Consequences of Mutations to the Phosphorylation Site of the α-Subunit of Na,K-ATPase for ATP Binding and E_1-E_2 Conformational Equilibrium†

Per Amstrup Pedersen, Jakob H. Rasmussen, and Peter L. Jørgensen*

Biomembrane Research Centre, August Krogh Institute, University of Copenhagen, 2100 Copenhagen OE, Denmark

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ABSTRACT: Expression of Na,K-ATPase in yeast allowed targeting of αβ-units with lethal substitutions at the phosphorylation site α1(D369N)β1 and α1(D369A)β1 at the cell surface at the same concentration of α-subunit and [^{3}H]ouabain binding sites as for wild type Na,K-ATPase. Phosphorylation and reaction with vanadate were abolished, and the mutations had no Na,K-ATPase or K-phosphatase activity. Binding of [^{3}H]-ATP at equilibrium revealed an intrinsic high affinity of the D369A mutation for ATP (K_D = 2.8 nM) that was 39-fold higher than for wild type Na,K-ATPase (K_D = 109 nM). The affinities for ADP were unaffected, indicating that the negative charge at residue 369 determines the contribution of the γ-phosphate to the free energy of ATP binding. Analysis of the K^+-ATP antagonism showed that the reduction of charge and hydrophobic substitution at Asp^{369} of the α-subunit caused a large shift in conformational equilibrium toward the E_2-form. This was accompanied by a large increase in affinity for [^{3}H]ouabain in Mg^{2+} medium with K_D = 4.9 nM for D369A compared to K_D = 51 nM for D369N and K_D = 133 nM for wild type, and [^{3}H]ouabain binding (K_D = 153 nM) to D369A was detectable even in absence of Mg^{2+}. In addition to its function as 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.

In the renal Na,K-pump, α1-subunits (1016 residues) and β1-subunits (302 residues) exist in a 1:1 stoichiometric ratio. The α-subunit may contain 10 transmembrane segments with sites for ATP binding and phosphorylation in the large central cytoplasmic protrusion between the fourth and fifth transmembrane segments, while the cation occlusion sites lie in the intramembrane domain. Residues of importance for formation of sites for cardiac glycosides are identified in the first and third extracellular loops of the α-subunit. The β-subunit has a single transmembrane segment, and the bulk of its hydrophilic residues are exposed to the extracellular surface with three N-linked glycosylation sites and three conserved disulfide bridges (Capasso et al., 1992; Jorgensen, 1992; Lingrel & Kuntzweiler, 1994; Lutsenko & Kaplan, 1995). In models of the reaction cycle, Figure 1, relatively large E_1-E_2 conformational changes in the α-subunit are thought to mediate long-range interactions between the ATP site and the cation occlusion sites in the membrane domain (Jorgensen, 1975; Jorgensen & Andersen, 1988; Lutsenko & Kaplan, 1995). In the initial step, binding of ATP with low apparent affinity ($K_m \approx 0.2-0.4$ mM) to the occluded E_2[2K]-conformation accelerates the E_2[2K]$-E_1$(2K) transition with deocclusion of K^+ at the cytoplasmic surface. ATP is bound with high affinity in the E_1-conformation ($K_D \approx 0.1-0.2$ μM) (Norby & Jensen, 1988), and the increase in

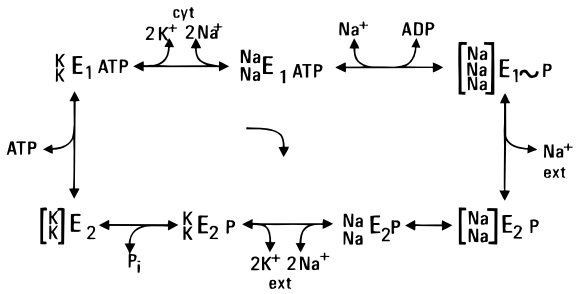

FIGURE 1: E_1-E_2 reaction cycle of the Na,K-pump with ping-pong sequential cation translocation. The phosphoforms can occlude Na^+, and dephosphoforms can occlude K^+ or Rb^+. [Na^+] or [K^+] within brackets are occluded and prevented from exchanging with medium cations. Binding studies with ^{22}Na showed that E_1P[3Na] occluded 3 Na^+ ions, while the capacity of E_2P[2Na] corresponded to 2 Na^+ ions per α-subunit (Jorgensen, 1991).

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 (Laüger, 1991). 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]$-E_2$P[2Na] phosphoforms in coupling with reorientation of cation sites and release of Na^+-ions at the extracellular surface.

Asp^{369} in a conserved Cys−Ser−Asp(P)−Lys−Thr segment (Walderhaug et al., 1985) is essential for the enzymatic activity as replacement of Asp^{369} by Asn, Glu, Thr, or Ala abolished ATP hydrolysis (Ohtsubo et al., 1990; Kuntzweiler et al., 1995; Pedersen et al., 1996) as do mutations to the phosphorylation site of Ca-ATPase from sarcoplasmic reticulum (Maruyama & MacLennan, 1988) or the H-ATPase from

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[1] Abbreviations: ClR-ATP, α-(4-(*N*-(2-chloroethyl)-*N*-methylamino)-benzoyl-amide-ATP; FITC, fluorescein 5-isothiocyanate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; K-pNPPase, potassium dependent *p*-nitrophenyl phosphatase activity; PCR, polymerase chain reaction; TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine.

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yeast (Rao & Slayman, 1992). It has however been difficult to assess the catalytic consequences of replacements to the phosphorylation site of Na,K-ATPase and other cation pumps because mutations to the phosphorylated aspartate cause retention in intracellular membranes of Na,K-ATPase in *Xenopus* oocytes (Ohtsubo et al., 1990) or NIH3T3 cells (Kuntzweiler et al., 1995) and of H-ATPase in yeast (Rao & Slayman, 1992). The mutation D369N of the α-subunit of Na,K-ATPase could be recovered from NIH3T3 cells allowing analysis of the influence of phosphate, Na^+, K^+, and ATP or ADP on [^{3}H]ouabain binding (Kuntzweiler et al., 1995).

In yeast, fully active pig α1β1 Na,K-ATPase and the lethal mutation D369N could be expressed in high yield with respect to protein and [^{3}H]ouabain binding sites. The absence of endogenous activity allowed assay of [^{3}H]-ATP binding at equilibrium with demonstration of a large increase in affinity for ATP of D369N in consequence of reducing the negative charge of Asp^{369} (Pedersen et al., 1996). This development opens the opportunity of examining several aspects of the transfer of energy from the ATP binding domain to cation translocation. Exact determinations of intrinsic affinities for binding of ATP and ADP and examination of the K^+−ATP antagonism are important for evaluating the role of nucleotide binding as a driving force for translocation of K^+. It is also important to examine if the amino acid replacements at the phosphorylation site have long-range effects on the cation binding sites in the transmembrane region of the protein and on the extracellular binding site for cardiac glycosides.

