Contribution to Tl^+, K^+, and Na^+ Binding of Asn^{776}, Ser^{775}, Thr^{774}, Thr^{772}, and Tyr^{771} in Cytoplasmic Part of Fifth Transmembrane Segment in α-Subunit of Renal Na,K-ATPase†

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ABSTRACT: The sequence Y^{771}TLTSNIPEIT^{781}P in the fifth transmembrane segment of the α-subunit of Na,K-ATPase is unique among cation pump proteins. Here, in search of the molecular basis for Na,K specificity, alanine and conservative substitutions were directed to six oxygen-carrying residues in this segment. The contribution of the residues to cation binding was estimated from direct binding of Tl^+ [Nielsen, et al. (1998) *Biochemistry 37*, 1961−1968], K^+ displacement of ATP binding at equilibrium, and Na^+-dependent phosphorylation from ATP in the presence of oligomycin. As an intrinsic control, substitution of Thr^{781} had no effect on $Tl^+(K^+)$ or Na^+ binding. There are several novel observations from this work. First, the carboxamide group of Asn^{776} is equally important for binding $Tl^+(K^+)$ or Na^+, whereas a shift of the position of the carboxamide of Asn^{776} (Asn^{776}Gln) causes a large depression of Na^+ binding without affecting the binding of $Tl^+(K^+)$. Second, Thr^{774} is important for Na^+ selectivity because removal of the hydroxyl group reduces the binding of Na^+ with no effect on binding of $Tl^+(K^+)$. Removal of the methyl groups of Thr^{774} or Thr^{772} reduces binding of both $Tl^+(K^+)$ and Na^+, whereas the hydroxyl group of Thr^{772} does not contribute to cation binding. Furthermore, the hydroxyl groups of Ser^{775} and Tyr^{771} are important for binding both $Tl^+(K^+)$ and Na^+. The data suggest that rotating or tilting of the cytoplasmic part of the fifth transmembrane segment may adapt distances between coordinating groups and contribute to the distinctive Na^+/K^+ selectivity of the pump.

The (α1β1) Na,K-pump is constitutively expressed in most mammalian cells, and the hormonal regulation of active Na^+ transport driven by this pump in kidney tubules and other epithelia is a key process controlling the amount of Na^+ and the volume of the extracellular fluid. The β1-subunit consists of 302 residues with a single transmembrane segment, three N-linked glycosylation sites, and three disulfide bonds. The α1-subunit has 1016 residues, and topological models suggest the presence of 10 transmembrane segments with sites for ATP binding and phosphorylation in the large central cytoplasmic protrusion between the M4 and M5. The cavity for occlusion of cations is formed within the intramembrane domain of the α1β1-unit (*1*−*3*). In the model linking long-range E_1−E_2 conformational transitions to primary active cation transport, the Na,K-pump couples ATP hydrolysis to the exchange of 3 Na^+ for 2 K^+ in a ping-pong sequence while alternating between two occluded E_1P[3Na] and E_2-[2K] conformations (*1*). 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. The apparent Na^+ affinity at the cytoplasmic surface of the E_1 form is about 1000-fold higher than the affinity for extracellular Na^+ binding, and the Na^+ site of the E_1P form is highly selective for Na^+ ions. The affinity for K^+ at the extracellular surface of the E_2 form is about 50-fold higher than at the cytoplasmic face, and the site for K^+ binding shows a broader specificity because it accommodates K^+, Rb^+, Tl^+, and NH_4^+ (*4*). The contribution of protein engineering to understanding the Na,K-pump mechanism can be to identify residues involved in coordinating cations in the E_1 and E_2 forms, thus defining the orientation of cation sites and the Na/K specificity of the system. This requires direct assays of cation binding for estimation of the change in binding energy after substitution of side chains contributing to binding of cations. The dissociation constants for binding of K^+-ion analogues Rb^+ or Tl^+ are equilibrium constants that are directly related to the Gibbs free energy change for cation binding to the protein (*5*).

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 the sarcoplasmic reticulum (*6*−*8*). Additional residues in M5 and M6 were shown to be important for the affinity of Ca^{2+} binding (*7*). Homologous counterparts to these residues in the α-subunit of Na,K-ATPase are Glu^{327}, Glu^{779}, Asp^{804}, and Asp^{808}, and direct measurements of Rb^+ and Tl^+ occlusion in recombinant Na,K-ATPase at equilibrium showed that each of the four carboxylate residues are essential for high-affinity occlusion of K^+ in the E_2 [2K] form (*9*, *10*). Reduced affinities for Na^+ dependence of

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Table 1: Sequences of Fifth Transmembrane Segments of Na,K-ATPase, H,K-ATPase (Gastric and Nongastric), Ca-ATPase from Sarcoplasmic Reticulum (SR), and Ca-ATPase from Plasma Membranes (PM)

Enzyme	Sequence	Reference
Na,K-ATPase	IAY TLTS**NIP**EIT **P**FLIFIIANI PLPL	*20*
H,K-ATPase,g.	IAY TLTK**NIP**ELT **P**YLIYITVSV PLPL	*21*
H,K-ATPase,non g	IAY TLTK**NIP**ELT **P**FLIYIVAGL PLPI	*22*
Ca-ATPase, SR	IRY LISS**N**VGEVV CIFLTAALGL PEAL	*7*
Ca-ATPase, PM	LQF QLTV**N**VVAVI VAFTGACITQ DSPL	*23*

phosphorylation in the presence of oligomycin indicated that these residues also contribute to coordination of Na^+ in the E_1P[3Na] form (*11*, *12*). The carboxylate groups may therefore be involved in alternate interaction with Na^+ or K^+ in the ping-pong sequence of Na,K exchange, but these findings do not explain the Na/K selectivity of the system.

In several instances these results are at variance with previous data that are based mainly on determination of $K_{0.5}$ values for Na^+ or K^+ stimulation of Na, K-ATPase activity. After expression of ouabain-resistant mutations in HeLa (*13*, *14*) or COS cells (*15*, *16*) and assays of Na,K-ATPase activity and Na^+-dependent phosphorylation, it was concluded that Glu^{327} is not essential for active transport (*16*), and whether Glu^{327}, Glu^{779}, and Asp^{808} are cation-coordinating residues was questioned (*13*, *14*, *17*). Only Asp^{804} appeared to be essential for enzyme function (*18*), but studies of K^+-ouabain antagonism showed that substitutions of Asp^{808} and Asp^{804} disrupted K^+ interactions with Na,K-ATPase (*19*).

Residues of importance for Na/K specificity should be located in amino acid sequence segments that are unique for the α-subunit of Na,K-ATPase among the known sequences of cation pumps. In the fifth transmembrane segment, the sequence (Y^{771}TLTSNIPEIT^{781}P) is unique to Na,K-ATPase, cf. Table 1. Mutations of Ser^{775} in M5 showed that this residue is part of the K^+ binding site (*18*, *24*) and the mutations Asn^{776}Ala and Tyr^{771}Ala appeared to be inactive, because they did not support growth of HeLa cells in ouabain medium (*25*). Substitution of Pro^{778} for Ala caused a 6-fold increase in apparent affinity for K^+ without altering Na^+ affinity (*26*), and the Tyr^{771}Leu mutation interfered with Na^+ interactions (*27*). Mutations of Glu^{779} in M5 also showed that this residue is important for Na/K selectivity (*15*, *28*). It is therefore probable that direct cation-binding assays of mutations of oxygen-containing residues in M5 may reveal important information about contributions of this segment to cation binding and Na/K selectivity.

