Importance of Intramembrane Carboxylic Acids for Occlusion of K^+ Ions at Equilibrium in Renal Na,K-ATPase[†]

Jesper M. Nielsen,[‡] Per Amstrup Pedersen,[‡] Steven J. D. Karlish,[§] and Peter L. Jorgensen*,[‡]

Biomembrane Research Center, August Krogh Institute, Copenhagen University, 2100 Copenhagen OE, Denmark, and Biochemistry Department, Weizmann Institute of Science, Rehovot 76100, Israel

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ABSTRACT: Site-directed mutagenesis and assay of Rb^+ and Tl^+ occlusion in recombinant Na,K-ATPase from yeast were combined to establish structure−function relationships of amino acid side chains involved in high-affinity occlusion of K^+ in the E_2[2K] form. The wild-type yeast enzyme was capable of occluding 2 Rb^+ or Tl^+ ions/ouabain binding site or $\alpha1\beta1$ unit with high apparent affinity ($K_{d(Tl^+)} = 7 \pm 2$ μM), like the purified Na,K-ATPase from pig kidney. Mutations of Glu^{327}(Gln,Asp), Asp^{804}(Asn, Glu), Asp^{808}(Asn, Glu) and Glu^{779}(Asp) abolished high-affinity occlusion of Rb^+ or Tl^+ ions. The substitution of Glu^{779} for Gln reduced the occlusion capacity to 1 Tl^+ ion/$\alpha1\beta1$-unit with a 3-fold decrease of the apparent affinity for the ion ($K_{d(Tl+)} = 24 \pm 8$ μM). These effects on occlusion were closely correlated to effects of the mutations on $K_{0.5(K^+)}$ for K^+ displacement of ATP binding. Each of the four carboxylate residues Glu^{327}, Glu^{779}, and Asp^{804} or Asp^{808} in transmembrane segments 4, 5, and 6 is therefore essential for high-affinity occlusion of K^+ in the E_2[2K] form. These residues either may engage directly in cation coordination or they may be important for formation or stability of the occlusion cavity.

The Na,K pump catalyzes the exchange of 3 Na^+ for 2 K^+ ions per ATP hydrolyzed, while alternating between two occluded E_1P[3Na] and E_2[2K] conformations with binding and release of Na^+ and K^+ in a ping-pong sequence (*1*). The occluded E_2[2K] conformation is considered to be an intermediate in the translocation of K^+ across the membrane. K^+ ions are bound with high affinity to the E_2P form to promote dephosphorylation and subsequent occlusion in the E_2[2K] form. Binding of ATP with low apparent affinity drives the E_2[2K] → E_1(2K) conformational change in the direction of E_1 with deocclusion and release of K^+ at the cytoplasmic surface.

On the basis of evidence from kinetic studies (*2*), Rb^+ occlusion was first measured directly in Na,K-ATPase from pig kidney using Dowex columns for removal of exchangeable cations (*3, 4*). In the absence of other ligands, occlusion of Rb^+ in purified renal Na,K-ATPase can be measured at equilibrium with a capacity of 2 Rb^+ ions/ouabain binding site or $\alpha1\beta1$ unit and a dissociation constant (K_d) of 7−9 μM (*5, 6*). This is an equilibrium constant, which is directly related to the standard free energy change for cation binding to the protein (*7*). In contrast, $K_{0.5}$ values for K^+ dependence of the rate of Na,K-dependent ATP hydrolysis reflect all reaction steps in the cycle, including the conformational equilibria (*8*). For identification of amino acid residues involved in high-affinity occlusion of cations, it is therefore important to develop assays of cation occlusion in recombinant Na,K-ATPase.

The cavity for occlusion of cations is formed within the structure of the $\alpha1\beta1$ unit (*6*), and extensive proteolysis has shown that the cation occlusion sites lie in the intramembrane domain (*9*−*11*). Chemical labeling has suggested that carboxylic acids are part of the cation binding sites within the membrane (*12*−*15*). 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 in Ca-ATPase of sarcoplasmic reticulum (SR) (*16*−*18*). The homologous counterparts to residues containing carboxylic acid side chains in the α-subunit of Na,K-ATPase are Glu^{327}, Glu^{779}, Asp^{804}, and Asp^{808}. A number of mutations of these residues are lethal in the sense that they do not confer ouabain resistance to cells (*19, 20*). However, the mutations Glu^{327}Gln, Glu^{327}Leu, Glu^{779}Ala, and Asp^{808}Glu were expressed in HeLa (*19, 21*) or COS cells (*20, 22*) with relatively high activity and moderate alterations of kinetic parameters in assays of Na,K-ATPase activity and Na^+-dependent phosphorylation. It was concluded that Glu^{327} is not essential for active transport (*22*) and it was questioned whether Glu^{327}, Glu^{779}, and Asp^{808} are cation-coordinating residues (*19, 23*). In contrast, after expression in ouabain-resistant NIH 3T3 cells, K^+ competition of ouabain binding to mutations of Glu^{327} to Ala, Asp, Glu, and Leu displayed severely altered interactions between these proteins and K^+ (*24*). Major alterations were also observed after analysis of cation or membrane potential dependence of pump current of mutations of Glu^{327} and Glu^{779} in transfected cells (*25, 26*). Moderate yields of expression and the presence of endogenous activity have so far prevented measurements of cation occlusion.

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* Address correspondence to this author: Tel. (45) 3532 1670; Fax (45) 3532 1567; E-mail PLJorgense@AKI.KU.DK.

