Importance of Na,K-ATPase Residue α1-Arg^{544} in the Segment Arg^{544}−Asp^{567} for High-Affinity Binding of ATP, ADP, or MgATP†

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ABSTRACT: To identify residues involved in ATP binding in the N-domain of the α1-subunit of Na,K-ATPase, mutations were directed to the segment Arg^{544}−Asp^{567}, a β-strand−loop−helix structure with Arg^{544} positioned at the mouth of the ATP-binding pocket near the interface to the P-domain. Substitution of Arg^{544} with Gln abolished high-affinity binding of free ATP, while substitution with lysine reduced ADP affinity with minor effects on ATP binding. The contribution of Arg^{544} to the change in free energy of ATP binding was estimated to 6.9 kJ/mol ($\Delta\Delta G_b$) from double mutations with Asp^{369} and to 7.8 kJ/mol from the MgATP dependence of phosphorylation. The phosphorylation data show that binding of Mg^{2+} may increase the apparent affinity of wild-type enzyme for ATP [$K_{1/2}$(ATP) 12 nM]. Moderately reduced affinities for ATP were seen after mutations of Asp^{555}, Glu^{556}, Asp^{565}, or Asp^{567} with $\Delta\Delta G_b \approx 0.5-3$ kJ/mol. Mutations of Cys^{549} did not affect ATP binding. In conclusion, Arg^{544} is important for binding of ATP or ADP, probably by stabilizing the β- or γ-phosphate moieties and aligning the γ-phosphate for interaction with the carboxylate group of Asp^{369}.

In mammals, Na,K-ATPase transforms 20−30% of the current ATP production at rest to active Na,K-transport in the kidney, central nervous system, and other cells of the body. Na,K gradients across cell membranes are required for maintaining membrane potential, cell volume, and secondary active transport processes of other solutes. Renal [α1β1γ]Na,K-ATPase consists of three subunits, α-subunit with 1016 residues, β-subunit with 302, and γ-subunit with 66−68 amino acids, and it is the largest protein complex in the family of cation pump proteins (*1*, *2*). Sites for ATP binding and phosphorylation are located in the large central cytoplasmic protrusion of the α-subunit between the fourth and fifth transmembrane segment, while the cation-binding sites lie in the intramembrane domain (*3*−*5*). The cytoplasmic protrusion is split in three domains (*6*) with a single nucleotide-binding N-domain separated from the P-domain comprising the phosphorylated residue, Asp^{369}, and the C-terminal 708-TGDGVND segment, which is involved in binding of Mg^{2+} (*7*).

Na,K-ATPase is unique among cation pumps in its ability to bind one molecule of free ATP per α1β1 unit in EDTA-containing buffer with high affinity (K_D = 30−100 nM), while the apparent affinity of the K-bound form is very low (*1*, *8*). The increase in binding energy of ATP associated with the E_2[2K] ↔ E_1(2K) conformational transition constitutes the driving force for transport of K^+ across the membrane (*9*). Binding of MgATP and phosphoryl transfer involves coordination of Mg^{2+} to Asp^{710} in the 708-TGDGVND segment (*7*). Ca-ATPase does not bind free ATP with high affinity, and MgATP is bound with moderate affinity (K_D = 5.6 μM) (*10*). The nucleotide-binding N-domain in the high-resolution structure of Ca-ATPase is inserted as a subdomain of the P-domain in a Rossman fold (*11*). It is formed by residues Gln^{360}−Arg^{604} (*6*) corresponding to Arg^{383}−Arg^{589} in the α-subunit of Na,K-ATPase. The high-resolution structure (*6*) confirms the involvement of Lys^{492} (Lys^{480} in Na,K-ATPase) known to be the receptor for 8-azido-ATP (*12*, *13*), of Lys^{515} (Lys^{501}), the site of FITC[1] labeling (*14*, *15*), and of Phe^{487} (Phe^{475}) (*16*), but the involvement of Thr^{441} and Arg^{560} (Arg^{544}) has not been predicted by chemical labeling. Lys^{515} (Lys^{501} in Na,K-ATPase) and Phe^{487} (Phe^{475}) lie deep in the pocket around the adenosine moiety of ATP, while Arg^{560} (Arg^{544}) is positioned near the mouth of the pocket. This region has not previously been examined in structure−function studies.