The purpose of this study therefore has been to determine the consequences for the binding of Tl^+(K^+) and Na^+ of substitutions of oxygen-containing residues Thr^{781}, Asn^{776}, Ser^{775}, Thr^{774}, Thr^{772}, and Tyr^{771} in the unique sequence (Y^{771}-TLTSNIPEIT^{781}P) of the cytoplasmic part of M5. The high-yield expression in yeast without endogenous activity (*9*) allowed assay of the equilibrium binding of Tl^+ as an adequate estimate of the influence of mutations on the affinity and capacity for occlusion of K^+ in the E_2[2K] form (*10*). K^+ displacement of ATP binding at equilibrium was monitored to determine the consequences of mutations for the equilibrium between the E_2[2K] form and the E_1ATP conformations in media containing K^+ ions and ATP (*29*). The apparent affinity for binding Na^+ in the E_1P[3Na] complex was assayed as a dead-end reaction in the presence of oligomycin which prevents E_1P-E_2P transition (*11*). Using the principles of Fersht et al. (*30*) we estimated the contribution of the carboxamide group of Asn or the hydroxyl groups of Ser, Thr, or Tyr residues of M5 to the free energy of binding of Tl^+ ions or Na^+ ions in the Na,K-pump protein.

MATERIALS AND METHODS

Site-Directed Mutagenesis. The α1-pig cDNA sequence was engineered so that the nucleotide sequence encoding TM5 is flanked by a *Mfe*I site and a *Bsa*BI site generating plasmid pPAP1945. Introduction of the *Mfe*I and *Bsa*I sites does not alter the primary structure of the α1-subunit. Mutagenesis was performed by insertion of the oligonucleotides shown in Table 2 into a *Mfe*I and *Bsa*BI digest of this plasmid. The nucleotide sequences of the mutations were confirmed by dideoxy sequencing. A restriction fragment carrying the desired mutation was subsequently inserted into the expression vector pPAP1666 (*9*).

Transformation of Yeast Cells. Yeast cells were transformed according to Ito et al. (*31*).

Growth of Yeast and Expression of Na,K-ATPase. Growth of transformed yeast cells in an Applikon fermentor equipped with an ADI 1030 Bio Controller and galactose induction of recombinant Na,K-pump protein synthesis were performed as before (*9*). 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, assay of Na,K-ATPase activity, and protein analysis were performed as described previously (*9*, *10*). Both crude and gradient membranes were used for the various assays.

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

Equilibrium Ouabain Binding. Aliquots of TDS-treated membranes containing 0.1−0.2 mg protein were incubated at 37 °C for 1 h in 3 mM $MgSO_4$, 1 mM $NaTris_2VO_4$, 1 mM EGTA, 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 standing on ice for 20 min, bound ouabain was isolated by centrifugation in the Beckman 100A centrifuge at 265 000*g* for 30 min at 4 °C, and the pellets were washed and counted as before (*9*).

Occlusion of Tl^+. After TDS treatment, the membranes were sedimented at 265 000*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, 25 mM Tris-SO_4 pH 7.4) and centrifuged for 10 min as before, followed by resuspension in wash-buffer. The

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylene-bis(oxyethylenenitrilo)]-tetraacetic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TDS, tris dodecyl sulfate; M4, M5, M6, transmembrane segments nos. 4, 5, and 6; Tris, tris(hydroxymethyl)-aminomethane.

Table 2: DNA Sequence of the Coding Strand of Nucleotides 2319−2372 in Wild-Type α1 Pig Kidney cDNA and Synthetic Oligonucleotides Used to Generate Mutations in Transmembrane Segment 5[a]

allele	DNA sequence of the coding strand of nucleotides 2319−2372 in pig kidney α1 cDNA alleles (5′−3′)
WT	AATTGCCTACACCCTCACCAGTAACATTCCAGAGATCACCCCCTTCCTGATAT
Y771F	AATTGCCT**T**CACCCTCACCAGTAACATTCCAGAGATCACCCCCTTCCTGATAT
T772S	AATTGCCTAC**T**CCCTCACCAGTAACATTCCAGAGATCACCCCCTTCCTGATAT
T772A	AATTGCCTAC**G**CCCTCACCAGTAACATTCCAGAGATCACCCCCTTCCTGATAT
T774S	AATTGCCTACACCCTC**T**CCAGTAACATTCCAGAGATCACCCCCTTCCTGATAT
T774A	AATTGCCTACACCCTC**G**CCAGTAACATTCCAGAGATCACCCCCTTCCTGATAT
S775T	AATTGCCTACACCCTCACCA**C**TAACATTCCAGAGATCACCCCCTTCCTGATAT
S775A	AATTGCCTACACCCTCACC**G**CTAACATTCCAGAGATCACCCCCTTCCTGATAT
T781S	AATTGCCTACACCCTCACCAGTAACATTCCAGAGATC**T**CCCCCTTCCTGATAT
T781A	AATTGCCTACACCCTCACCAGTAACATTCCAGAGATC**G**CCCCCTTCCTGATAT

[a]Nucleotides altered compared with wild type are in **bold**. The reading frame carrying the altered nucleotide is underlined. Synthetic oligonucleotides were cloned into the *Mfe*I and *Bsa*BI sites flanking the DNA sequence encoding TM5 in plasmid pPAP1945.

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 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 separated from free cations on Dowex-Tris 50W-X8 columns (50−100 mesh), and $^{204}Tl^+$ was measured by liquid scintillation counting (*10*). Data points are average values of duplicate determinations. Curves of Tl^+ binding versus ion concentration were fitted by nonlinear least-squares regression analysis to the Hill/Michaelis−Menten equations using Sigmaplot (Jandel Scientific): Tl^+ binding /ouabain binding ratio $= a \cdot [TlNO_3]^n/(c^n + [TlNO_3]^n)$, where a is the maximum Tl^+ binding/ouabain binding ratio, c is $K_{0.5(Tl^+)}$, and n is the Hill coefficient. For the Michaelis−Menten equation $n = 1$.

ATP Binding at Equilibrium and *K^+ Ion Displacement of ATP Binding.* Aliquots of TDS-treated gradient membranes containing 150−200 μg membrane protein were incubated on ice for 30 min in 1 mL of 13 nM [^{3}H]ATP (Amersham, specific activity 36 Ci/mmol), 10 mM MOPS-Tris pH 7.2, 10 mM EDTA-Tris, and 0.1−10 mM KCl. Choline chloride was added to maintain constant ionic strength. 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 remaining buffer removed with a paper towel. The pellet was resuspended for determination of protein and bound [^{3}H]ATP by scintillation counting. Kinetic constants were calculated using the Hill equation of the Sigmaplot 4.0 program (Jandel Scientific).

Na^+-Dependent Phosphorylation from [γ-^{32}P]ATP. Aliquots of TDS-treated gradient membranes containing 150 μg membrane protein were incubated for 10 min at 20 °C in Eppendorf tubes containing 190 μL of 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 (*11*). The reaction was initiated by adding [γ-^{32}P]ATP to 2 μM, the tubes were shaken continuously at 2200 min^{-1}, and the reaction was stopped after 4−20 s by adding 1 mL 4.8% PCA, 0.6 mM P_i, 0.6 mM ATP, and incubation for 10 min at 0 °C. The mixture was centrifuged for 10 min at 14 000 rpm, and the pellet was washed 4 times in stop solution. The pellet was resuspended in 200 μL 1 M NaOH and incubated for 30 min at 60 °C to dissolve the protein. Aliquots of 150 μL were transferred to scintillation counting and aliquots of 10−20 μL for protein analysis as described previously (*11*). Maximum phosphorylation and $K_{0.5(Na^+)}$ values were estimated using the Hill equation of the Sigmaplot 4.0 program (Jandel Scientific).