In the present work we therefore examined if lethal mutations to Asp^{369}, D369N or D369A, allow targeting of binding sites for [^{3}H]ouabain to the yeast cell surface. Preparations of the mutations were obtained in amounts sufficient to allow analysis of equilibrium binding of [^{3}H]-ATP and ADP. The effects of K^+ on [^{3}H]-ATP binding were monitored to determine the influence of the mutations on the equilibrium constant for the $E_2[2K]-E_1(2K)$ conformational transition. Binding of [^{3}H]ouabain at equilibrium was also used to assess the conformational equilibrium of the mutant proteins and their interactions with cations.

MATERIALS AND METHODS

Site-Directed Mutagenesis. Site-directed mutagenesis was performed according to Ho et al. (1989). The nucleotide sequences of the two D369A mutagenic primers were 5′ CAT CTG CTC AGC CAA AAC CGG 3′ and 5′ CCG GTT TTG TCG GAG CAG ATG 3′. The mismatched nucleotides changing D369 to A369 are underlined. A fragment containing the mutation was subsequently cloned into the expression plasmid pPAP1466. The DNA sequence of the entire PCR fragment was confirmed by dideoxy sequencing. The sequences of the D369N primers were reported before (Pedersen et al., 1996).

Transformation of Yeast Cells. Yeast cells were transformed by electroporation according to Becker & Guarante (1991).

Growth of Yeast and Expression of Na,K-ATPase. A single colony of transformed yeast cells was selectively propagated until saturation in 10 mL of glucose minimal medium supplemented with leucine. Further propagation and large-scale production in an Applicon fermentor equipped with an ADI 1030 Bio Controller and galactose induction of Na,K-ATPase synthesis were performed as before (Pedersen et al., 1996).

[^{3}H]Ouabain Binding to Intact Yeast Cells. Aliquots containing 10 mg of intact yeast cells were incubated at 37 °C for 1 h in 1 M sorbitol, 3 mM $MgSO_4$, 1 mM $Tris_3VO_4$, 1 mM EGTA, 10 mM MOPS−Tris pH 7.2 in the presence of 10 nM [^{3}H]ouabain (Amersham) and varying concentrations of cold ouabain. Solutions of $Tris_3VO_4$ were prepared by dissolving $NaVO_3$ (Merck) in Tris and passing $NaTris_2VO_4$ over Dowex 50W-X8 columns equilibrated with TrisCl. After the solution stood at 4 °C for 20 min, bound and unbound ouabain were separated by centrifugation in an Eppendorf centrifuge at 3000 rpm for 5 min at 4 °C. To prevent proteolysis, 1 mM PMSF, 1 μg/mL chymostatin, 1 μg/mL pepstatin, and 1 μg/mL leupeptin were added. Samples were washed once in ice-cold binding buffer. The amount of bound [^{3}H]ouabain was determined by scintillation counting.

Isolation of yeast membranes, assay of protein, ATPase and phosphatase activities, and phosphorylation from [γ^{32}P]-ATP were performed as before (Pedersen et al., 1996). For preparation of gradient membranes, crude membranes were fractionated on step gradients consisting of 15 mL of 40% plus 15 mL of 15% (w/v) sucrose in lysis buffer with proteolysis inhibitors. A sample of 6−8 mL containing 40 mg of protein was layered on top, and the gradient was centrifuged for 2 h at 50 000 rpm in the Ti-60 Beckman rotor. The membrane band at the 15/40% interface containing 20−30% of sample protein in 6 mL was collected in a syringe, diluted 5-fold in lysis buffer, and collected by centrifugation at 2 h at 50 000 rpm.

SDS-Treatment of Gradient Membranes. Gradient membranes were incubated for 30 min at 20 °C with 0.3 mg/mL SDS, 2 mg/mL protein, 25 mM imidazole, 1 mM EDTA, 1 mM EGTA, pH 7.5 and 1 mM PMSF, 1 μg/mL chymostatin, 1 μg/mL pepstatin, and 1 μg/mL leupeptin to prevent proteolysis. The SDS-treated membranes were sedimented for 30 min at 265000*g* in the Beckman 100A centrifuge and resuspended in the buffers for binding of [^{3}H]-ATP or [^{3}H]-ouabain.

Molecular sieve HPLC of soluble complexes of αβ-units with [^{3}H]ouabain in $C_{12}E_8$ were done as before (Pedersen et al., 1996) on TSK 3000 SW (7.5 × 300 mm) Toyo Soda gel filtration columns with a TSK SW guard column (7.5 × 75 mm).

Equilibrium [^{3}H]-ATP Binding. Aliquots of SDS-treated gradient membranes containing 150−200 μg of membrane protein were incubated on ice with 10 mM MOPS−Tris pH 7.2, 10 mM EDTA−Tris, [^{3}H]-ATP (Amersham, specific activity 36Ci/mmol) to final concentrations of 0.6−13 nM plus varying amounts of cold Tris−ATP and either 0.1−10 mM NaCl or 0.1−10 mM KCl. To prevent proteolysis, 1 mM PMSF, 1 μg/mL chymostatin, 1 μg/mL pepstatin and 1 μg/mL leupeptin were added. Bound and unbound [^{3}H]-ATP were separated by centrifugation at 265000*g* for 30 min at 4 °C. The supernatant was discarded and the remaining buffer removed with a paper towel. The pellet was resuspended for determination of protein and bound [^{3}H]-ATP by scintillation counting, and specific binding was calculated as binding in presence of NaCl minus binding in presence of KCl. Kinetic constants were calculated using the ENZFIT program (Biosoft, Elsevier) and Michaelis−Menten kinetics.

