residues after double lysine substitution favors Tl^+ binding by −18.3 kJ/mol. It is probable that electrostatic repulsion between the two substituted lysines rearranges the structure thus favoring the binding of a single Tl^+ ion. Although both the first and second lysine substitutions reduce Na^+-dependent phosphorylation from ATP, 10–20% of Na,K-ATPase activity remains in the double mutation.

The salt gland of *A. salina* is unique as it allows the shrimp to survive in salt brine with concentrations rising to 20–30% salt. In these conditions, the work required to transport Na^+ from hemolymph to sea water will rise from 10–12 kJ/mol to >20 kJ/mol Na^+ [8]. Transport ceases when the work required to transport three Na^+ ions exceeds the free energy of ATP hydrolysis (−60 kJ/mol [8]). The observation that 10–20% Na,K-ATPase activity remains after double lysine substitutions suggests that the Na,K pump stoichiometry has been altered to one K and one to two Na per ATP in this isozyme thus adapting to the energy requirements of the transport process. The function of lysines as fixed internal monovalent cation analogues for K^+ ions may therefore explain why brine shrimp survive as the only animal species in the extreme conditions in salt lakes.

9. Structure–function relationships of ATP binding

9.1. Assay of high affinity ATP binding at equilibrium and identification of coordinating residues

The E_1 forms of Na,K-ATPase in NaCl medium or in Tris buffer have high affinities for ATP (K_D = 30–100 nM) while the K^+-bound E_2 forms have very low affinities for ATP (K_{ap} = 200–400 μM) [7]. Specific binding of ATP or ADP to wild type and mutant Na,K-ATPase from yeast can therefore be determined as the binding at 10 mM NaCl minus that in medium containing 10 mM KCl [22,30].

Several residues have earlier been implicated in binding of ATP by mutagenesis or chemical labelling in Na,K-ATPase [58–64]. Chemical labeling experiments suggested that an arginine residue is part of the ATP binding domain of Na,K-ATPase [65], but the specific residue has not been identified. Mutagenesis screening is applied to identify charged residues of importance for high affinity ATP binding in the segment Arg^{544}–Asp^{567} in the cytoplasmic protrusion of the α1 subunit of porcine Na,K-ATPase. This segment is selected for mutagenesis because it has partial sequence homology to nucleotide binding domains in adenylate kinase [1,66] and the ADP/ATP exchange protein [67]. The segment between Gly^{547} and the negatively charged Asp^{555} is hydrophobic and labeled by probes that are designed for tagging of intramembrane segments [68]. Other observations also indicate that this region is important. The neighboring Cys^{549} is a receptor for ATP-protectable labeling with 2-[4′-maleimidylanilino]naphthalene-6-sulfonic acid [69] or erythrosin isothiocyanate [70] and both modifications block catalysis. The data in Table 4 show that substitution of Arg^{544} to Gln interferes selectively with the binding of ATP or ADP.

Double mutation with Asp^{369}Ala shows that the contribution of the basic groups of Arg^{544} to the change in free energy of ATP binding is 6 kJ/mol ($\Delta\Delta G_b$). Arg^{544} is therefore essential for high affinity binding of ATP or ADP and for catalysis. In an alignment with Ca-ATPase of SR, Arg^{544} in the α subunit is positioned two residues upstream of Arg^{560} in Ca-ATPase and this residue is part of the single nucleotide binding site in the N domain [4]. As Arg^{544} is essential for high affinity binding of both ATP and ADP in Na,K-ATPase the data suggest that Arg^{544} has a role in stabilizing the α and β phosphates of ATP and ADP. Reduced affinities for ATP are seen after mutation of Asp^{555}, Glu^{556}, Asp^{565}, or Asp^{567} corresponding to contributions of 2–4 kJ/mol to the free energy of binding of free ATP [71].

9.2. *Role of the phosphorylated residue Asp^{369} in control of ATP binding and conformational E_1–E_2 transition*

Binding of [^{3}H]ATP at equilibrium reveals an intrinsic affinity of the Asp^{369}Ala mutation for ATP (K_D = 1.5 nM) that is 30–40-fold higher than for wild type Na,K-ATPase (K_D = 39 nM) (Fig. 6). This mutation of the site for transient phosphorylation of Na,K-ATPase and other cation pumps effectively blocks phosphorylation and ATPase activity [39,72]. The free energy required to overcome the electrostatic interaction between the carboxylate group of Asp^{369} and the γ phosphate of ATP is equal to the difference in free energy of ATP binding between wild type and the Asp^{369}Ala mutation and it amounts to 7.9 kJ/mol since the alanine side chain is without charge [30]. The reduction of the electrostatic repulsion between Asp^{369} and the γ phosphate upon exchanging this residue to Asn or Ala reveals an intrinsic high affinity binding of ATP that has not previously been observed in Na,K-ATPase. A similar increase in affinity for MgATP has been observed after the Asp^{351}Ala mutation in Ca-ATPase [73]. These results suggest that it is an important functional aspect of the negative charge at the phosphorylation site to balance out these very tight associa-