Table 3: Capacities and Affinities for Ouabain Binding and Na,K-ATPase Activities after Substitutions of Thr^{781}, Asn^{776}, Ser^{775}, Thr^{774}, Thr^{772}, Tyr^{771}, and Wild Type[a]

	Na,K-ATPase activity (%)	Ouabain binding (pmol/mg pr)	Ouabain binding K_d (nM)
T781A	100	19 ± 3	5 ± 1
T781S	43	3 ± 1	3 ± 2
N776A	0	10 ± 3	6 ± 4
N776Q	7	7 ± 1	7 ± 5
S775A	12	4 ± 2	4 ± 2
S775T	1	8 ± 5	6 ± 5
T774A	25	9 ± 4	7 ± 5
T774S	62	5 ± 1	6 ± 4
T772A	16	8 ± 1	10 ± 2
T772S	51	8 ± 2	6 ± 2
Y771F	27	10 ± 1	8 ± 6
WT	100	10 ± 2	5 ± 1

[a] Data of Na,K-ATPase activities are average values of two or three determinations. The capacities and affinities of ouabain binding are average values for 3−5 consecutive preparations. Data are estimated by nonlinear regression analysis of binding at a series of [^{3}H]ouabain concentrations.

RESULTS

Expression in Yeast of Mutations of Thr^{781}, Asn^{776}, Ser^{775}, Thr^{774}, Thr^{772}, and Tyr^{771}. The Na,K-ATPase activities and ouabain binding characteristics of the mutant proteins are shown in Table 3. The expression levels as monitored by ouabain binding were variable, between 3 and 19 pmol/mg protein with affinities for ouabain in the range of K_d 3−10 nM. Assays of Na,K-ATPase activities revealed large reductions after substitution of Asn^{776} and Ser^{775} to 0−12% of wild type. Intermediate activities in the range of 16−62% were observed after mutation of Thr^{772} and Thr^{774}, whereas the activities after substitutions to Thr^{781} were close to that of wild type. All equilibrium binding and phosphorylation data were expressed relative to parallel determinations of the ouabain binding capacity for each membrane preparation to cancel variations among different fermentations and preparations of individual mutant proteins.

The Interaction of Tl^+, K^+, or Na^+ with Recombinant, Wild-type Na,K-ATPase. The maximum binding of wild-type

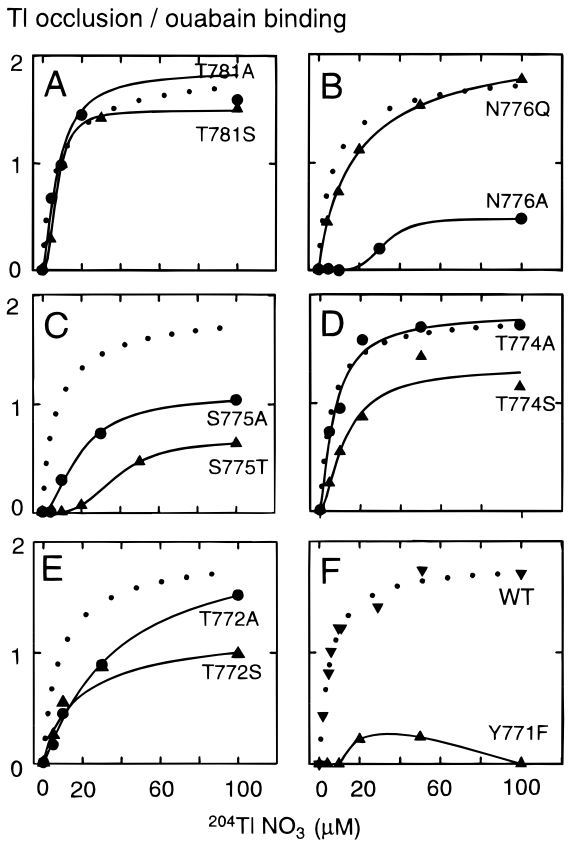

FIGURE 1: Effect of substitutions of Thr^{781} (A), Asn^{776} (B), Ser^{775} (C), Thr^{774} (D), Thr^{772} (E), or the mutation Tyr^{771}Phe (F) on the concentration dependence of ^{204}Tl$^+$ occlusion. Data of occlusion of wild-type Na,K-ATPase are shown in frame F (▼), and the fitted dotted line (····) is shown for comparison in all six frames. Occlusion was expressed as the ratio of ^{204}Tl$^+$ occlusion (picomoles/mg protein) to [^{3}H] ouabain-binding capacities as determined in each experiment. Data points are average values of two separate experiments with double determinations at each cation concentration. The average values of ouabain-binding capacities for wild type and mutations are shown in Table 3. Assays were as described in Materials and Methods. Using Sigmaplot 4.0 (Jandel Scientific) lines were fitted by nonlinear least-squares regression analysis to the Hill/Michaelis−Menten equations: occluded Tl$^+$/ouabain binding ratio $= a\cdot[TlNO_3]^n/(c^n + [TlNO_3]^n)$, where 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 estimated maximum capacities and $K_{0.5(Tl^+)}$ values are shown in Table 4.

gradient membranes from yeast was close to two Tl$^+$ occlusion sites per ouabain binding sites (1.9 ± 0.3) with $K_{0.5(Tl^+)}$ 7 ± 3 μM (Figure 1F and Table 4) in agreement with earlier data (*10*). These values are identical with those of purified pig kidney Na,K-ATPase (1.9 ± 0.1; $K_{0.5(Tl^+)}$ 6.3 ± 0.9 μM), not shown. In parallel, K$^+$-nucleotide antagonism was assayed to monitor the consequences of amino acid substitutions for K$^+$ displacement of ATP binding from the E_1ATP form. This reflects the ability of K$^+$ ions to stabilize the alternative conformation, E_2[2K]. Figure 2c and Table 4 show that K$^+$ ions displaced ATP from wild type with $K_{(0.5,K^+)} = 0.064 \pm 0.004$ mM. The curves of Na$^+$-dependent phosphorylation of wild type from ATP in the presence of oligomycin are shown in Figure 3 F, and the $K_{0.5(Na^+)}$ value was fitted to 0.62 ± 0.06 mM (Table 5).

Mutations of Thr^{781} Had No Effect on Binding of Tl$^+$, K$^+$, or Na$^+$. After substitution of Thr^{781} for Ala or Ser, the values

Table 4: Consequences of Mutations of Thr^{781}, Asn^{776}, Ser^{775}, Thr^{774}, Thr^{772}, or Tyr^{771}, for Tl$^+$ Occlusion and K$^+$ Displacement of ATP[a]

	Tl$^+$ occl/ouab[b] ratio	Tl$^+$ occl $K_{0.5(Tl^+)}$ (μM)	K$^+$ displacement of ATP $K_{0.5(K^+)}$ (mM)
T781A	1.9 ± 0.3	8 ± 3	0.04 ± 0.005
T781S	1.5 ± 0.02	7.4 ± 0.2	0.075 ± 0.04
N776A	0.48 ± 0.02	32 ± 4	3.3 ± 1.8
N776Q	2.2 ± 0.1	19 ± 3	0.25 ± 0.02
S775A	1.09 ± 0.09	19 ± 3	1.83 ± 0.04
S775T	0.67 ± 0.02	38 ± 1	1.2 ± 0.004
T774A	1.9 ± 0.6	8.5 ± 6.4	0.03 ± 0.001
T774S	1.3 ± 0.2	12.5 ± 3.5	0.11 ± 0.01
T772A	2.0 ± 0.3	37 ± 13	0.21 ± 0,08
T772S	1.3 ± 0.1	20 ± 4	0.106 ± 0,003
Y771F	0.24	16	0.55 ± 0.001
WT	1.9 ± 0.3	7 ± 3	0.064 ± 0.004

[a] Occlusion of ^{204}Tl$^+$ was determined and maximum values of Tl$^+$occlusion/ouabain binding and $K_{0.5(Tl^+)}$ were fitted by nonlinear least-squares regression as in Figure 1. $K_{0.5\ (K^+)}$ values for displacement of ATP were estimated by nonlinear regression analysis as in Figure 2. [b] occl, occlusion; ouab, ouabain.

for Tl$^+$ occlusion and the $K_{0.5(K^+)}$ values for K$^+$ displacement of ATP were close to those for wild type (Figure 1 and 2 and Table 4). As seen in Figure 3 and Table 5, the mutations of Thr^{781} had almost no effect on Na$^+$ dependence of phosphorylation from ATP. The observation that substitutions of this residue did not interfere with binding of K$^+$ or Na$^+$ serves as an intrinsic control of the subsequent experiments. The consequences of mutations of the Thr^{781} have not been determined previously.