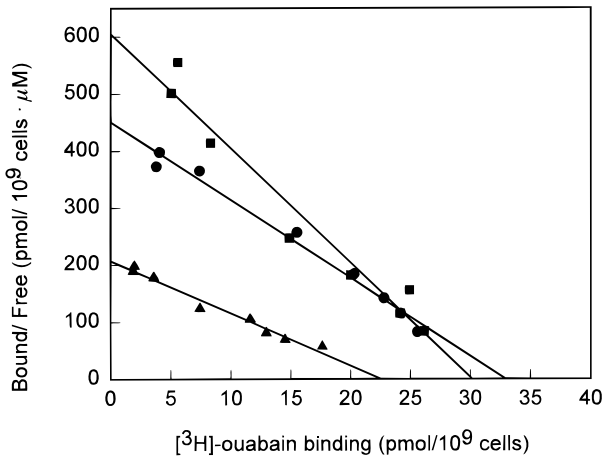

FIGURE 2: [^{3}H]Ouabain binding assay of the expression of wild type $\alpha1\beta1$ (●) and the mutations $\alpha1$(D369N)$\beta1$ (▲) and $\alpha1$-(D369A)$\beta1$ (■) in cell membranes of intact yeast cells. Transformed yeast cells were produced in the fermentor, and aliquots containing 10 mg of cells were incubated for [^{3}H]ouabain binding as described under Materials and Methods. The data were fitted by nonlinear least-squares regression to the lines with $K_D = 72 \pm 4$ nM for wild type, 106 ± 7 nM for $\alpha1$(D369N)$\beta1$, and 48 ± 4 nM for $\alpha1$(D369A)$\beta1$.

Displacement of [^{3}H]-ATP with ADP. Aliquots of SDS-treated gradient membranes containing 100−200 μg of protein were incubated at 0−4 °C for 1 h in 1 mL containing either 10 mM NaCl or 10 mM KCl, 10 mM MOPS−Tris pH 7.2, 10 mM EDTA−Tris, and 13.9 nM [^{3}H]-ATP (Amersham, specific activity 36Ci/mmol), and ADP−Tris concentrations of 0−4000 nM. To prevent proteolysis, 1 mM PMSF, 1 μg/mL chymostatin, 1 μg/mL pepstatin, and 1 μg/mL leupeptin were added. Protein and bound [^{3}H]-ATP were determined as above.

Equilibrium [^{3}H]Ouabain Binding. Aliquots containing 100−200 μg of SDS-treated gradient membrane protein were incubated at 37 °C for 1 h in 3 mM $MgSO_4$, 1 mM $Tris_3$-VO_4, 1 mM EGTA, 10 mM MOPS−Tris pH 7.2 in the presence of 10 nM [^{3}H]ouabain (Amersham, specific activity 36 Ci/mmol), varying concentrations of cold ouabain, and 1 mM PMSF, 1 μg/mL chymostatin, 1 μg/mL pepstatin, and 1 μg/mL leupeptin to prevent proteolysis. After the mixtures were allowed to stand at 4 °C for 20 min, bound and unbound ouabain were separated by centrifugation in the Beckman 100 A centrifuge at 265000*g* for 30 min at 4 °C. Samples were washed twice in ice-cold binding buffer and centrifuged for 10 min at 265000*g*. The pellet was resuspended for analysis of protein and bound [^{3}H]ouabain by scintillation counting.

RESULTS

Expression in Saccharomyces cerevisiae of Mutations D369N, D369A, and Wild Type Na,K-ATPase. After transformation with the cDNA of $\alpha1\beta1$-pig kidney Na,K-ATPase, the yeast cells expressed both the wild type Na,K-ATPase and the D369N and D369A mutations. From the Scatchard plots of [^{3}H]ouabain in Figure 2, it is seen that the capacities for binding to intact cells expressing the D369N or D369A mutations were within the same range as the capacities for binding to cells expressing wild type Na,K-ATPase, suggesting that both wild type and the mutant $\alpha\beta$-units expose their sites for [^{3}H]ouabain binding to the extracellular medium.

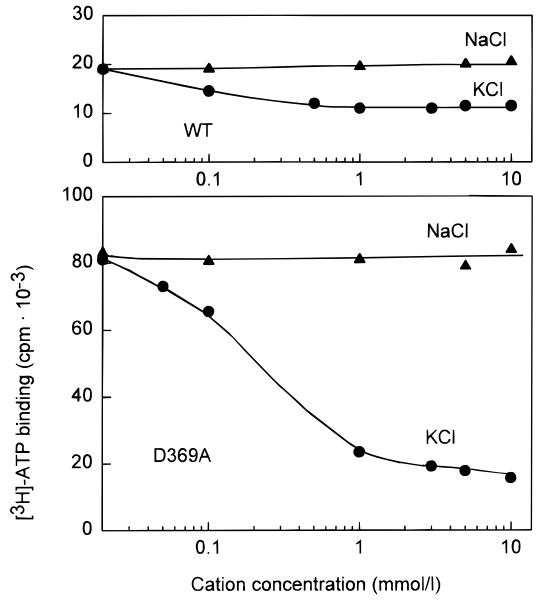

FIGURE 3: Effect of NaCl (▲) or KCl (●) on [^{3}H]-ATP binding to wild type $\alpha1\beta1$ Na,K-ATPase (upper frame) or to mutation D369A (lower frame). Aliquots of about 200 μg of SDS-treated gradient membranes were incubated on ice with 10 mM MOPS−Tris pH 7.2, 10 mM EDTA, [^{3}H]-ATP (Amersham, specific activity 36Ci/mmol) at concentrations of 13 nM and either 0.1−10 mM NaCl or 0.1−10 mM KCl. Estimation of [^{3}H]-ATP binding as described in Materials and Methods.

In intact cells or crude membrane preparations, the affinities for [^{3}H]ouabain were apparent with K_D values for the mutations approaching those found for wild type, Figure 2. All subsequent experiments on binding of [^{3}H]ouabain or [^{3}H]-ATP, including the titrations of the effects of KCl or NaCl, were performed with membrane preparations that had been treated with detergent. The effects of unmasking vesicles with detergent on the affinities for [^{3}H]-ATP or [^{3}H]-ouabain can readily be explained by facilitating the access of the ligands to their respective sites (Jorgensen & Andersen, 1988).

In line with previous results (Pedersen et al., 1996), the [^{3}H]ouabain complexes of solubilized, mutant, Na,K-ATPase were stable during chromatography in $C_{12}E_8$ at 2−4 °C with elution volumes (8.5−9.0 mL), not shown, corresponding to those of wild type and of the purified renal Na,K-ATPase (8.5 mL) (Pedersen et al., 1996). This provides evidence for organization of the phosphorylation mutations in $\alpha\beta$-units with hydrodynamic properties similar to those of wild type recombinant enzyme and the native renal Na,K-ATPase.

Effect of Mutations D369N and D369A on Binding of ATP and ADP. Assays on both crude and detergent treated membranes showed that the Na,K-ATPase and K-phosphatase activities were absent in the D369N and D369A mutations. Na-dependent phosphorylation from [γ^{32}P]-ATP was abolished in the mutations whereas phosphorylation of wild type (data not shown) was comparable to the capacities for binding of [^{3}H]-ATP and [^{3}H]ouabain.