The purpose of the present work has therefore been to identify specific amino acid residues contributing to high-affinity binding of ATP in Na,K-ATPase in the segment Arg^{544}−Asp^{567}. Mutagenesis screening was combined with high-yield expression in yeast (*17*, *18*) and assay of ATP binding at equilibrium (*7*, *19*). Attention was drawn to the Arg^{544}−Asp^{567} segment (*3*) because it resembles the Walker B consensus sequence in the nucleotide-binding domains of adenylate kinase (*20*), ADP/ATP exchange protein (*21*), and ABC transporters (*22*). When modeled on the fold of the high-resolution structure of Ca-ATPase (*6*), the Arg^{544}−Asp^{567} segment forms a β-strand−loop−helix in the N-domain with Arg^{544} positioned near the mouth of the pocket and the interface to the P-domain (Figure 1). The mutations

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[1] Abbreviations: FITC, fluorescein isothiocyanate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride.

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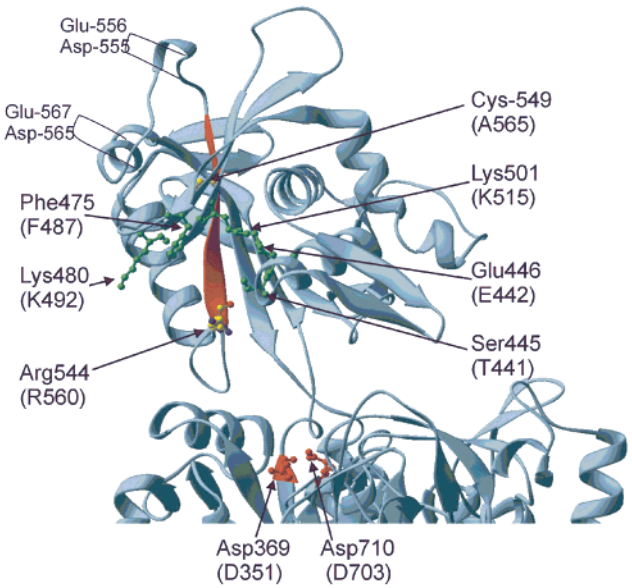

FIGURE 1: Ribbons model of the position of residues of importance for ATP binding in the N-domain in the α-subunit of Na,K-ATPase in the backbone structure of Ca-ATPase (*6*). Numbers of residues in the α-subunit of Na,K-ATPase are given with residue numbers in Ca-ATPase in parentheses. Lines indicate the approximate positions of Asp^{555}-Glu^{556} and Asp^{565}-Glu^{567}. The text of the introduction gives reference to the evidence for involvement of the marked residues in ATP binding. Mutations of Arg^{544}, Cys^{549}, Asp^{565}, Glu^{567}, Asp^{555}, and Glu^{556} are examined in the present work.

were expressed in high yield as estimated by ouabain binding (*17*), and contributions to the free energy of binding were estimated from assays of free ATP binding at equilibrium (*7*, *19*). To determine the free energy contribution of the basic groups of Arg^{544} to binding of free ATP, double mutations with Asp^{369}Ala were produced to exploit the high ATP affinity after removal of the phosphorylated carboxylate group of Asp^{369} (*7*, *19*). Binding constants for ADP were determined to examine if Arg^{544} interacts with the γ-phosphate or with other moieties of the ATP molecule. Phosphorylation assays at low concentrations of ATP in the presence of oligomycin were exploited to determine the influence of mutations on apparent affinities for binding of MgATP.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. Mutations were constructed by site-directed mutagenesis according to Ho et al. (*23*) using the primers shown in Table 1. The resulting PCR fragments were digested with *Bam*HI and *Eag*I and inserted into similarly digested pPAP1933 (*17*). The nucleotide sequence of all PCR fragments was confirmed by dideoxy sequencing.