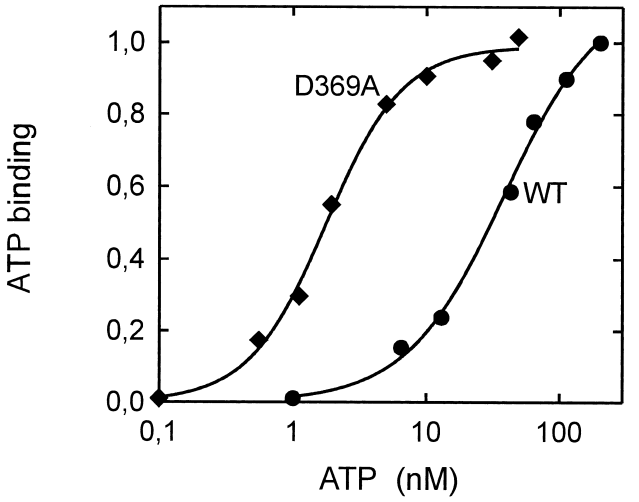

Fig. 6. High affinity free ATP binding of wild type Na,K-ATPase and the Asp^{369}Ala mutation. Yeast membranes were incubated on ice with 5 mM MOPS–Tris pH 7.2, 10 mM EDTA–Tris, protease inhibitors, [^{3}H]ATP (Amersham, specific activity 36 Ci/mmol) to a final concentration of 6–21 nM plus cold Tris–ATP to final total ATP concentrations of 6–200 nM. Either 10 mM NaCl or 10 mM KCl was added to allow estimation of specific binding. Bound and unbound [^{3}H]ATP were separated by centrifugation at 265 000×g for 30 min at 4°C. The supernatant was discarded and the pellet was resuspended for measurement of bound [^{3}H]ATP by scintillation counting and determination of protein content [30]. K_D was estimated from the equation ATP binding = a×[ATP]/(c+[ATP]) in which a is the maximum ATP binding and c is the dissociation constant. K_D for wild type (●) is 38 ± 5 nM and for Asp^{369}Ala (◆) 1.8 ± 0.1 nM.

Table 4
Dissociation constants for ATP or ADP for mutations R544K and D369A/R544Q compared to wild type and D369A

	ATP/ouabain binding ratio	K_D ATP (nM)	K_D ADP (μM)
WT	0.81 ± 0.06	39 ± 7	0.083
R544K	0.75 ± 0.04	29 ± 7	1.26
R544Q	0.02	n.d.	n.d.
D369A	0.9 ± 0.1	1.5 ± 0.2	0.12
D369A/R544Q	0.86 ± 0.06	28 ± 6	0.74

Values for ATP binding are calculated from curves similar to those in Fig. 6. Values of K_D ADP were estimated as before [30] using the equation: $K_{ADP} = K_{1/2}(ADP)\ K_{ATP}/(K_{ATP}+[ATP])$.

tions of ATP with the binding domain in order to prevent the formation of an energy well within the reaction cycle. As proposed by Jencks [74], high energy intermediates are likely to act as barriers to rapid turnover of transport systems.

Analysis of the K^+–ATP antagonism shows that the reduction of charge and hydrophobic substitution at Asp^{369} of the α subunit causes a large shift in conformational equilibrium towards the E_2 form. Substitution of Asp^{369} or Asn^{369} to 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 the absence of Mg^{2+} [30]. In addition to its function as a receptor of the γ phosphate of ATP, Asp^{369} has important short-range catalytic functions in modulating the affinity for ATP and long-range functions in governing the E_1–E_2 transitions which are coupled to reorientation of cation sites and changes in affinity for digitalis glycosides. The position of the free charge of the carboxylate group of Asp^{369} may be such that it contributes to preventing the E_1–E_2 transition in the absence of phosphate and magnesium. Removal of the charge and hydrophobic substitution in Asp^{369}Ala favors transmission of the conformational transition originating in the phosphorylated segment through the transmembrane segment to the ouabain site at the extracellular surface. Substitution to a hydrophobic residue may in part substitute for the requirements for Mg^{2+} and phosphate, since mutation of Asp^{369} or Asn^{369} to 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 the absence of Mg^{2+} [30]. Mutations in the region around Asp^{369} may answer questions concerning the role of charge and relative hydrophobicity of the peptide segment around Asp^{369} for the E_1–E_2 transitions and the coupling to cation translocation and changes in affinity of Na,K-ATPase for digitalis glycosides.