The conditions for demonstrating specific binding of [^{3}H]-ATP or [^{3}H]-ADP are shown in Figure 3. Addition of NaCl up to 10 mM did not alter binding at 13 nM [^{3}H]-ATP since both the $Tris^+$-bound and the Na^+-bound E_1-forms of wild type and mutant proteins have high affinities for ATP (Table 1). In contrast, addition of 0.1−10 mM KCl displaces [^{3}H]-ATP binding because K^+ stabilizes the E_2[2K] form of the

Table 1: Effect of Mutations D369N and D369A on Capacities and Dissociation Constants for [^{3}H]-ATP and ADP

	[^{3}H]-ATP K_{ATP} (nM)	capacity (pmol/mg of Pr)	ADP[a] K_{ADP} (nM)	$\Delta G°(\gamma$-phosphate)[b] (kJ/mol)
wild type	109 ± 11	14.1 ± 0.8	152 ± 10	−0.75
D369N	5.9 ± 0.4	21 ± 1	196 ± 33	−8.0
D369A	2.8 ± 0.5	13.1 ± 1	122 ± 30	−8.6

[a] Values were estimated from the displacement curves in Figure 5 using the equations in Appendix A. [b] $\Delta G°(\gamma$-phosphate), the contribution of γ-phosphate to ATP binding at 0 °C, was calculated as $\Delta G°(\gamma$-phosphate) = $\Delta G°$(ATP binding) − $\Delta G°$(ADP binding).

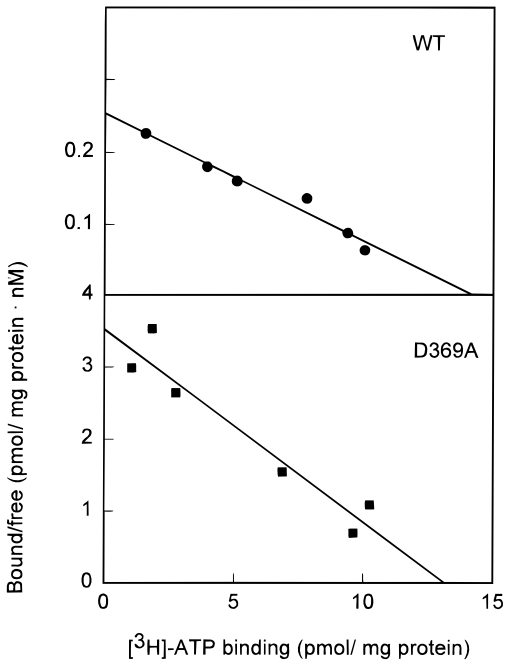

FIGURE 4: Scatchard plot of [^{3}H]-ATP binding to SDS-treated gradient membranes of wild type (●) and mutation α1(D369A)β1 (■) recombinant Na,K-ATPase. Procedures for preparation of gradient membranes, incubation with SDS, and assay of [^{3}H]-ATP binding in 10 mM NaCl or 10 mM KCl medium were as described in Figure 3 and in Materials and Methods. The data were fitted by nonlinear least-squares regression to the lines with K_D = 109 ± 11 nM and a binding capacity of 14.1 ± 0.8 pmol/mg of protein for wild type and K_D = 3.4 ± 0.5 nM and a binding capacity of 13 ± 1 pmol/mg of protein for D369A.

protein with an affinity for ATP (K_{ap} 200−400 μM) more than an order of magnitude lower than that of the E_1-form (Jorgensen & Andersen, 1988; Norby & Jensen, 1988). Specific binding of ATP or ADP to wild type and mutant proteins can therefore be determined as the binding at 10 mM NaCl minus that in medium containing 10 mM KCl. For D369A, the specific binding at 13 nM [^{3}H]-ATP formed a much larger fraction (80%) of total binding than for wild type (50%). For determination of the dissociation constants and total binding capacities, the assays were performed with increasing concentrations of [^{3}H]-ATP to provide the data for the Scatchard plots in Figure 4. It is seen that the mutation D369A altered the dissociation constant from 109 ± 11 nM to 2.8 ± 0.5 nM or by 39-fold. A similar plot for D369N gave a dissociation constant of 5.9 ± 0.4 nM.

In sheep α1-Na,K-ATPase expressed in NIH 3T3 cells (Kuntzweiler et al., 1995), [^{3}H]ouabain binding to wild type was stimulated by MgATP (AC_{50} 18−21 μM) and MgADP (AC_{50} 7 μM), while both nucleotides inhibited [^{3}H]ouabain

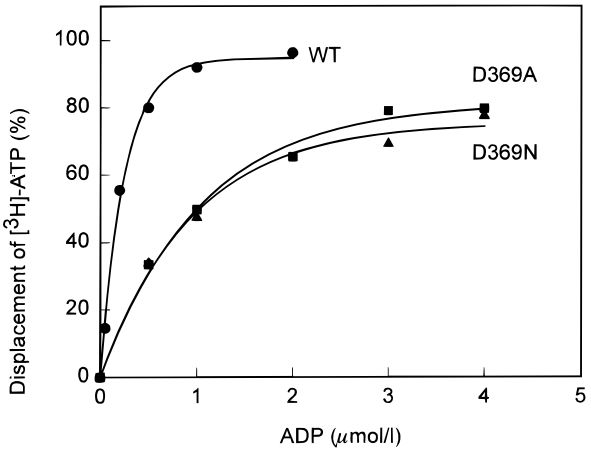

FIGURE 5: ADP displacement of [^{3}H]-ATP binding at equilibrium to SDS-treated gradient membranes of wild type (●) and mutation α1(D369N)β1 (▲) and α1(D369A)β1 (■). Procedures for preparation of gradient membranes, incubation with SDS, and assay of [^{3}H]-ATP binding in NaCl or KCl medium with increasing concentrations of ADP were as described in Materials and Methods. Binding data were fitted according to hyperbolic saturation curves.

binding to the D369N mutation with IC_{50} 2.5 μM for ATP and 5 μM for ADP. These half-maximum values for ATP are 180−400-fold higher than the K_D values in Table 1 for the binding of [^{3}H]-ATP at equilibrium to wild type and D369N. The indirect approach of using [^{3}H]ouabain as a probe of nucleotide interactions may therefore not allow monitoring of the interaction of ATP with the E_1-conformation.