[‡] Copenhagen University.

[§] Weizmann Institute of Science.

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Here direct measurements of Rb^+ and Tl^+ occlusion in recombinant Na,K-ATPase from yeast (*27*−*29*) were performed after conservative substitutions to Glu^{327}, Glu^{779}, Asp^{804}, and Asp^{808} to examine if these residues are involved in occlusion of K^+ in the E_2[2K] form. Due to the reduced Na,K-ATPase activity in yeast membranes, conditions for measurement of cation occlusion were optimized to reduce the unspecific background (*30, 31*). To determine the stoichiometry of Rb^+ or Tl^+ occlusion in recombinant Na,K-ATPase, the data were expressed relative to the ouabain binding capacity (*27*).

Preservation of high-affinity ATP binding (*27, 29*) allowed for titration of the K^+−nucleotide antagonism to monitor the consequences of the mutations for the equilibrium between the E_2[2K] and the E_1ATP conformations in medium containing K^+ ions and ATP. The present work includes titrations of the effect of mutations of Glu^{779} on $K_{0.5(K^+)}$ for displacement of ATP binding, for comparison with available data for mutations of Asp^{804}, Asp^{808}, and Glu^{327} (*29, 32*).

MATERIALS AND METHODS

Site-Directed Mutagenesis. Site-directed mutagenesis was performed according to Ho et al. (*33*). The nucleotide sequences of the mutagenic primers were as follows: 5′AAC GTG CCT GAC GGT TTG CTG G 3′ and 5′CCA GCA AAC CGT CAG GCA CGT T 3′ (Glu^{327}Asp), 5′ CAA CGT GCC TCA AGG TTT GCT 3′ and 5′ AGC AAA CCT TGA GGC ACG TT 3′ (Glu^{327}Gln), 5′ AAC ATT CCA GAC ATC ACC CCC T 3′ and 5′ AGG GGG TGA TGT CTG GAA TGT T 3′ (Glu^{779}Asp), 5′ AGG GGG TGA TTT GTG GAA TGT TA 3′ and 5′ TAA CAT TCC ACA AAT CAC CCC CT 3′ (Glu^{779}Gln). The mismatched nucleotides causing the mutations are underlined. A PCR fragment containing the mutation was subsequently inserted into the expression plasmid pPAP1666 (*27*). Nucleotide sequences of all PCR fragments were confirmed by dideoxy sequencing. The sequences of the mutagenic primers for Asp^{804}Asn, Asp^{804}Glu, Asp^{808}Asn, and Asp^{808}Glu (*27, 29*) have been reported before.

Transformation of Yeast Cells. Yeast cells were transformed by electroporation according to Becker and Guarante (*34*).

Growth of Yeast and Expression of Na,K-ATPase. Growth of transformed yeast cells in an Applicon fermenter equipped with an ADI 1030 Bio Controller and galactose induction of recombinant Na,K pump protein synthesis were performed as before (*27*). From each fermentation 100−200 g of yeast and 1−2 g of crude membrane protein were produced.

Isolation of Yeast Membranes. Isolation of crude membranes, fractionation of membranes on sucrose step gradients, and assay of protein were performed as before (*27, 28*). Both crude and gradient membranes were used for the various assays.

TDS Treatment of Membranes. Membranes were incubated for 30 min at 20 °C with 0.3 mg of TDS[1]/mL, 5 mg of protein/mL, 10% (w/v) sucrose, 1 mM EDTA, 1 mM EGTA, 25 mM imidazolehydrochloride, pH 7.5, supplemented with 1 mM PMSF, and 1 mg/mL of chymostatin, pepstatin, and leupeptin to prevent proteolysis. These protease inhibitors were added in all further steps described below.

Occlusion of Rb^+. After addition of TDS, the membranes were divided into two portions, which were supplemented with 2 mM $MgCl_2$, with or without 0.5−1 mM ouabain and 0.1 mM $Tris_3VO_4$, followed by incubation as described above. EDTA−Tris (3 mM), pH 7.3, was added to the membranes and incubation was continued for 10 min at 20 °C. Membranes were placed on ice for at least 20 min, before aliquots containing 0.4−0.5 mg of protein were incubated with 25−400 μM ^{86}RbCl[$(0.4-7) \times 10^6$ cpm/sample] and 100 mM choline chloride for 7 min at 0 °C. Free cations were separated from occluded cations on Dowex-Tris 50W−X8 columns (50-100 mesh) as previously described (*30*). Protein was determined in samples applied to the columns, since it has been shown that protein is eluted essentially quantitatively (*30*). $^{86}Rb^+$ was measured by its Cerenkov radiation. SDS and ammonium vanadate were converted to their Tris salts by passing them over Dowex-Tris 50W-X8 columns (50−100 mesh).

Occlusion of Tl^+. After TDS treatment, the membranes were sedimented at 265000*g* in the Beckman 100A centrifuge for 30 min at 4 °C. Membranes were washed twice with ice-cold wash buffer [10% (w/v) sucrose, 0.5 mM EDTA, and 25 mM Tris-SO_4, pH 7.4] and centrifuged for 10 min as before, followed by resuspension in wash buffer. The membranes were then divided into two portions and incubated with 1 mM $MgSO_4$, with or without 0.5 mM ouabain for 20 min at 20 °C. The membranes were cooled on ice for at least 20 min, before aliquots containing 0.4−0.5 mg of protein were incubated with 2−30 μM $^{204}TlNO_3$ [$(0.5-7) \times 10^6$ cpm/sample] and varying concentrations of unlabeled $TlNO_3$ for 7 min at 0 °C. Occluded $^{204}Tl^+$ ions were isolated as described for $^{86}Rb^+$ occlusion. $^{204}Tl^+$ was measured by liquid scintillation counting.