Substitution of Asn^{776} with Ala Severely Depressed Binding of both K$^+$ and Na$^+$, Whereas Substitution with Gln Only Depressed Na$^+$ Binding. Substitution of the carboxamide group for a hydrogen atom in the Asn^{776}Ala mutation caused a large reduction of Tl$^+$ occlusion capacity with a 4-fold decrease in affinity for Tl$^+$ ($K_{0.5} = 32 \pm 4$ μM). The mutation also caused a large 52-fold increase of $K_{0.5(K^+)}$ for K$^+$ displacement of ATP (Table 4), and the displacement of ATP was incomplete at 10 mM KCl (Figure 2). In parallel, the $K_{0.5(Na^+)}$ for Na$^+$-dependent phosphorylation was 19-fold higher than for wild type. The substitution for Ala thus showed that the carboxamide group of Asn^{776} contributes almost equally to the binding of Tl$^+$(K$^+$) or Na$^+$ in the E_2 or E_1 conformations, respectively. In contrast, a change of the geometry of the carboxamide group in the mutation Asn^{776}Gln caused a 32-fold increase of $K_{0.5(Na^+)}$ to 20 ± 1 mM. In this mutation, the $K_{0.5(K^+)}$ for K$^+$ displacement of ATP was increased 4-fold compared with wild type (Table 4). This dissociation of the two parameters for binding K$^+$ suggests that the mutation causes a shift of the conformational E_1-E_2 equilibrium in the direction of the E_1 form.

Analysis of the rate of Na$^+$-dependent phosphorylation showed that the reduced affinities for Na$^+$ seen after mutation of Asn^{776} for Ala or Gln reflect severely reduced initial rates of phosphorylation. A phosphorylation time of 20 s was required to reach the maximum levels seen in Figure 4 in the presence of oligomycin. With a phosphorylation time of 4 s, the levels that reached 0−150 mM NaCl in the presence of oligomycin were only 0.33 ± 0.05 or 0.42 ± 0.03 for the mutations of Asn^{776} for Ala or Gln, respectively, compared

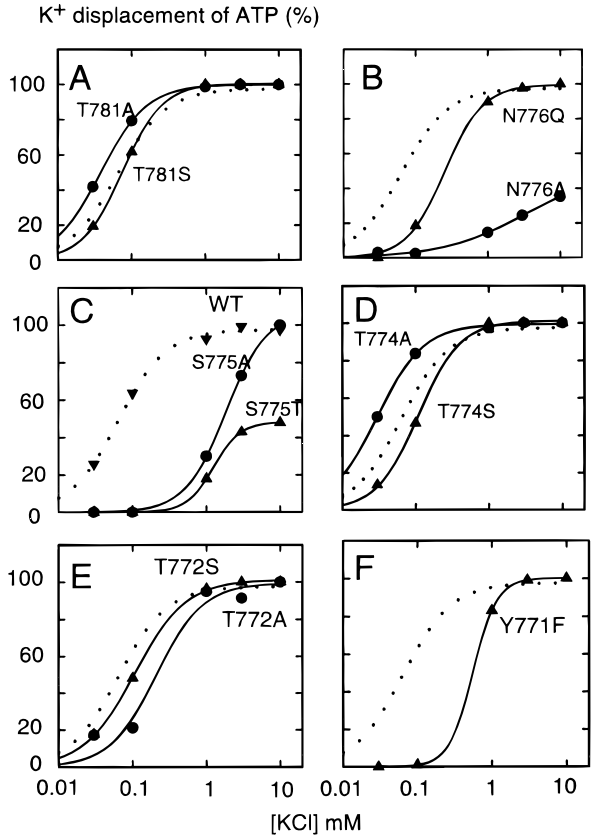

FIGURE 2: Effect of substitutions of Thr[781] (A), Asn[776] (B), Ser[775] (C), Thr[774] (D), Thr[772] (E), or the mutation Tyr[771]Phe (F) on K^+ displacement of ATP binding. Data for wild-type Na,K-ATPase are shown in frame C (▼), and the fitted dotted line (····) is shown for comparison in all six frames. 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 in Materials and Methods. Data points are average values of two separate experiments. The lines were fitted, and $K_{0.5(K^+)}$ values (Table 4) were estimated by nonlinear least-squares regression analysis using the equation: Displaced ATP = a·[KCl]^n/(c^n + [KCl]^n), where a is the maximum ATP displacement, c is $K_{0.5(K^+)}$, and n is the Hill coefficient.

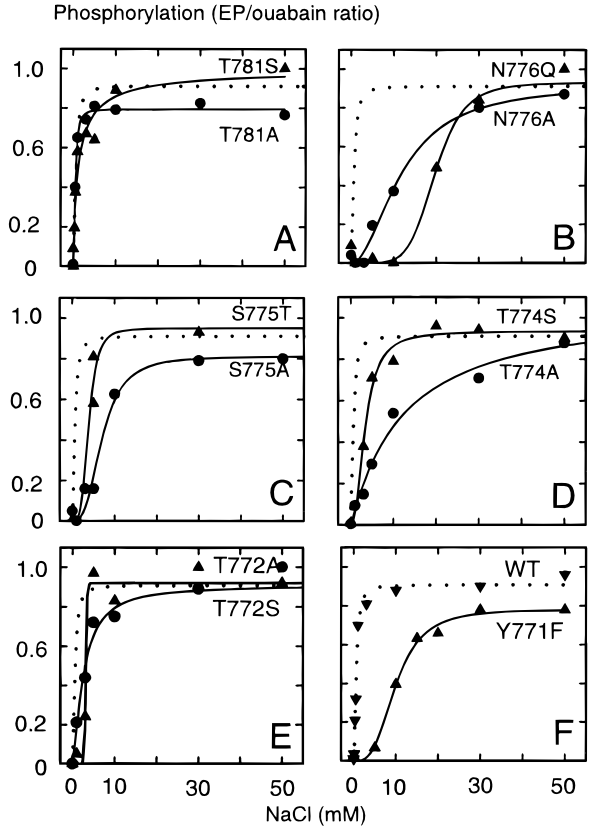

FIGURE 3: Effect of substitutions of Thr[781] (A), Asn[776] (B), Ser[775] (C), Thr[774] (D), Thr[772] (E), or the mutation Tyr[771]Phe (F) on Na^+-dependent phosphorylation from [γ-^{32}P]ATP. Data for phosphorylation of wild-type Na,K-ATPase are shown in frame F (▼), and the fitted dotted line (····) is shown for comparison in all six frames. The procedure was as described in Materials and Methods. Data points are average values of two separate experiments. The lines were fitted, and maximum phosphorylation levels and $K_{0.5(Na^+)}$ values (Table 5) were estimated by nonlinear least-squares regression analysis using the equation: Phosphorylation/ouabain ratio = a·[NaCl]^n/(c^n + [NaCl]^n), where a is the maximum phosphorylation level, c is $K_{0.5(Na^+)}$, and n is the Hill coefficient.

with a level of 1.0 for wild type (not shown). Analysis of the time course of phosphorylation at 50 mM NaCl showed that the half-time for phosphorylation was 4.0 ± 0.4 s for the Asn[776]Ala mutation (Figure 4) and 3.2 ± 1.3 s for the Asn[776]Gln mutation (not shown). The half-time of 0.34 ± 0.06 s for wild type (Figure 4) probably represents the minimum mixing time of the manual assay.