Estimates of the dissociation constants (K_{ADP}) for equilibrium binding between recombinant Na,K-ATPase and ADP were calculated from the curves for equilibrium displacement of [^{3}H]-ATP by ADP in Figure 5. It is seen that the hyperbolic saturation curves fit the data and eq 1 from the Appendix gives the relationship between the $K_{1/2}$ value for ADP displacement and the equilibrium dissociation constants, K_{ATP} and K_{ADP}, for the Na,K-ATPase−nucleotide complexes.

$$K_{ADP} = K_{1/2}(\text{ADP})K_{ATP}/(K_{ATP} + [\text{ATP}]) \qquad (1)$$

The data in Table 1 shows that the calculated K_{ADP} values were in the same range for wild type as for the mutations D369N and D369A. The value for the wild type agreed well with an estimate of the K_{ADP} of 178 ± 51 nM calculated from a Scatchard plot of a direct assay of [^{3}H]-ADP binding (not shown) and with previous estimates for pig kidney Na,K-ATPase (Norby & Jensen, 1988). The contribution of the γ-phosphate to ATP binding at 0 °C was calculated from the experimentally determined equilibrium binding constants for ATP and ADP binding to Na,K-ATPase. Table 1 shows that replacement of the carboxylate group of Asp^{369} with the carboxamide group of Asn (D369N) caused a much larger change in free energy of binding (−7.3 kJ/mol) than the replacement of Asn^{369} with Ala (N369A) (−0.6 kJ/mol). The partial charge of the carbonyl oxygen of Asn is about −0.3 relative to a charge of −1 for the carboxylate group of Asp (Creighton, 1993), and the data suggest that the changes in free energy are related to the reduction in charge of residue 369. In the wild type the charge repulsion is canceled by strong attractive forces between the γ-phosphate of ATP and other residues of the protein (Discussion).

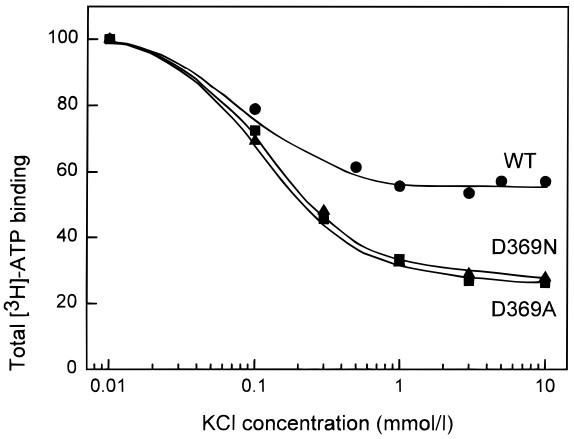

FIGURE 6: Effect of KCl on [^{3}H]-ATP binding to wild type $\alpha1\beta1$ Na,K-ATPase (●) and to mutations $\alpha1$(D369N)$\beta1$ (▲) and $\alpha1$-(D369A)$\beta1$ (■). Aliquots of about 200 μg of SDS-treated gradient membranes were incubated on ice with 10 mM MOPS−Tris pH 7.2, 10 mM EDTA, [^{3}H]-ATP (Amersham, specific activity 36 Ci/mmol) at concentrations of 13 nM and 0.1−10 mM KCl. Estimation of [^{3}H]-ATP binding as described in Materials and Methods.

Table 2: Effects of Mutations D369N or D369A on K^+−ATP Antagonism and on Equilibrium Constants (K_c) for the $E_1(K)$ − $E_2(K)$ Transition as Calculated from $K_{1/2}$ Values Using Eq 2 of Appendix B[a]

	ATP $K_{1/2}$ K^+ (mM)	K_c
wild type	0.11 ± 0.02	769
D369N	0.14 ± 0.01	1798
D369A	0.14 ± 0.02	3195

[a] $K_{1/2}$-values were determined from curves in Figure 6.

Effect of Mutations D369N and D369A on K^+−ATP Antagonism. The K^+−nucleotide antagonism (Norby & Jensen, 1988) is a classic feature of Na,K-ATPase that can be utilized for estimating relative affinities for the ions and their effects on the conformational state of the enzyme protein (Jorgensen & Karlish, 1980). In Figure 6 titration curves for the effects of KCl on [^{3}H]-ATP binding are compared for wild type and the mutations D369N and D369A. It is seen that addition of KCl reduces [^{3}H]-ATP binding until a level is reached at 3−10 mM KCl for both wild type and the two mutant preparations. The data confirm that both of the mutations, D369N and D369A, have retained the ability to form the alternative conformations E_1Na in NaCl or E_2K in KCl medium where specific binding of [^{3}H]-ATP or [^{3}H]-ADP is abolished.

The $K_{1/2}$ values for displacement by KCl of [^{3}H]-ATP were 0.11 mM for wild type and 0.14 mM for the two mutations (Table 2). This limited variation may appear surprising in view of the pronounced differences in affinities for ATP (Table 1), but the interaction with KCl reflects 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 (Jorgensen & Karlish, 1980), see Appendix B.

Mathematical analysis of the equilibrium binding of ATP to Na,K-ATPase with respect to K^+ concentration leads to eq 2 for the $K_{1/2}$ value for K^+ inhibition of ATP binding. (See Appendix B for derivation of the equation.) K_{ATP} has

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

been determined experimentally by equilibrium binding in this work (Figure 4 and Table 1). Previous estimates are available of the potassium affinity for the E_1-conformation of the enzyme, K_p = 75 mM, and the conformational equilibrium between E_1K- and E_2K-forms, K_c = 1000, from experiments on the wild type pig kidney Na,K-ATPase (Karlish, 1980, Jorgensen & Karlish, 1980). Given the experimentally determined $K_{1/2}$ values in Table 2 and the affinities for ATP (K_{ATP} in Table 1), K_c was calculated for the mutations and the wild type under the assumption that K_p is unaltered by the mutations, Table 2. It can be seen that K_c (D369N) has increased 2-fold while K_c (D369A) has increased 4-fold compared to wild type, suggesting that the conformations of these mutations are even more poised in the direction of E_2 than observed for the wild type enzyme.

Table 3: Effect of Mutations D369N and D369A on Dissociation Constants (K_D) for Binding of [^{3}H]Ouabain in Media Containing Mg^{2+}, Vanadate Plus Mg^{2+}, or EDTA[a]

medium	Mg^{2+} 3 mM, vanadate 1 mM, K_D (nM)	capacity (pmol/mg)	Mg^{2+} 3 mM, K_D (nM)	EDTA 1 mM, K_D (nM)
wild type	1.8 ± 0.9	13.4 ± 0.4	133 ± 35	*b*
D369N	58 ± 8	5.8 ± 0.4	51 ± 7	*b*
D369A	4.7 ± 1.2	9.4 ± 0.3	4.9 ± 0.9	153 ± 34

[a] K_D values were estimated from Scatchard plots as in Figure 7. [b] In medium containing 1 mM EDTA and no Mg^{2+}, the counts of [^{3}H]ouabain in the pellet of wild type or D369N were not different from background as determined in medium with 1 mM cold ouabain.