Equilibrium Ouabain Binding. Aliquots of TDS-treated membranes containing 0.1−0.2 mg of protein were incubated at 37 °C for 1 h in 3 mM $MgSO_4$, 1 mM $NaTris_2VO_4$, 1 mM EGTA, and 10 mM MOPS−Tris, pH 7.2, in the presence of 2−10 nM [^{3}H]ouabain (18 Ci/mmol) and varying concentrations of unlabeled ouabain. After the reaction was allowed to stand on ice for 20 min, bound ouabain was isolated by centrifugation in the Beckman 100A centrifuge at 265000*g* for 30 min at 4 °C and the pellets were washed and counted as before (*27*).

Assay of Na,K-ATPase activity, ATP binding at equilibrium, and *K^+-ion displacement of ATP binding* were performed as before (*27*−*29*).

RESULTS

Expression in Yeast of Mutations of Glu^{327}, Glu^{779}, Asp^{804}, and Asp^{808}. After expression in yeast, the Na,K-ATPase activity was severely reduced in mutations of Asp^{804} and Asp^{808}, while 5−37% of the activity was preserved in mutations of Glu^{327} and 51−85% in mutations of Glu^{779} (Table 1). The capacities for binding of [^{3}H]ouabain varied

[1] Abbreviations: TDS, tris(dodecyl) sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DEAC, 4-(diazomethyl)-7-(diethylamino)-coumarin; TM, transmembrane segment; $C_{12}E_8$, octa(ethylene glycol) mono-*n*-dodecyl ether.

Table 1: Na,K-ATPase Activity, [^{3}H]Ouabain Binding, and $^{86}Rb^+$ Occlusion in Mutants of Na,K-ATPase[a]

		[^{3}H]ouabain binding		$^{86}Rb^+$ occlusion	
mutation	Na,K-ATPase activity (%)	capacity (pmol/ mg of protein)	K_d (nM)	pmol/ mg of protein	%
E327D	5	9.3	4	0.3 ± 0.2	7
E327Q	37	10	4	0.1 ± 0.1	2
E779D	51	6.6	14	0.0 ± 0.4	0
E779Q	85	12	23	2.0 ± 0.6	33
D804E	13	4.1	14	0.1 ± 0.2	6
D804N	17	21	10	0.0 ± 0.1	0
D808E	0	6.6	8	0.4 ± 0.1	10
D808N	0	12	9	0.6 ± 0.1	10
WT	100	8.9	8	4.6 ± 0.2	100

[a] The capacities and affinities for [^{3}H]ouabain binding were determined by extrapolation in Scatchard plots. Occlusion was determined at 100 μM $^{86}Rb^+$, and was expressed as the mean of 3−4 (mutants) or 21 (wild-type) experiments ± SEM Data for Na,K-ATPase activities are average values of two or three determinations. Assays were as described in Materials and Methods. Percentage occlusion was calculated after the amount of occlusion was normalized to the ouabain binding capacity.

randomly around the level found for wild type (Table 1). Western blots of yeast membrane protein (*27*) showed that mutations of Glu^{327}, Glu^{779}, Asp^{804}, and Asp^{808} were expressed at a concentration close to 1 α1 unit/[^{3}H]ouabain binding site (*29*; unpublished observations).

Capacity of Recombinant Enzyme for Rb$^+$ Occlusion. Prior to the occlusion assay, the yeast membranes were treated with an optimum concentration of TDS to open vesicles and thus expose both surfaces of the protein to ligands. To determine the stoichiometry of occlusion in recombinant Na,K-ATPase, the ouabain-sensitive Rb^+ occlusion was assayed in parallel to the capacity for ouabain binding. As seen from Figure 1, the capacity for Rb^+ occlusion was 2-fold higher than the capacity for ouabain binding. This corresponds to a stoichiometry of 2 Rb^+ ions/ α1β1 unit. The apparent affinity of the yeast Na,K-ATPase for Rb^+ ions (K_d of 154 ± 70 μM Rb^+) measured at high ionic strength was in the same range as for the enzyme of renal origin (*4*, *30*).

Measurement of Rb^+ occlusion in the wild-type Na,K-ATPase at saturating concentrations of Rb^+ ions was near the limit of assay sensitivity. This was due to the relatively low apparent affinity for Rb^+ in conditions of optimal signal-to-noise ratio and the relatively low concentration of Na,K pumps in yeast membranes. In the mutants (Table 1), relative levels of occlusion were therefore determined at 100 μM Rb^+ where the signal-to-noise ratio was optimal.

Occlusion of Rb$^+$ in Mutant Enzymes. The ability of enzymes with substitutions of Glu^{327}, Glu^{779}, Asp^{804}, and Asp^{808} to bind ouabain with high affinity (Table 1) allowed the use of ouabain to define the background in assays of cation occlusion and to correct for variations of expression levels of mutant and wild-type Na,K pump proteins. In Table 1, it is seen that 33% ± 10% of the Rb^+ occlusion level was preserved in Glu^{779}Gln, while the ability to occlude at 100 μM Rb^+ ions was almost eliminated in mutations of Asp^{804} and Asp^{808} to Asn or Glu, of Glu^{327} to Asp or Gln, and of Glu^{779} to Asp.