The functional consequences of the mutation of Asn[776] had not been determined previously. The observation that mutations of Asn[776] in the ouabain-resistant isoform could not support growth of HeLa cells (*25*) agreed with our identification of the carboxamide group of Asn[776] as being essential for binding of both K^+ and Na^+ ions.

Substitution of Ser[775] for Ala or Thr Interfered with Binding of Both K^+ and Na^+. After substitution of Ser[775] for Ala the capacity for Tl^+ occlusion was reduced from two to about one site per ouabain site with a 2.7-fold increase of the $K_{0.5(Tl^+)}$ value (Table 4). The replacement of Ser[775] with Thr caused a further reduction of the capacity for occlusion of Tl^+ and a 5.5-fold increase of the $K_{0.5(Tl^+)}$ (Table 4). Changes of similar magnitude with 19- or 29-fold higher values of $K_{0.5(K^+)}$ for displacement of ATP were observed after substitution of Ser[775] for Thr or Ala (Figure 2 and Table 4), and displacement of ATP was incomplete for the Ser[775]Thr mutation. The data reflect a close correlation between the consequences of the amino acid substitutions for high-affinity

Table 5: Consequences of Mutations of Thr[781], Asn[776], Ser[775], Thr[774], Thr[772], or Tyr[771] for Phosphorylation and Binding of Na^+[a]

	EP/ouabain ratio	$K_{0.5\,(Na^+)}$ (mM)
T781A	0.8 ± 0.1	0.49 ±0.03
T781S	1.0 ± 0.1	1.2 ± 0.6
N776A	0.92 ± 0.08	12 ± 2
N776Q	0.93 ± 0.04	20 ± 1
S775A	0.81 ± 0.04	7.0 ± 0.7
S775T	0.95 ± 0.07	3.8 ± 0.4
T774A	1.06 ± 0.08	12.3 ± 2.7
T774S	0.94 ± 0.03	3.4 ± 0.3
T772A	0.92 ± 0.03	3.1 ± 0.3
T772S	0.91 ± 0.05	2.6 ± 0.5
Y771F	0.78 ± 0.03	10.0 ± 0.5
WT	0.91 ± 0.02	0.62 ± 0.06

[a] Na^+-dependent phosphorylation from [γ-^{32}P]ATP was determined, and maximum phosphorylation levels and $K_{0.5(Na^+)}$ values were fitted by nonlinear least-squares regression analysis as in Figure 3.

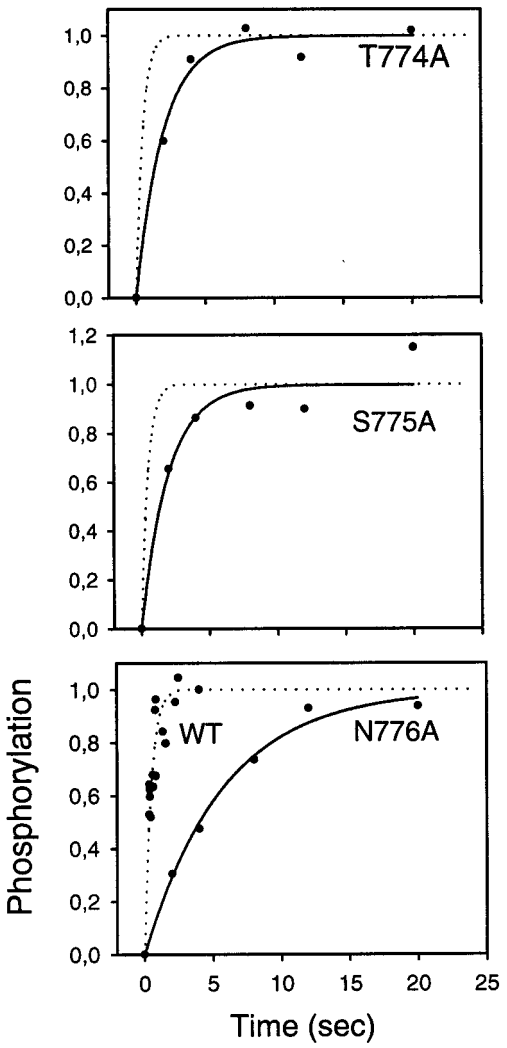

FIGURE 4: Consequences of alanine substitutions of Thr^{774} (top), Ser^{775} (middle), and Asn^{776} (bottom) for the rate of Na^+-dependent phosphorylation from ATP. Data for wild-type Na,K-ATPase are shown in the bottom frame and the fitted dotted line (···) is shown in all three frames. Reactions were initiated by adding [γ-^{32}P]ATP to continuously shaken (2200 min^{-1}) Eppendorff tubes at 0−4 °C containing 200 μL of 2 μM [γ-^{32}P] ATP, 3 mM $MgCl_2$, 30 μM oligomycin, 50 mM NaCl, 100 mM choline chloride, 20 mM TES-Tris, pH 7.5, protease inhibitors, and 150 μg TDS-treated gradient membrane protein. Incubation times were 0.4−4 s for wild-type membranes and 2−20 s for the mutations. The lines were fitted, and $T_{0.5}$ values were estimated by nonlinear least-squares regression analysis using the equation: Phosphorylation = $a\,(1 - e^{-bx})$, where a is the maximum phosphorylation level, $b = -\ln(0.5)/(x50)$, and x is the incubation time in seconds. The estimated $T_{0.5}$ values were 0.34 ± 0.06 s for wild type, 4.0 ± 0.4 s for Asn^{776}Ala, 1.4 ± 0.3 s for Ser^{775}Ala, and 1.4 ± 0.2 s for Thr^{774}Ala.

Tl^+-ion occlusion and the effects on the K^+-nucleotide antagonism.

The data in Figure 3 and Table 5 show that $K_{0.5(Na^+)}$ for Na^+-dependent phosphorylation was increased 11-fold after substitution of Ser^{775} for Ala (7.0 mM), but the increase was smaller, 6-fold, after substitution for Thr (3.8 mM) compared with the value for wild type (0.62 mM, Table 5). Analysis of phosphorylation rates showed that the reduced affinities for Na^+ after mutation of Ser^{775} for Ala reflected reduced initial rates of phosphorylation with half-time 1.4 ± 0.3 s at 50 mM NaCl compared with 0.34 ± 0.06 s for wild type (Figure 4).

Mutations of Thr^{774} Severely Reduced Binding of Na^+ with Little Effect on Binding of K^+. The mutations of Thr^{774} had a differential effect on Tl^+ occlusion and Na^+-dependent phosphorylation. Tl^+ occlusion was unaffected by the Thr^{774}-Ala mutation (Figure 1 and Table 4). The substitution for Ser caused a moderate reduction of occlusion (Figure 1 and Table 4). The $K_{0.5(K^+)}$ for ATP displacement was moderately increased after substitution for Ser, but the $K_{0.5(K^+)}$ for the Thr^{774}Ala mutation (0.03 mM) was lower than for wild type (0.064 mM) (Figure 2 and Table 4). This suggests that interference of the mutation with the binding of Na^+ may destabilize the E_1 conformation and thus reduce the concentration of K^+ required for stabilization of the E_2 [2K] form. Removal of the hydroxyl and methyl groups of Thr^{774} by mutation to Ala caused a large, 20-fold reduction of the apparent affinity for Na^+ stimulation of phosphorylation for 20 s (Figure 3 and Table 5). The altered Na^+ affinity reflects reduced initial rates of phosphorylation of the Thr^{774}Ala mutation as the half-time for phosphorylation at 50 mM NaCl was 1.4 ± 0.2 s compared with 0.34 ± 0.06 for wild type (Figure 4). With a phosphorylation time of 4 s, the phosphorylation level at 150 mM Na^+ was only 0.63 ± 0.03 for the Thr^{774}Ala mutation, whereas the level for Thr^{774}Ser was 0.95 ± 0.04 and near that of wild type (not shown). Replacement of the methyl group for a hydrogen atom by substituting Thr^{774} for Ser resulted in a smaller, 5-fold reduction of apparent Na^+ affinity (Figure 3 and Table 5). The specific effect of the Thr^{774}Ala mutation on Na^+ binding was therefore caused by removal of the hydroxyl group. The consequences of these mutations of Thr^{774} had not been determined previously.