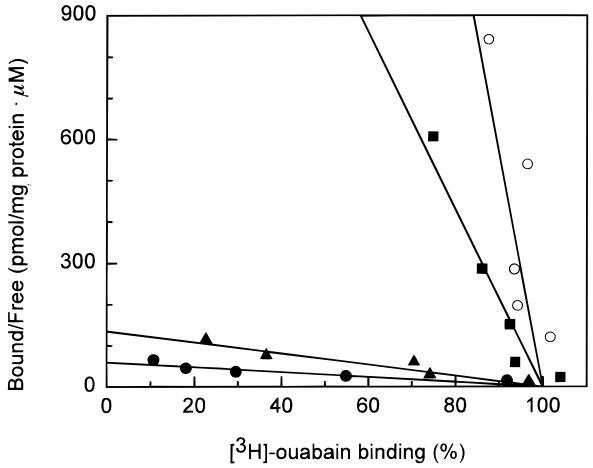

FIGURE 7: Scatchard plots of [^{3}H]ouabain binding in medium containing $MgSO_4$ to wild type Na,K-ATPase (●) and the mutations $\alpha1$(D369N)$\beta1$ (▲) and $\alpha1$(D369A)$\beta1$ (■) and in medium containing $MgSO_4$ plus vanadate to wild type (○). Aliquots of the preparations containing 150 μg of protein were incubated for 60 min at 37 °C with [^{3}H]ouabain as described under Materials and Methods. The data were fitted by nonlinear least-squares regression (lines) to give the capacities and dissociation constants (K_D) in Table 3.

Effect of Mutations D369N and D369A on Ouabain Binding. Also in isolated cell membrane preparations, the concentrations of α-subunit protein (cf. Pedersen et al., 1996) and the capacities for [^{3}H]ouabain binding (Table 3) were in the same range for wild type and mutant preparations. Although vanadate had no influence on [^{3}H]ouabain binding to the mutations, D369N or D369A, the data in Figure 7 and Table 3 show that the affinity of D369A for [^{3}H]ouabain in Mg^{2+} medium (K_D = 4.9 nM) was approaching the range of the K_D for [^{3}H]ouabain binding to wild type (K_D = 1.8 nM) in medium containing both Mg^{2+} and vanadate ($Tris_3$-VO_4 without Na^+). Even in the absence of Mg^{2+}, in medium containing EDTA, binding of [^{3}H]ouabain to D369A was

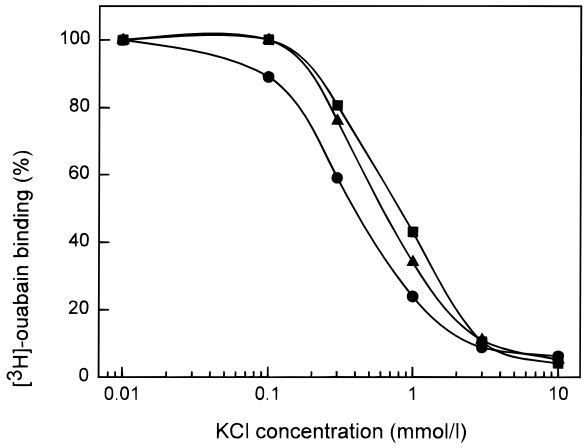

FIGURE 8: Effect of KCl on [^{3}H]ouabain binding to wild type $\alpha1\beta1$ Na,K-ATPase (●) and to mutations $\alpha1$(D369N)$\beta1$ (▲) and $\alpha1$-(D369A)$\beta1$ (■). Aliquots of about 200 μg of SDS-treated membranes were incubated with 3 mM $MgSO_4$, 1 mM EGTA, 10 mM MOPS−Tris pH 7.2 in the presence of 10 nM [^{3}H]ouabain (Amersham, specific activity 36 Ci/mmol) and 0.1−10 mM KCl. Estimations of [^{3}H]ouabain binding were as described in Materials and Methods.

Table 4: Effects of Mutations D369N or D369A on K^+−Ouabain and Na^+−Ouabain Antagonism and on Equilibrium Constants (K_c*) for the $E_1 - E_2$ Transition in the Presence of Mg^{2+} as Calculated from $K_{1/2}$ Values Using Eq 3 of Appendix C[a]

	ouabain			
	$K_{1/2}$ K^+ (mM)	$K_{1/2}$ Na^+ (mM)	$K_{1/2}$ $Tris^+$ (mM)	K_c*
wild type	0.39 ± 0.03	6.8 ± 0.3	30 ± 3	0.46
D369N	0.65 ± 0.09	5.5 ± 0.6	23 ± 2	2.2
D369A	0.83 ± 0.10	14.5 ± 1.4	57 ± 25	5.2

[a] $K_{1/2}$ values were determined from curves as in Figure 8.

detectable with relatively low affinity (K_D = 153 nM) while there was no binding to wild type or D369N. An altered poise of the MgE_1−MgE_2 conformational equilibrium may at least in part explain these consequences of the mutations D369N and D369A for the affinity for [^{3}H]ouabain in Mg^{2+} medium (Table 3).

Effect of Mutations D369N and D369A on K^+−Ouabain and Na^+−Ouabain Antagonism. It is known from previous studies (Hansen & Skou, 1973; Johnson et al. 1995) that K^+ or Na^+ stabilizes conformations with relatively low affinities for ouabain. These effects can be useful for estimating relative affinities for the ions and their effects on the conformational state of the enzyme protein. In Mg^{2+} medium, the addition of K^+ stabilizes the E_2K-conformation with a reduced binding affinity for ouabain (Hansen & Skou, 1973). From the curves of [^{3}H]ouabain binding at increasing KCl concentrations in Figure 8, it is seen that the mutations increase the $K_{1/2}$ values for KCl 2-fold from 0.39 mM in wild type to 0.65 and 0.83 mM for D369N and D369A, Table 4. It is of interest to see how these experimental data fit with the equilibrium constants derived above from the analysis of the interactions of wild type and mutant preparations with ATP and KCl.

In Appendix C, eq 3 is deduced to estimate K_c^*, the equilibrium constant between the E_1-form and the E_2-form at saturating Mg^{2+} concentration, from $K_{1/2}$ for K^+ inhibition of ouabain binding to Na,K-ATPase; K_D, the equilibrium binding constant for ouabain binding to wild type (1.8 nM, Table 3); K_c, the equilibrium constant for E_1(K) and E_2(K) (Table 2), and K_p, the equilibrium constant for K^+ binding to the E_1-form and the ouabain concentration. It is assumed that K_p is unaltered by mutation and the addition of Mg^{2+}.

$$K_c^* = K_D\{K_{1/2}(1 + K_c) - K_p\}/K_p([OU] + K_D) \quad (3)$$

From the estimated K_c* values in Table 4 it can be seen that the K_c* values for the D369N and D369A mutations have increased 5−11-fold in the same direction as the values obtained from analysis of the K^+−ATP antagonism (Table 2).