Since Rb^+ is the only ligand required for stabilization of the E_2[2Rb] occluded conformation, the severe effects of conservative substitutions of Glu^{327}, Asp^{804}, and Asp^{808} on Rb^+ occlusion suggested that these carboxylic acids are involved in high-affinity occlusion of Rb^+ ions in the E_2-[2Rb] complex. Although mutation of Glu^{779} to a residue with the shorter side chain Asp completely abolished Rb^+ occlusion, an appreciable amount of occlusion was conserved when the carboxylate group of Glu^{779} was replaced with the carboxamide of Gln. It was important to decide if the mutation Glu^{779}Gln only had reduced the apparent affinity of the enzyme for Rb^+ ions or if the capacity for the ions also was affected. For this purpose the sensitivity of the Rb^+ occlusion assay was too low and Tl^+ occlusion had to be developed as an alternative assay.

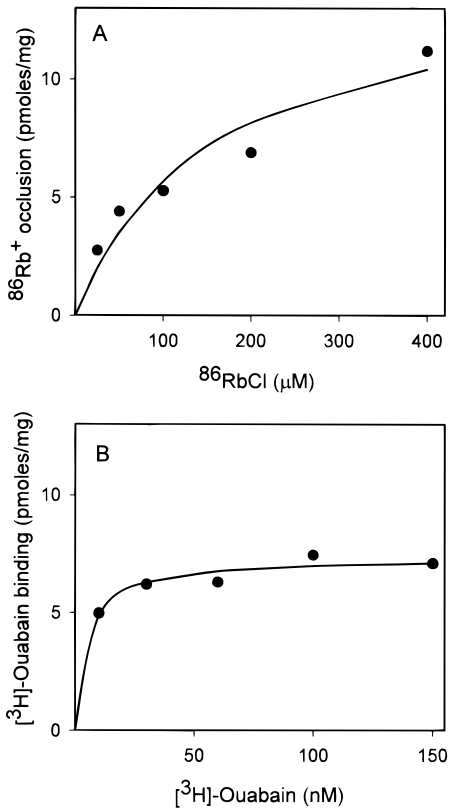

FIGURE 1: Concentration dependence of $^{86}Rb^+$ occlusion (A) and [^{3}H]ouabain binding (B) to wild-type recombinant Na,K-ATPase from yeast. Assays were as described in Materials and Methods. Data were fitted by nonlinear least-squares regression resulting in capacities of 14 ± 3 pmol/mg of protein and $K_{0.5}$ = 154 ± 70 μM for $^{86}Rb^+$ occlusion and 7.3 ± 0.3 pmol/mg of protein and K_d = 5.0 nM for [^{3}H]ouabain binding.

Affinity and Capacity of Recombinant Enzyme for Tl$^+$ Occlusion. In Figure 2 is shown the ouabain-sensitive occlusion of wild type as a function of Tl^+ concentrations at 2−200 μM. The binding capacity for ouabain was determined in parallel for each TDS-treated membrane preparation of this series and the results were expressed as the ratio of Tl^+ occlusion to ouabain binding capacity. The K_d was fitted to 7 ± 2 μM, which is close to the apparent affinity at low ionic strength for Tl^+ occlusion in purified kidney Na,K-ATPase (*31*). The Tl^+ occlusion capacity was 17 ± 1 pmol/mg of protein. The extrapolated ouabain binding capacity of the yeast membranes was 9.2 ± 0.1 pmol/ mg of protein, and data from four separate experiments gave an average value of 1.9 ± 0.2 Tl^+ occlusion sites/ouabain binding site. These data suggested that it would be feasible to determine Tl^+ occlusion in mutant enzymes at saturating

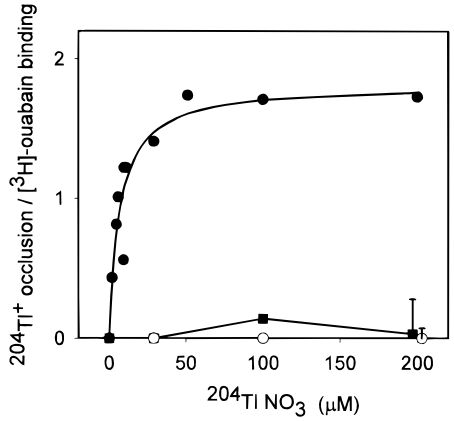

FIGURE 2: Concentration dependence of $^{204}Tl^+$ occlusion to wild-type (●) Na,K-ATPase, α1(Glu^{327}Gln)β1 (■), and α1(Glu^{327}Asp)β1 (○). Occlusion was expressed as the ratio of $^{204}Tl^+$ occlusion (picomoles per milligram of protein) to [^{3}H]ouabain binding capacities of 9.2, 7.5, and 10.0 pmol/mg of protein for wild type, Glu^{327}Gln, and Glu^{327}Asp, respectively. Assays were as described in Materials and Methods. Data for wild type were fitted by nonlinear least-squares regression with capacities of 1.8 ± 0.1 $^{204}Tl^+$ occlusion sites/[^{3}H]ouabain binding site and a K_d value of 7 ± 2 μM for $^{204}Tl^+$ occlusion. At 200 μM Tl^+, mean values ± SEM were calculated for three consecutive preparations of membranes.