Substitution of the Methyl Group of Thr^{772} for a Hydrogen Atom Interfered Equally with Binding of K^+ and Na^+. Substitution of Thr^{772} for Ala or Ser caused an almost equal reduction of Tl^+ occlusion (Figure 1E and Table 4). Because the change of binding was the same after substitution of the methyl group for a hydrogen atom (Thr^{772}Ser) as after substitution of both the methyl and hydroxyl group for a hydrogen atom (Thr^{772}Ala), the hydroxyl group of Thr^{772} does not appear to contribute to the binding of $Tl^+(K^+)$. A similar argument applies to the binding of Na^+, because the 5- and 4.2-fold reductions of affinities for Na^+-dependent phosphorylation were overlapping for the Thr^{772}Ala and Thr^{772}-Ser mutations (Figure 3E and Table 5). Cation binding of mutations of Thr^{772} had not been assayed before.

Removal of the Hydroxyl Group of Tyr^{771} Severely Depressed the Binding of both K^+ and Na^+. The conservative substitution of Tyr^{771} for Phe caused a large depression of the capacity for occlusion to 13% of the wild-type value and a reduction of the affinity for Tl^+ (Figure 1F and Table 4). Similarly a large 9-fold increase of $K_{0.5(K^+)}$ for K^+ displacement of ATP displacement was observed (Figure 2F and Table 4). The mutation also caused a 16-fold increase of $K_{0.5(Na^+)}$ for Na^+-dependent phosphorylation from ATP (Figure 3F and Table 5), indicating that the hydroxyl group of Tyr^{771} contributes equally to binding of Na^+ in the E_1 form and binding of $Tl^+(K^+)$ in the E_2 form. Our phosphorylation data for the Tyr^{771}Phe mutation are similar to those seen after expression of the ouabain-resistant mutation Tyr^{773}-Leu in COS-1 cells (*27*); but in contrast to our data on binding of Tl^+ or K^+ to the Trp^{771}Phe mutation, the $K_{0.5(K^+)}$ for K^+ stimulation of Na,K-ATPase activity of the Tyr^{773}-

Table 6: Change in Gibbs Free Energy of Tl^+ and Na^+ Binding to Wild-Type Recombinant Na,K-ATPase[a]

	$\Delta G_b(Tl^+)$[a] kJ/mol	$\Delta G_{b(Na^+)}$[b] kJ/mol
wild type	−28.8	−16.7

[a] Gibbs free energy of Tl^+-ion binding was estimated from the data in Table 4 using the equation: $\Delta G_{b(Tl^+)} = -RT$ ln (Tl^+ occl/ouab/$K_{0.5(Tl^+)}$)$_{WT}$. [b] Gibbs free energy of Na^+-ion binding was estimated from the data in Table 5 using the equation: $\Delta G_{b(Na^+)} = -RT$ ln(EP/ouab/$K_{0.5(Na^+)}$)$_{WT}$.

Table 7: Alterations in Gibbs Free Energy ($\Delta\Delta G_b$) of Tl^+- or Na^+-ion binding at 4 °C of Mutations of Thr^{781}, Asn^{776}, Ser^{775}, Thr^{774}, Thr^{772}, or Tyr^{771} in M5 of the α-Subunit of Na,K-ATPase[a]

allele	$\Delta\Delta G_b(Tl^+)$ kJ/mol	$\Delta\Delta G_b(Na^+)$ kJ/mol
T781A	+0.3	−0.3
T781S	+0.6	+1.3
N776A	+6.7	+6.8
N776Q	+1.9	+8.0
S775A	+3.6	+5.9
S775T	+6.3	+4.1
T774A	+0.4	+6.5
T774S	+2.2	+3.8
T772A	+3.7	+3.7
T772S	+3.3	+3.3
Y771F	+6.7	+6.6

[a] $\Delta\Delta G_b$ values were calculated from data in Tables 4 and 5 using the equation of Fersht et al. (*30*): $\Delta\Delta G_b = -RT$ ln [(maximum binding/$K_{0.5}$)$_{mut}$/(maximum binding/$K_{0.5}$)$_{WT}$].

Leu mutation was unaffected in the COS cell preparation (*27*).

Estimation of the Contribution of Residues of M5 to Changes in Free Energy of Cation Binding. In the presence of Mg^{2+} to stabilize the E_2 form, the curves of Tl^+ binding to wild type fit hyperbolic curves as demonstrated previously for binding Rb^+ (*32*) and Tl^+ (*33*) to purified renal Na,K-ATPase. The two Tl^+ binding sites per α-subunit are therefore equivalent and independent (cf. ref *4*). At low temperatures, the rate of deocclusion of Rb^+ or Tl^+ from renal Na,K-ATPase is negligible (*2*), and the $K_{0.5(Tl^+)}$ for Tl^+ occlusion is an equilibrium constant that is directly related to the standard free energy change for cation binding to the protein (*5*, *10*). 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 Gibbs free energy of binding between Tl^+ and Na,K-ATPase is estimated at $\Delta G_b = -28.8$ kJ/mol at 4 °C for each of two ions (Table 6). To take into account both the changes in affinity and capacity of Tl^+ binding, the consequences of the substitutions for the changes in Gibbs free energy of binding ($\Delta\Delta G_b$) were estimated from the ratios of maximum binding to $K_{0.5(Tl^+)}$ as shown in Table 7. After substitution of Asn^{776} and Ser^{775}, the curves of Tl^+ binding were sigmoid (Figure 1, B and C), indicating 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. Further analysis is required to determine whether the mutated residues provide coordinating groups for the cations or whether they are important for the stability of the binding cavity.

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 $K_{0.5(Na^+)}$ for Na^+ dependence of phosphorylation for wild type is identical with the K_d for $^{22}Na^+$ ion binding at equilibrium to the oligomycin complex of purified pig kidney Na,K-ATPase (*34*). 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^+ (cf. ref *35*). For wild type, the $K_{0.5\ (Na^+)}$ value of 0.62 mM (Figure 3, Table 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 (Table 6). After the substitutions several sigmoid-shaped curves were observed (Figure 3), 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.

For the mutations of Thr^{781}, the binding curves were overlapping those of wild type, and the estimated values of the free energies of Tl^+ binding ($\Delta\Delta G_b$) in Tables 6 and 7 were near zero. The costs of removing the carboxamide group of Asn^{776} and the hydroxyl group of Tyr^{771} were of the same magnitude for Tl^+ and Na^+ ($\Delta\Delta G_b = 6.5-6.8$ kJ/mol; Table 7). Alteration of the position of the carboxamide group (Asn^{776}Gln) caused a large change in binding energy for Na^+ ($\Delta\Delta G_b = 8$ kJ/mol), whereas the binding energy for K^+ was barely affected (Table 7). The differential changes in free energy of binding after substitutions of Ser^{775}, Thr^{774}, and Thr^{772} are evaluated in the Discussion.