In Table 4 the values for K^+ are compared to similar titrations of the effects of Na^+ or $Tris^+$ on the binding at a low concentration of [^{3}H]ouabain (10 nM) in the presence of Mg^{2+}. In agreement with the shift of conformational equilibrium, higher concentrations of Na^+ or $Tris^+$ were required for preventing [^{3}H]ouabain binding to D369A than to wild type, while the concentrations for D369N were lower for reasons that are not fully understood. The $K_{1/2}$ value for wild type in Table 4 is much higher than that for ^{22}Na occlusion in the E_2P−ouabain complex of renal Na,K-ATPase ($K_{1/2}$ = 1.5 mM) (Jorgensen, 1991). This may reflect the fact that, in addition to binding of Na^+, a series of reactions is involved in the exclusion of [^{3}H]ouabain in presence of Mg^{2+} and stabilization of the E_1Na-conformation of Na,K-ATPase (Hansen & Skou, 1973; Johnson et al., 1995).

DISCUSSION

Characterization of lethal Na,K-ATPase mutations with respect to enzyme−ligand interactions and conformational equilibria is shown to be a fruitful approach at establishing structure−function relationships for the phosphorylated residue of the Na,K-pump. This approach is facilitated after expression of the mutations $\alpha1$(D369N)$\beta1$ and $\alpha1$(D369A)$\beta1$ and wild type Na,K-ATPase in high yield in yeast cell surface membranes devoid of endogenous activity. Assay of binding of [^{3}H]-ATP and ADP at equilibrium to these mutations led to identification of an intrinsic site for very tight binding of ATP and determination of the relative affinities for nucleotides. The data show that Asp^{369} has important short-range functions as a major determinant of the equilibrium affinity for ATP. The pronounced effects of the substitutions on the K^+−ATP antagonism and [^{3}H]ouabain binding demonstrate that Asp^{369} also has important long-range functions in regulation of the E_1−E_2 conformational transitions.

Size exclusion chromatography of the soluble [^{3}H]ouabain complexes shows that the hydrodynamic properties of the mutated $\alpha1$(D369A)$\beta1$ and $\alpha1$(D369N)$\beta1$ units are similar to those of recombinant wild type or pig kidney $\alpha1\beta1$ Na,K-ATPase. This, together with the observations that high-affinity ligand binding sites for nucleotides and ouabain are preserved along with interactions with Na^+ and K^+ and the transitions between E_1- and E_2-conformations, supports the notion that unspecific perturbations of tertiary structure are indeed limited after mutations to the phosphorylation site. Both cytoplasmic and extracellular aspects of the mutant proteins appear to be correctly folded and structurally organized in the membrane.

Appearance of [^{3}H]ouabain binding sites at the surface of yeast cells expressing either wild type or mutated $\alpha1\beta1$-units may appear surprising, since previous data have shown that mutations to the transiently phosphorylated aspartate residue

cause retention in intracellular membranes and failure in targeting of Na,K-ATPase (Ohtsubo et al., 1990; Kuntzweiler et al., 1995) or H-ATPase (Rao & Slayman, 1992) to the plasma membrane. This could be related to the role of the β-subunit in assembly and targeting of Na,K-ATPase (Geering, 1990). In NIH3T3 cells (Kuntzweiler et al., 1995) and *Xenopus* oocytes (Ohtsubo et al., 1990) the exogenous α-subunits must compete with endogenous α-subunits for assembly with the endogenous β-subunits in the endoplasmic reticulum. Preference of the endogenous β-subunit for wild type subunits over proteins with lethal mutations may thus explain the retention of the mutated protein in the intracellular membranes in these cells. Competition for assembly or targeting appears to be avoided in the present yeast expression system, because the α1- and β1-subunits are coexpressed from the same plasmid and competing endogenous α-subunits are absent.

Role of the Charge of the Carboxylate Group of Asp^{369} in ATP Binding. The analysis of the D369N and D369A mutations showed that residue Asp^{369} is of fundamental importance in determining the affinity for ATP relative to ADP through interaction with the γ-phosphate from ATP, since the drastic increase in affinity for ATP in consequence of mutation of Asp^{369} to Asn or Ala was not accompanied by significant alterations in the affinities for ADP (Table 1).

It is of interest to learn if the changes in affinity for ATP observed after mutagenesis are correlated to removal of the negative charge of the side chain of residue 369 with subsequent reduction of the electrostatic repulsion between the side chain of Asp and the γ-phosphate of ATP. 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 the wild type (D369) and the D369A mutation, and it amounts to 7.9 kJ/mol (Table 1) since the alanine side chain is without charge. The free energy expense needed to position ATP in the binding pocket of the D369N mutation with respect to charge repulsion between γ-phosphate and D369N amounts to 0.6 kJ/mol (Table 1). This corresponds to 7% of the expense in free energy required to overcome the repulsion from a carboxylate group. This value is lower than expected since the carbonyl oxygen of Asn is known to have a partial charge of about −0.3 relative to a charge of −1 for the carboxylate group of Asp (Creighton, 1993). The position of the Asn side chain in the local environment of the protein may therefore be relatively unfavorable for contributing to the electrostatic repulsion of the γ-phosphate of ATP.

The reduction of the electrostatic repulsion between Asp^{369} and the γ-phosphate upon exchanging this residue with Asn or Ala reveals an intrinsic high-affinity binding of ATP that has not previously been observed in Na,K-ATPase. Further analysis is required to determine if this high intrinsic affinity for ATP can play a role as a driving force for uptake of K^+ ions into the cell [cf. Laüger (1991)]. The organization of the protein in the membrane and the function of ligand sites for cations and ouabain with preservation of the conformational change argue against the possibility that the mutations have created an artifactual site for ATP. In contrast, the effect of the substitutions of Asp for Asn or Ala on the conformational equilibrium with a shift toward the E_2-form would rather contribute to reducing the affinity for ATP binding. The calculations of binding energies therefore provide minimum estimates of the intrinsic binding affinity and the work required to overcome the electrostatic repulsion between Asp^{369} and the γ-phosphate in ATP. Our results suggest that it is an important functional aspect of the negative charge at the phosphorylation site to balance out this very tight association of ATP with the binding domain in order to prevent the formation of an energy well within the reaction cycle. As proposed by Jencks (1989), high-energy intermediates are likely to act as barriers to rapid turnover of transport systems.