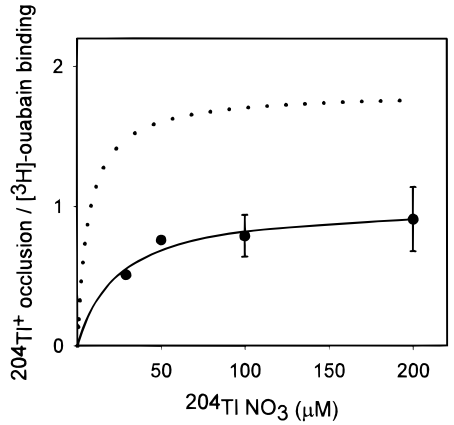

FIGURE 3: $^{204}Tl^+$ occlusion in α1(Glu^{779}Gln)β1 (●) compared to wild-type (dotted line) Na,K-ATPase. Occlusion was expressed as the ratio of $^{204}Tl^+$ occlusion (picomoles per milligram of protein) to an [^{3}H]ouabain binding capacity of 10.7 pmol/mg of protein for Glu^{779}Gln. Assays were as described in Materials and Methods. Data for Glu^{779}Gln were fitted by nonlinear least-squares regression resulting in a capacity of 1.0 ± 0.1 $^{204}Tl^+$ sites/[^{3}H]ouabain binding site and K_d = 24 ± 8 mM. Data points are average values of duplicate determinations. At 100 and 200 μM Tl^+, mean values ± SEM were calculated for three or four consecutive preparations of membranes. Data for wild-type Na,K-ATPase correspond to the calculated line in Figure 2.

ion concentrations up to 30-fold higher than the K_d value for Tl^+ occlusion in wild-type enzyme.

Figure 2 also shows that occlusion of Tl^+ was completely abolished in the conservative Glu^{327}Gln or Glu^{327}Asp mutations. In the Glu^{779}Gln mutation, Figure 3, occlusion was reduced to a level of 1.0 ± 0.1 Tl^+ ion/ouabain binding site with $K_{d(Tl^+)}$ = 24 ± 8 μM. This affinity was reduced to a level about 3-fold lower than in wild-type. From Figure 4 it is seen that occlusion of Tl^+ was completely abolished after the conservative substitutions of Asp^{804} to Glu or Asn. In the Glu^{779}Asp mutation and in both mutations of Asp^{808}, occlusion levels were very low in the range 30−200 μM Tl^+, but in these cases the determination of the capacity at

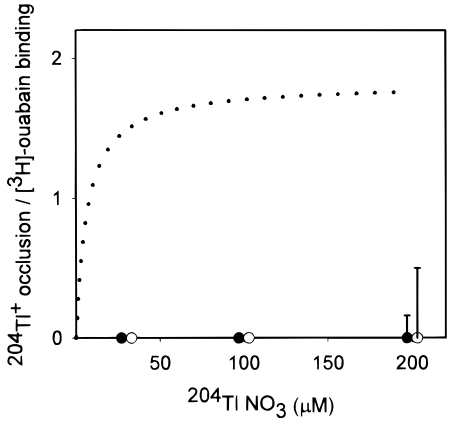

FIGURE 4: $^{204}Tl^+$ occlusion in α1(Asp^{804}Glu)β1 (●) and α1(Asp^{804}Asn)β1 (○) mutations of Na,K-ATPase. Occlusion was expressed as the ratio of $^{204}Tl^+$ occlusion (picomoles per milligram of protein) to [^{3}H]ouabain binding capacities of 4.1 and 21 pmol/mg of protein for Asp^{804}Glu and Asp^{804}Asn, respectively. At 200 μM Tl^+, mean values ± SEM were calculated for three consecutive preparations of membranes. Assays were as described in Materials and Methods. Data for wild-type Na,K-ATPase (dotted line) correspond to the calculated line in Figure 2.

Table 2: $^{204}Tl^+$ Occlusion in Mutants of Na,K-ATPase[a]

mutation	[^{3}H]ouabain binding capacity (pmol/mg of protein)	$^{204}Tl^+$ occlusion/ [^{3}H]ouabain binding
E779D	8.0	0.055 ± 0.65
D808E	5.5	0.16 ± 0.39
D808N	9.8	−0.37 ± 0.18
WT	9.1	2.05 ± 0.39

[a] The capacities for [^{3}H]ouabain binding were determined by extrapolation in Scatchard plots. Assays were as described in Materials and Methods. Occlusion was determined at 100 μM $^{204}TlNO_3$ and was expressed as the mean of three or four determinations ± SEM. Occlusion was expressed as the ratio of $^{204}Tl^+$ occlusion (picomoles per milligram of protein) to the [^{3}H]ouabain binding capacity (picomoles per milligram of protein).

200 μM was uncertain due to a high background value. A series of determinations was therefore done at 100 μM and the data in Table 2 show that the occlusion levels at this concentration were close to zero for the mutations Glu^{779}Asp, Asp^{808}Asn, and Asp^{808}Glu.