10. The P domain and energy transduction in Na,K-ATPase

The structure of the P domain has been solved at high resolution for Ca-ATPase of SR [4]. Alignment shows that the amino acid sequences of Na,K-ATPase and Ca-ATPase are homologous around the phosphorylation site and the C-terminal TGDGVND sequence. The sequences connecting the phosphorylation site to M4 and the TGDGVND to M5 show 50–60% amino acid homology between the two proteins. It is therefore justified to use the P domain structure in Ca-ATPase [4] as a model for that in Na,K-ATPase. The aspartates of the 708-TGDGVND segment are essential for function [75] and they were thought to engage in ATP binding on the basis of chemical labeling with FSBA of Lys^{719} [76] or with ClRATP at Asp^{710} or Asp^{714} [77].

Recently, mutations of charged residues in the TGDGVND sequence were expressed in yeast using modified fermentation protocols with reduced temperatures to prevent the unfolded protein response and proteolysis of mutant α subunit protein [78]. Assay of ATP binding at equilibrium shows that in mutations of Asp^{710} and Asn^{713}, ATP affinity is preserved or increased, while Na,K-ATPase activity is severely reduced. Assay of phosphorylation of Asp^{369} with formation of the MgE_1P[3Na] intermediate in the presence of oligomycin shows that the affinity for Mg^{2+} is reduced seven-fold in the Asn^{713}Ala mutation, 27-fold in Asp^{710}Asn, and that Asp^{710}Ala almost abolishes Mg^{2+} binding (Table 5). Asp^{710} and Asn^{713} are therefore important for coordination of

Table 5
Consequences of mutations of Asp^{710} and Asn^{713} for ATP binding and Mg^{2+} binding in the E_1P or E_2P phosphorylated forms

	ATP/ouabain binding ratio	K_D ATP (nM)	$K_{1/2(Mg^{2+})}$ E_1P[3Na] (μM)	$K_{1/2(Mg^{2+})}$ E_2P–ouabain (μM)
WT	1.4 ± 0.1	38 ± 5	24 ± 5	11 ± 2
D710N	1.2 ± 0.1	19 ± 2	648 ± 88	5 ± 2
D710A	1.2 ± 0.1	16 ± 1	> 10 000	2 ± 1
N713A	0.7 ± 0.1	34 ± 2	168 ± 76	4 ± 1

Values for ATP binding are calculated from curves similar to those in Fig. 4. $K_{1/2(Mg^{2+})}$ values were determined from curves of Mg^{2+} activation of Na^+-dependent phosphorylation from ATP in the presence of oligomycin or for curves of [^{3}H]ouabain binding with excess phosphate in the medium [11,14].

Mg^{2+} in the phosphorylated E_1P complex. Mutations of Asp^{714} are expressed in yeast, but they are completely devoid of catalytic activity and their properties have not yet been analyzed. Mutation with removal of the charge of Asp^{710} and Asn^{713} facilitates ATP binding and causes a shift of the conformational equilibrium towards the E_2 form, as described above for Asp^{369}. It is a surprise that the data in Table 5 show that Asp^{710} and Asn^{713} do not contribute to coordination of Mg^{2+} in the E_2–ouabain complex. The E_1P–E_2P transition must therefore involve a shift of Mg^{2+} coordination, but the new coordinating residues have not been identified by mutagenesis. Inferences have been made from Fe^{2+}-induced cleavage that involves the TGES segment in the second cytoplasmic loop [79]. In Ca-ATPase, this segment is part of the A domain consisting of the second cytoplasmic loop between M3 and M4 and the N-terminus [4]. It is known from previous experiments with chymotryptic cleavage that the second cytoplasmic loop is essential for the E_1P–E_2P transition [1]. The mutagenesis data show that the 708-TGDGVND segment has different functions in the E_1 and E_2 conformations. Asp^{710} is essential and Asn^{713} is important for transfer of γ phosphate from ATP to the Asp^{369} residue and for coordination of Mg^{2+} in E_1P[3Na]. The transition to the E_2P form involves a shift of Mg^{2+} coordination away from these two residues and they become more important for K^+-induced hydrolysis of the acyl–phosphate bond at Asp^{369}. This role of the 708-TGDGVND segment in Na,K-ATPase is commensurate with its position in a strongly negatively charged domain in close proximity to the phosphorylated Asp^{351} in Ca-ATPase [4].

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