DISCUSSION

Mutagenesis screening of six oxygen-carrying residues of the specific M5 sequence Y^{771}TLTSNIPEITP^{782} shows that the carboxamide group of Asn^{776} or the hydroxyl groups of Ser^{775} and Tyr^{771} contributes 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 ≥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 K^+ or Na^+ in the occluded $E_1P[3Na]$ or $E_2[2K]$ conformations. An important differential effect is observed for Thr^{774}, because its hydroxyl group contributes to the binding of Na^+ ions, but it is not required for high-affinity occlusion of K^+ ions. Differential effects on binding of Na^+ or K^+ are also observed after changing the geometry of the carboxamide group of Asn^{776} (Asn^{776}Gln) or the hydroxyl of Ser^{775} (Ser^{775}-Thr). Combined with our previous analysis of the contribution to K^+- and Na^+-ion binding of the carboxylate groups of Glu^{327}, Glu^{779}, Asp^{804}, and Asp^{808} in the α-subunit of Na,K-ATPase (*10*), the present results are relevant for understanding Na/K selectivity. They also provide information about the structural differences between the ion-binding cavities of the $E_2[2K]$ and $E_1P[3Na]$ conformations.

All mutations in this work have preserved the ability to bind ouabain with high affinity to the complex with Mg^{2+} and vanadate, an indication that they are capable of adopting the E_2 conformation. Preservation of high-affinity ATP binding indicates that the mutations also can undergo transition to the E_1 form. The observation that mutations of Thr^{781} near the center of M5 has no effect on the interactions

with K^+ or Na^+ serves as an intrinsic control of the mutagenesis experiments on the remaining residues. The mutations therefore appear to interfere selectively with cation binding without causing unspecific perturbation of the folding of cytoplasmic or extracellular domains for high-affinity binding of ATP or ouabain. This specificity of the responses and the limited structural consequences of substitution of amino acid residues in presumptive intramembrane segments is a common experience for several membrane proteins (*36*), including Ca-ATPase of the sarcoplasmic reticulum (*7*).

Identification of the carboxamide group of Asn^{776} as important for binding of both K^+ and Na^+ is a novel observation. Previously, it was noted that the Asn^{776}Ala substitution yielded apparently nonfunctional enzyme in HeLa cells (*25*), but the consequences of mutations of Asn^{776} previously have not been examined in Na,K-ATPase. In Ca-ATPase of the sarcoplasmic reticulum, the substitution of the homologous counterpart, Asn^{768} for Ala, leads to reduced Ca^{2+} affinity (*7*), whereas the Asn^{768}-Ile mutation has the same Ca^{2+} affinity as wild type with a reduced rate of transport (*27*). It is referred to as an E_2P-dephosphorylation mutant which is blocked in a low-energy conformation of the phosphoenzyme, but the role of this residue in Ca^{2+} binding has not been defined. In our analysis of the Asn^{776}-Ala mutation, 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. In contrast 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); Table 7). The increase of side-chain volume and the altered geometry of the carboxamide group of Asn^{776} are therefore much more critical for binding Na^+ than for binding K^+.

The conditions seem to be different for the neighbor, Ser^{775}, although this residue 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). It is of interest to compare our results with those of Blostein et al. (*24*) using an indirect assay of K^+ occlusion by the change in Na^+-dependent phosphorylation from ATP after incubation with KCl. Blostein et al. (*24*) found that the $K_{0.5(K^+)}$ = 2.7 mM for the Ser^{775}Ala mutation was 5-fold higher than for the wild type and that the capacity for occlusion was reduced to 66% of control. The magnitude of the changes after mutation are similar in the two experiments, although the stoichiometry of the Ser^{775}Ala mutation was reduced to about one Tl^+ ion per ouabain site in our direct assay of Tl^+ binding and our $K_{0.5(Tl+)}$ value of 19 μM was more than 100-fold lower than the $K_{0.5(K^+)}$ value in the phosphorylation assay (2.7 mM). This probably reflects that the latter constant includes the conformational equilibrium and the binding of several ligands in addition to K^+. Our conclusion that the hydroxyl group of Ser^{775} is important for binding both K^+ and Na^+ disagrees with an earlier report based on expression of the α-subunit with substitution of Ser^{775} for Ala or Cys in HeLa cells at high extracellular K^+ at 8-fold higher levels of α-subunit protein concentration than in the wild type (*18*). In this preparation the $K_{0.5(Na^+)}$ values for catalyzing phosphorylation or ATPase activity were identical for the Ser^{775}Ala mutation and the wild type (*18*). In agreement with our data, a subsequent report on assays of Na-ATPase activity in absence of K^+ suggested an alteration in intrinsic Na^+ binding in the Ser^{775}Ala mutation (*24*).

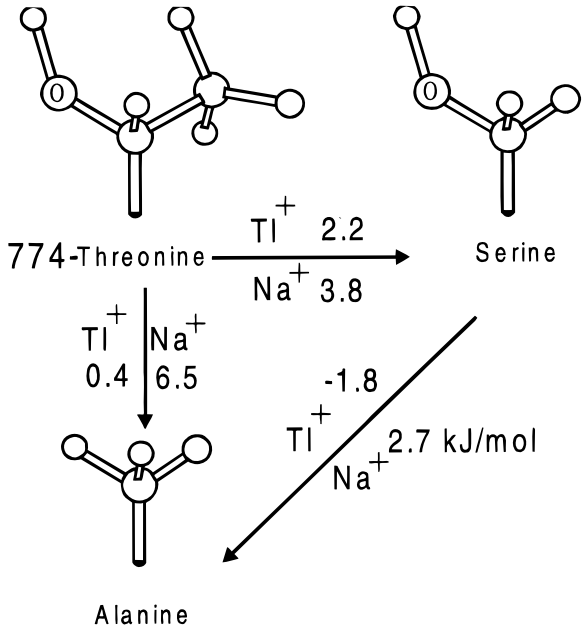

FIGURE 5: Contribution of methyl and hydroxyl groups of Thr^{774} to binding of Tl^+ or Na^+. The numbers are changes in free energy ($\Delta\Delta G_b$ kJ/mol) of Thr^{774}Ala or Thr^{774}Ser mutations from Table 7. The contributions of the hydroxyl group were estimated as the differences for the Ser-Ala substitution.

In Nature's own experiment Ser^{775} of Na,K-ATPase is replaced by Lys^{791} in H,K-ATPase (Table 1), and the outcome supports the conclusion that Ser^{775} contributes to binding K^+. Insertion of the large positively charged lysine residue can explain that the intrinsic affinity for binding Rb^+ is 80-fold lower in H,K-ATPase ($K_{0.5(Rb^+)}$ = 650 μM; ref *37*) than in Na,K-ATPase ($K_{0.5(Rb^+)}$ = 9 μM; ref *32*).

The specific effect of the Thr^{774}Ala mutation on Na^+ interactions is of particular interest for understanding Na^+/K^+ selectivity. Measurements of the changes in free energy of binding of Tl^+ or Na^+ for the substitution of Thr^{774} for Ser or Ala allow separation of the contributions of the hydroxyl and the methyl groups to cation binding as illustrated in Figure 5. 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^+ (Figure 5). The hydroxyl group in Thr^{774} is therefore strongly involved in the ability of the enzyme to discriminate between Na^+ and K^+. Substituting the methyl group for a hydrogen atom (Thr^{774}-Ser) costs 3.8 kJ/mol for Na^+ binding and 2.2 kJ/mol for Tl^+ binding. Similar effects of removing a methyl group were observed for Thr^{772}, because substitution for Ala or Ser caused almost equal changes of the free energy of binding of Tl^+(K^+) and Na^+ (3.7 and 3.3 kJ/mol, Table 7). These contributions of the methyl groups of Thr^{774} and Thr^{772} are close to the experimentally determined cost of transfer of a

methyl group from water to *n*-octanol (2.9 kJ/mol) (38). Rather than contributing to cation binding, the methyl groups of Thr^{774} and Thr^{772} may therefore be important for stabilizing the side chain in the hydrophobic environment of the transmembrane segment.