K^+ Displacement of ATP Binding. The K^+−nucleotide 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. The antagonism can therefore be utilized to monitor the functional consequences for the E_1−E_2 conformational equilibrium of amino acid substitutions that interfere with high-affinity occlusion of K^+ (Tl^+) ions. Titrations of K^+ displacement of ATP binding of mutations of Glu^{779} are shown in Figure 5. It is seen that, in the wild type, K^+ ions displaced ATP from the E_1-ATP form with high apparent affinity ($K_{0.5(K^+)}$ = 0.11 mM). In the Glu^{779}Gln mutation the apparent affinity of K^+ for displacement of ATP was reduced 3-fold to $K_{0.5(K^+)}$ = 0.34 mM, while the Glu^{779}Asp mutation abolished the K^+ induced displacement of ATP from the protein, Figure 5. These results are listed in Table 3 for comparison with $K_{0.5(K^+)}$ values obtained in previous titrations of ATP displacement from the mutations of Glu^{327} (*32*) and Asp^{804} and Asp^{808} (*29*). The ATP binding capacities and affinities at equilibrium after substitutions of Glu^{327} (Gln or Asp, 12−16 pmol/mg of

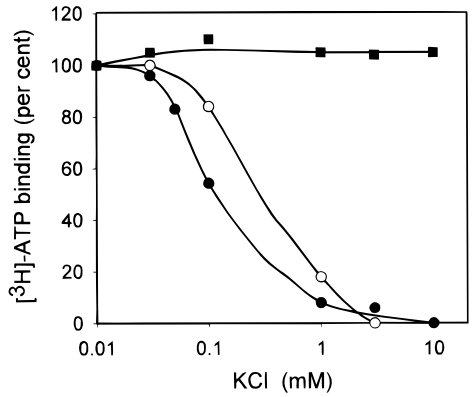

FIGURE 5: Effect of KCl on [^{3}H]ATP binding to recombinant wild-type (●) $\alpha1\beta1$ Na,K-ATPase and to mutations $\alpha1(Glu^{779}Gln)\beta1$ (○) and $\alpha1(Glu^{779}Asp)\beta1$ (■). Membranes were incubated with 13 nM [^{3}H]ATP in the presence of 0−10 mM KCl. Choline chloride was added to maintain constant ionic strength. The procedure was as previously described (*27*−*29*).

Table 3: Effect of Mutations on K^+ Displacement of ATP Binding[a]

mutation	K^+-ATP $K_{0.5}(K^+)$ (mM)
E327D	>10
E327Q	5.2
E779D	>10
E779Q	0.34
D804E	>10
D804N	>10
D808E	2.3
D808N	>10
WT	0.11

[a] The effect of K^+ ions on ATP equilibrium binding was determined by incubation 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 previously described (*27*−*29*). For substitutions to Glu^{779} the data are from Figure 5. The remaining data are compiled from refs *29* and *32*.

protein; K_d = 50−73 nM) and Glu^{779} (Gln or Asp: 6−9 pmol/mg of protein; K_d = 69−89 nM) were in the range for wild type (7 pmol/mg of protein; K_d = 52 nM). In mutations where occlusion of Tl^+ ions was abolished (Glu^{327}Asp, Asp^{804}Glu, Asp^{804}Asn, Asp^{808}Asn, and Asp^{808}Glu), the $K_{0.5(K^+)}$ values for ATP displacement were 2.3−5.2 mM or higher than 10 mM as compared with 0.11 mM for wild type. In the case of Glu^{779}Gln, the $K_{0.5(K^+)}$ value for ATP displacement was increased in proportion to the change in affinity for Tl^+ occlusion. The data reflect a close correlation between the consequences of the amino acid substitutions for high-affinity Tl^+ ion occlusion and their effects on the K^+−nucleotide antagonism.

DISCUSSION

The recombinant Na,K pump of yeast membranes was capable of occluding 2 Rb^+ or Tl^+ ions/$\alpha1\beta1$-unit with high apparent affinity, like the purified Na,K-ATPase from pig kidney. Ouabain binding in the presence of Mg^{2+} provided a precisely defined background, since all examined mutants had retained the ability to bind ouabain with high affinity. The data establish the assay of Tl^+ ion occlusion as a sensitive tool for detecting altered capacities and apparent affinities for occlusion in the E_2[2K] complex of recombinant Na,K-ATPase.

Substitution of carboxylate side chains in transmembrane domains could conceivably cause structural distortions of the protein, but unspecific perturbations appear to be limited. The hydrodynamic properties as judged by high-resolution chromatography of the mutations of Asp^{804}, Asp^{808} (*27*, *29*), and Glu^{327} (unpublished observations) were not altered. Preservation of high-affinity ouabain binding shows that binding sites for Mg^{2+} and vanadate (phosphate) are functional (*27*). Furthermore, the mutants examined in this work had preserved the ability to bind ATP with high affinity. The mutant proteins have thus retained the structure of ligand binding domains at the cytoplasmic and extracellular surfaces, as well as the ability to undergo transitions between E_1 and E_2 conformations when elicited by ligands other than Na^+ or K^+.

Consequences of Substitutions to Glu^{327}. Conservative substitutions of Glu^{327} for Asp or Gln abolished high-affinity Rb^+ or Tl^+ occlusion as well as K^+-induced displacement of ATP. The affinity of K^+ for displacement of ATP from the Glu^{327}Gln mutation ($K_{0.5(K^+)}$ = 5.2 mM) was reduced considerably. The V_{max} of Na,K-ATPase was reduced to $^1/_3$ of wild-type levels. These observations are unexpected in view of previous conclusions that both conservative (Glu^{327}Gln) and nonconservative substitutions (Glu^{327}Leu) are functional with minor alterations in cation affinity (*19*, *22*, *23*). These authors showed that the V_{max} of Na,K-ATPase was 81−100% for Glu^{327}Gln and 26−73% for Glu^{327}Leu, while Glu^{327}Asp was a lethal mutation. Recently Vilsen (*35*) observed that the V_{max} of Na,K-ATPase and the phosphorylation level of Glu^{327}Gln were reduced to about 33% of wild-type and ascribed the discrepancy to overexpression of the mutant protein. A similar 4−8-fold increase in expression of α-subunit protein was observed for mutations of Ser^{775} (*36*). The appearance of values for V_{max} of Na,K-ATPase near 100% for mutations of Glu^{327} may therefore be due to compensation for the decrease in molecular activity by selection of cells with a high copy number of the mutant gene during growth in medium containing ouabain.