In spite of the large expense in free energy required to position the γ-phosphate in proximity to the carboxylate group in residue 369, the wild type enzyme has a high affinity for ATP. This points at strong interactions between other parts of the ATP molecule and putative residues in the α-subunit that form part of a presumptive pocket for nucleotide binding. In addition to the segment around the phosphorylation site, homologies with other ATP binding proteins argue for contribution of other segments of the α-subunit to the ATP binding domain (Jorgensen & Andersen, 1988). The segment 706−721 containing the sequence G^{711}DGXNDSP^{719} is highly conserved among cation pumps of the E_1−E_2 type. The residues Asp^{712} and Asp^{716} are labeled covalently by ClR-ATP (Ovchinnikov et al., 1987), and they are each essential for Na,K-ATPase activity as mutations to Asp^{712} and Asp^{716} do not support growth of HeLa cells in 10 μM ouabain (Lane et al., 1993). A hydrophobic segment (546−561) is conserved among the cation pumps and a number of other ATP binding proteins (Jorgensen & Andersen, 1988), and it is labeled by TID (Modyanov et al., 1991). Covalent attachment of FITC is prevented by ATP (Karlish, 1980), and Lys^{501} is labeled along with Lys^{482}. The residue Lys^{482} is also the site of labeling by 8-azido ATP, but neither of these lysines is essential for phosphorylation or ATP hydrolysis (Tran et al., 1994). As yet there is no observation of mutations in Na,K-ATPase or Ca-ATPase (Clarke et al., 1990) which cause a decrease in the affinity for ATP binding. The disclosure of the intrinsic high binding affinity for ATP raises the opportunity that expression in yeast of double-mutations based on the D369A mutation may serve as a tool in future attempts to identify side chains involved in ATP binding.

Effect of Mutations to Asp^{369} on E_1−E_2 Conformational Equilibrium

In addition to its function as receptor for the γ-phosphate from ATP and a major determinant of the ATP affinity, the side chain of residue 369 turned out to be a key residue for regulation of the conformational transition accompanying cation translocation. The evidence for this is estimation of the $E_2(K)$−$E_1(K)$ conformational equilibrium from K^+-titrations of equilibrium ATP binding for the wild type and the two mutations, showing a 2−4-fold increase in the E_2-(K) concentration compared to $E_1(K)$ for D369N and D369A. The K_c value of 769 obtained for the $E_2(K)$−$E_1(K)$ equilibrium for the wild type enzyme fits very well a K_c value of 1000 previously reported from studies on renal Na,K-ATPase (Jorgensen & Karlish, 1980). Quantitative estimations of E_2−E_1 conformational equilibrium from K-titration of ouabain binding also revealed a large increase in concentration of the E_2- compared to the E_1-conformation, Table 4. These estimates of the conformational equilibria are consistent with

the observation that the affinity for ouabain is greatly increased in Mg^{2+} medium for the D369A and D369N mutations, Table 3, since high-affinity ouabain binding is a recognized property of E_2-conformations (Jorgensen & Andersen, 1988).

On a hydropathy scale (Kyte & Doolittle, 1982), Asp (−14.6 kJ/mol) and Asn (−14.6 kJ/mol) are equally hydrophilic, while Ala (10.5 kJ/mol) is considerably more hydrophobic. In a hydropathy plot of the peptide sequence of 20 residues around residue 369, it can be seen that the profile of the plot is unaffected by the substitution of Asp for Asn, while the substitution of Asp for Ala causes a pronounced change in the profile with a considerable displacement from the hydrophilic to the hydrophobic section of the plot. While the substitution of Asn^{369} for Ala had only a moderate effect on the affinity for ATP, it had a major effect on the conformational equilibrium (Tables 2 and 4) and on the affinity for ouabain (Table 3). Our data suggest that the position of the free charge of the carboxylate group of Asp^{369} may be such that it contributes to prevent the E_1-E_2 transition in the absence of phosphate and magnesium. Removal of the charge and hydrophobic substitution (D369A) favor transmission of the conformational transition originating in the phosphorylated segment through the transmembrane segment to the ouabain site at the extracellular surface. Mutation to a hydrophobic residue may in part substitute for the requirements for Mg^{2+} and phosphate, since substitution of Asp^{369} or Asn^{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 the absence of Mg^{2+} (Table 3). The role of hydrophobic interactions and water activity in the E_1-E_2 conformational transitions have been discussed extensively before (DeMeis, 1981; Jorgensen & Andersen, 1988). The expression system and assay techniques developed in this work allow studies of a series of point mutations to answer questions concerning the role of charge and relative hydrophobicity of the peptide segment around Asp^{369} for the E_1-E_2 transitions which are coupled to cation translocation and changes in affinity of Na,K-ATPase for digitalis glycosides.

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APPENDIX A. ADP DISPLACEMENT OF ATP BINDING

The simultaneous equilibrium binding of ATP and ADP can be described by the following reactions:

$$E_1{\cdot}ATP \overset{K_{ATP}}{\leftrightarrow} E_1 + ATP$$

$$E_1{\cdot}ADP \overset{K_{ADP}}{\leftrightarrow} E_1 + ADP$$

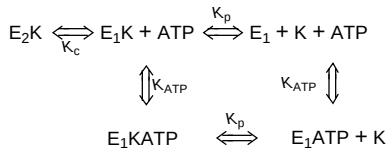
These equilibria lead to the following equation for K_{ADP}:

$$K_{ADP} = [ADP]_{1/2}\frac{K_{ATP}}{(K_{ATP} + [ATP])}$$

$[ADP]_{1/2}$ is the nucleotide concentration required to displace half of the initially bound ATP.

APPENDIX B. POTASSIUM−ATP ANTAGONISM

The simultaneous equilibrium binding of ATP and K^+ can be described by the following set of reactions. We consider binding of only one K^+ ion for reason of simplicity.



The following equation can be derived from the equilibrium situation:

$$K_c = \frac{K_p\left\{\frac{[ATP]}{K_{ATP}} + 1\right\}}{[K]_{1/2}}$$

$[K]_{1/2}$ is the potassium concentration required to displace half of the initially bound ATP.

APPENDIX C. POTASSIUM−OUABAIN ANTAGONISM

The exclusive binding of ouabain and potassium to the Na,K-pump can be described by the following equations:

$$E_1 \overset{K_c^*}{\leftrightarrow} E_2$$

$$E_1 + K \overset{K_p}{\leftrightarrow} E_1K \overset{K_c}{\leftrightarrow} E_2K$$

$$E_2oua \overset{K_D}{\leftrightarrow} E_2 + oua$$

The following equation may be derived for the equilibrium situation:

$$K_c^* = \frac{K_D\{[K]_{1/2}(1 + K_c) - K_p\}}{K_p([oua] + K_D)}$$

$[K]_{1/2}$ is the potassium concentration required to displace half of the initially bound ouabain.

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