The hydroxyl group of the aromatic residue, Tyr^{771}, contributes almost equally to binding of Tl^+ ($\Delta\Delta G_b = 6.7$ kJ/mol) or Na^+ ($\Delta\Delta G_b = 6.5$ kJ/mol). Mutations of the homologous residue Tyr^{763} of SR Ca-ATPase suggested a gate function for this residue (*39*). The equal contribution to binding of K^+ or Na^+ agrees with this role, but the present data do not contribute to distinguish this possibility from a direct contribution of Tyr^{771} to coordination of the cations in the occluded conformations.

Comparisons of the present data of Table 7 with the contributions to Tl^+ and Na^+ binding of intramembrane carboxylic acid residues Glu^{327}, Glu^{779}, Asp^{804}, and Asp^{808} are of considerable interest. However, in contrast to the partial effects of the mutations in the present study, the conservative substitutions of the carboxylic acids Glu^{327}, Glu^{779}, Asp^{804}, and Asp^{808} abolish the high-affinity occlusion of Rb^+ or Tl^+ (*10*) and they interfere severely with Na^+-dependent phosphorylation (*12*). This strong inhibition of binding precludes measurements of binding affinities for cations and estimation of the changes of free energy of cation binding. Mutation of each of the carboxylate side chains abolishes high-affinity binding of Tl^+ in the range of 0−200 μM (*10*). Assuming $K_{0.5(Tl^+)}$ values of ≥200 μM, the estimated change of the free energy of binding ($\Delta\Delta G_b$) is greater than 8 kJ/mol. Similarly, conservative substitution of each of the carboxylate residues reduces phosphorylation to less than 50% in the presence of 150 mM Na^+ (*11*). Assuming a $K_{(0.5\ Na^+)} \geq 150$ mM, it can be estimated that each carboxylate group contributes at least 12.4 kJ/mol to Na^+-ion binding. This approaches 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 (*40*). Comparison of the sum of individual side-chain contributions with the overall binding energies for Tl^+ and Na^+ must await development of procedures for more exact determination of the contributions of intramembrane carboxylic acid residues.

It is important to examine whether the contributions of residues in M5 to cation binding are relevant for the energies required for Na/K selectivity at the cytoplasmic and extracellular surfaces in intact cells. The apparent affinities for Na^+ and K^+ at the cytoplasmic surface have been estimated at 0.6 mM and 10 mM (*4*). This affinity ratio corresponds to a 16-fold preference for Na^+ over K^+ at the cytoplasmic surface and requires that Na^+ binding is stabilized by 6.4 kJ/mol more than K^+ binding. The specific contribution of Thr^{774} to binding Na^+ and contributions in the range of 4−7 kJ/mol from Agn^{776}, Ser^{775}, and Tyr^{771} in the cytoplasmic part of M5 could 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 (*4*). The extracellular K/Na affinity ratio of 3000-fold requires that K^+ binding is 18.4 kJ/mol more stable than binding of Na^+. This apparently requires the contribution of several residues, but this value is within the range of binding energies determined for the residues examined in this work and for each of the four carboxylate residues in M4, M5, or M6. As

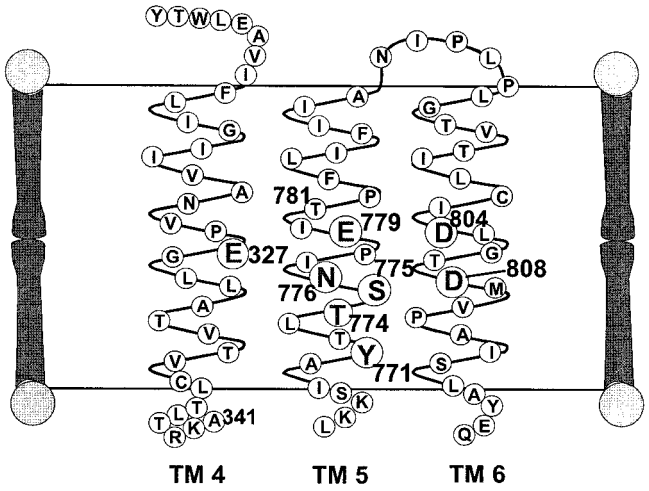

FIGURE 6: Position of residues of importance for binding of K^+ or Na^+ in a helical representation of residues in M4, M5, and M6. The drawing is based on a previous topological model for the α-subunit (*44*). With the indicated bilayer width (30 Å) and a pitch of 5.4 Å and 3.6 residues/turn, each intramembrane segment comprises 20 amino acid residues.

discussed below, the high-affinity cation binding in the two conformations E_1P[3Na] or E_2[2K] may be determined by the precise adjustments of distances between oxygen groups in transmembrane segments. In addition several residues in the hydrophilic cytoplasmic loop of the α-subunit may also be important for Na/K specificity, as suggested from the analysis of conserved core sequences in the superfamily of cation transport ATPases (*41*).

The consequences of the substitutions of the involved groups of Asn^{776}, Ser^{775}, Thr^{774}, Thr^{772}, and Tyr^{771} illustrate that precise positioning of the side chains is important for achieving the relatively high cation affinities of the ion-binding cavities of the E_2[2K] and the E_1P[3Na] conformations. At the level of M5, the transition from E_2 to E_1 forms appears to involve expansion in terms of the number of cation-coordinating residues. A topological model of the position of oxygen-carrying side chains of the specific sequence Y^{771}TLTSNIPEITP^{782} in transmembrane segments of the α-subunit of Na,K-ATPase is shown in Figure 6. The residues involved in binding K^+ in the E_2[2K] form, Asn^{776}, Ser^{775}, Tyr^{771}, and Glu^{779} (*12*), align as a patch to form a polar surface that spans about 140° of the 360° helix. 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 extend by about 100° to involve two thirds of the circumference of the helix. The nonpolar residues Ile^{780}, Ile^{777}, and Leu^{773} are located with Thr^{772} and Thr^{781} at opposite side of the cylinder of an amphipathic helix. The presence of proline in the helix may create a kink with positioning of these hydrophobic residues at the concave curvature, whereas Pro^{778} and its +1 (Glu^{779}), −3 (Ser^{775}), and −4(Thr^{774}) polar neighbors are exposed on the convex side (cf. ref *42*). In addition 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, Glu^{779} (cf., *42*). These effects of proline on the structure may serve to expose main-chain carbonyls for interaction with cations. With this in mind, it is interesting that the substitution of Pro^{778} for Ala has been shown to cause

a 5−6-fold increase in $K_{0.5(K^+)}$ without altering $K_{0.5(Na^+)}$ (*27*). This was proposed to be an indirect effect of the proline on the binding of K^+ to Glu^{779} (*27*). An alternative possibility is that the replacement of Pro^{778} with Ala allows formation of hydrogen bonds with the carbonyl groups of Ser^{775} or Thr^{774} and that this may interfere with the role of the carbonyl in coordinating K^+ ions.

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 the intramembrane segment. We previously proposed a model in which the structural change accompanying E_1−E_2 transition is transmitted indirectly to M5 via the interaction of Asp^{369}-phosphate with Mg^{2+} which is coordinated to other negatively charged groups of the segment (707−725) of the α-subunit (*12*). Evidence for interaction between these segments has also been obtained in experiments with iron catalyzed cleavage of the α-subunit (*43*).

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