The previous data for the effect of Glu^{327}Gln mutation on K^+ dependence of Na,K-ATPase activity varies from $K_{0.5}$ of 3.2 mM (*19*) to 4.8 mM (*22*), while $K_{0.5}$ for external K^+ dependence of pump current was 8.8 mM (*26*). In the latter two studies the curves of K^+ dependence of Na,K-ATPase activity or pump current show a strong sigmoidicity with very low activity in the concentration range of 0−1 mM K^+. The severely reduced K^+ stimulation of ATPase activity or pump current in the range of 0−1 mM K^+ in the external medium is compatible with loss of high-affinity Tl^+ occlusion and K^+ displacement of ATP after conservative substitutions to Glu^{327}. In line with this, K^+ competition of [^{3}H]ouabain binding to all four mutant forms of Glu^{327} (Gln, Asp, Ala, and Leu) displayed severely altered interactions between these proteins and K^+ (*24*). It is obvious that effects of mutations on $K_{0.5}$ values for K^+ activation of the Na,K-ATPase activity reflect not only the effects on the rate of interaction with K^+ ions but the rates of all reaction steps in the catalytic cycle. The large discrepancies between effects of mutations on kinetic constants for the overall Na,K-ATPase reaction and equilibrium constants for Tl^+ occlusion demonstrate the importance of measuring ligand binding at equilibrium.

The consequences of mutations for the equilibrium between E_1ATP and E_2[K] conformations have not been studied directly before. For the Glu^{327}Gln mutant, the observation of a 15-fold decrease of $K_{0.5}$ for ATP dependence of Na,K-ATPase activity (*22*), while the dissociation constant for ATP binding at equilibrium is unaltered (*32*), may be due to loss of the ability to occlude K^+ ions with high affinity. The large apparent increase in affinity for ATP in the catalytic assay may reflect a shift of the E_1-E_2 conformational equilibrium toward the E_1 form. The loss of high-affinity occlusion of K^+ may result in an increased E_2-E_1 transitional rate, with compensation for the effect of the K^+ occlusion step on the rate of the overall catalytic cycle.

Our study of conservative substitutions to Glu^{327} shows that this residue is essential for high-affinity occlusion of Tl^+ (K^+). While high-affinity occlusion of K^+ was abolished in the Glu^{327}Gln mutation, low-affinity binding and translocation of K^+ are preserved since loading of K^+ sites in the range of 5−20 mM extracellular K^+ is sufficient to activate the Na,K pump current of the Glu^{327}Gln mutant in HeLa cells (*26*).

Consequences of Substitutions to Glu^{779}. Substitutions to Glu^{779} caused partial disruption of occlusion. It is striking that the Glu^{779}Gln mutation reduced the capacity to 1 site for Tl^+/α-subunit with 3-fold lower affinity than in wild type. In the Glu^{779}Asp mutation, the affinity for Rb^+ and Tl^+ ions was further reduced in agreement with the abolishment of K^+ induced displacement of bound ATP. These changes in affinity for K^+ fit those previously observed (*20, 37*). Na,K-ATPase activity was also retained after substitutions to Glu^{779} (*20, 21, 37*), but with two major changes in catalytic properties. First, for the Glu^{779}Ala mutation the activity in the presence of Na^+ alone was increased to 40−50% of total Na,K-ATPase activity as compared to 2−3% in wild type (*20, 37*) as a possible indication of a change in cation specificity. Second, there was a large decrease of the K_m for ATP of the overall Na,K-ATPase reaction from 0.28 to 0.4 mM in the wild type to 0.08−0.12 mM in Glu^{779}Ala, 0.12 mM in Glu^{779}Asp, and 0.05 mM in Glu^{779}Lys (*20, 37*). As discussed above for Glu^{327}, these changes in apparent affinities for ATP may reflect a shift of the E_1-E_2 equilibrium, which is secondary to interference with occlusion of K^+.

Electrophysiological analysis has shown 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 (*25, 38*). 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. This proposal does not fit easily with our 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. Our data rather suggest that Glu^{779} contributes coordinating groups to K^+ ions in the occlusion cavity per se or is important for stability of the occlusion cavity.

The carboxylic residue Glu^{779} can be modified by DEAC, which inactivates Na,K-ATPase with loss of K^+ and Na^+ binding. Na^+ or K^+ ions can prevent the inactivation. The inactive enzyme can bind ATP in the E_1 form with high affinity and undergo transition to the E_2 form when it is induced by ligands other than cations (*14, 15*). Quantitatively, the present data differ from the results of labeling with DEAC since substitutions to Glu^{779} only caused partial disruption of Tl^+ occlusion. Qualitatively, the results are similar in the sense that mutation of Glu^{779} alters Tl^+ occlusion in parallel with Na^+ activation of phosphorylation (unpublished observations). On balance, the data discussed above show that Glu^{779} is important for Tl^+ occlusion either because the side chain provides coordinating groups for the cation or because it is of central importance for maintaining the stability of the occlusion cavity.

Consequences of Substitutions to Asp^{804} and Asp^{808}. The deleterious effects of substitutions to Asp^{804} and Asp^{808} on Rb^+ and Tl^+ ion occlusion are in agreement with previous results. Analysis of K^+ displacement of ATP binding, Na^+-dependent phosphorylation (*29*), and K^+ competition with ouabain binding (*39*) 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 (*40, 41*). The homologous carboxylates at position 820 and 824 in H,K-ATPase appear to be involved in binding of K^+ ions (*42*). However, the Ca-ATPase of plasma membranes (*43*) and sarcoplasmic reticulum (*44*) or H-ATPase from yeast (*45*) or plants (*46*) have Asn or Ala at the position of Asp^{804} in the α-subunit of Na,K-ATPase. Consensus is therefore that both Asp^{804} and Asp^{808} contribute coordinating groups to K^+ in the E_2[2K] conformation.

Contribution of Carboxylates in Transmembrane Segments TM4, TM5, and TM6 to the Occlusion Cavity. In principle, cation occlusion could be interfered with by mutation of carboxylates that directly ligate the cations or participate in cation access paths, in gating structures that prevent cation deocclusion, or in formation of bonds of importance for the stability of the occlusion cavity. These possibilities cannot be easily distinguished on the basis of occlusion measurements, although in general one would expect that mutation of direct coordinating residues would produce the strongest effect on occlusion.

The carboxylate groups are positioned in the vicinity of proline residues in the presumptive transmembrane segments, TM4 ($NVPE^{327}G$), TM5 ($TS^{775}NIPE^{779}ITP$), and TM6 ($ID^{804}LGTD^{808}MVP$). This may introduce kinks in α-helices and the presence of multiple charged residues may render the structures relatively unstable. The hydrophobic hairpin consisting of transmembrane segments TM5/TM6 ($Gln^{737}-Lys^{827}$, 8 kDa of the α-subunit) remains membrane associated after extensive proteolytic cleavage of the membrane bound Na,K-ATPase (*10*), but only when cation sites are occupied by K^+ during proteolysis. Upon removal of the occluded cations, the hairpin spontaneously leaves the membrane and it can be collected in the supernatant after sedimentation of the membrane residual (*47*). The release of the TM5/TM6 fragment follows thermal inactivation, which is protected against by Rb^+ occlusion (*48*). Previous findings are that Na,K-ATPase is most thermostable in KCl medium (*49*) and that K^+ ion occlusion prevents inactivation of soluble Na,K-ATPase in nonionic detergent ($C_{12}E_8$) (*6*). These observa-

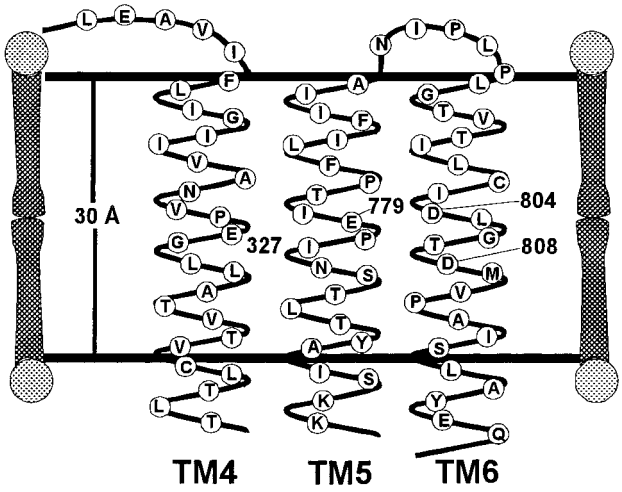

FIGURE 6: Model of the position of Glu^{327}, Glu^{779}, Asp^{804}, and Asp^{808} in putative transmembrane helices 4, 5, and 6 in α-subunit of Na,K-ATPase based on the topological model of Jorgensen (*52*). With the indicated bilayer width (30 Å) and a pitch of 5.4 Å and 3.6 residues/turn, each intramembrane segment comprises 20 amino acid residues.

tions on K^+ ion occlusion-dependent stabilization of the TM5/TM6 hairpin are consistent with the present findings that specific disruption of Tl^+ occlusion occurs upon mutation of each of four carboxylate residues in three presumptive transmembrane segments (TM4, TM5, and TM6).

The data fit into a model in which amphiphilic helices of TM4 and TM5-TM6 (Figure 6) are organized with the carboxylate side chains oriented toward a central cavity or ion channel, as proposed for the Ca-ATPase of sarcoplasmic reticulum (*18*). However, further analysis is required to identify additional side chains of importance for occlusion of K^+. It can be assumed that six oxygen groups may be required for coordination of one dehydrated K^+ ion with ion diameter 2.6 Å (*50, 51*). Ser^{775} may engage in interactions with K^+ (*36*). A number of other side chains containing oxygen, nitrogen, or sulfur (Cys, Asn, Gln, Thr, Ser, Met, Tyr), or main chain carbonyls may also contribute to coordination of K^+ in the occluded E_2[2K] complex. The assays of Tl^+ occlusion and titration of K^+ displacement of ATP binding in recombinant Na,K-ATPase from yeast provide sensitive tools for identification of additional residues that are engaged in high-affinity occlusion of K^+ ions.

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