Transformation of Yeast Cells. Yeast cells were transformed according to Gietz et al. (*24*).

Growth of Yeast and Expression of Na,K-ATPase. Transformed yeast cells were grown in an Applikon fermentor equipped with an ADI 1030 Bio Controller and connected to a computer running the BioXpert program. Growth of yeast cells expressing wild-type Na,K-ATPase or the mutation Arg^{544}Gln at 30 °C and Na,K-pump protein synthesis induced with galactose were performed as before (*17*). Yeast cells producing all other mutations were grown at 30 °C until all glucose was metabolized, transferred to 15 °C, and induced with 2% galactose (*18*). Yeast cells were harvested 48 h later.

Table 1: Sequence of Primers Used for Site-Directed Mutagenesis of Amino Acids R544−D567 and Double Mutant D369A/R544Q in the α_1-Subunit of Pig Kidney Na,K-ATPase[a]

allele	DNA sequence of the coding strand of primers (5′−3′)
D369A	CAT CTG CTC AGC CAA AAC CGG
R544K	GGC CTC GGG GAA AAG GTG CTG GGT TTC
R544Q	GGC CTC GGG GAA CAG GTG CTG GGT TTC
C549A	GCT GGG TTT CGC CCA CCT TTT C
C549S	GCT GGG TTT CTC CCA CCT TTT C
D555E	CTT TTC CTG CCG GAA GAG CAG TTC C
D555N	CTT TTC CTG CCG AAC GAG CAG TTC
E556D	CTG CCG GAC GAC CAG TTC CCC G
E556Q	CCT GCC GGA CCA ACA GTT CCC CG
D565E	CTT CCA GTT TGA AAC CGA CGA TG
D565N	CTT CCA GTT TAA CAC CGA CGA TG
D567E	GTT TGA CAC CGA AGA TGT GAA TTT C
D567N	GTT TGA CAC CAA CGA TGT GAA TTT C

[a] The altered nucleotides are underlined.

Isolation of crude membranes, western blotting, and protein analysis were performed as described previously (*17*).

Membrane Preparation and Assays. Treatment of membranes with SDS, equilibrium binding of ouabain, or assay of Na,K-ATPase activity was performed as described earlier (*17*, *19*). Inhibitors of proteolysis [leupeptin (1 μg/mL), chymostatin (1 μg/mL), pepstatin (1 μg/mL), and PMSF (1 mM)] were added during all assays (*17*).

Equilibrium [^{3}H]ATP Binding. Aliquots containing 300−500 μg of membrane protein were incubated on ice for 30 min with 5 mM MOPS−Tris, pH 7.2, 10 mM EDTA−Tris, and [^{3}H]ATP (Amersham, specific activity 36 Ci/mmol) to a final concentration of 6−21 nM plus an increasing amount of cold Tris−ATP resulting in final concentrations of ATP of 6−200 nM. Either 10 mM NaCl or 10 mM KCl was added to allow specific binding to be calculated as binding in the presence of NaCl minus binding in the presence of KCl (*7*, *19*). To prevent proteolysis protease inhibitors were added to a concentration of 1 mM PMSF, 1 μg/mL chymostatin, 1 μg/mL pepstatin, and 1 μg/mL leupeptin. Bound and unbound [^{3}H]ATP were separated by centrifugation at 265000*g* for 30 min at 4 °C. The supernatant was discarded, and the pellet was resuspended for measurement of bound [^{3}H]ATP by scintillation counting and determination of protein content.

Na^+-Dependent Phosphorylation as a Function of MgATP. Aliquots of 200−300 μg of TDS-treated wild-type yeast membranes were incubated for 10 min at 20 °C in Eppendorf tubes containing 190 μL of 3 mM $MgCl_2$, 0.2 mM EDTA, 30 μM oligomycin, 10 mM NaCl or KCl, 20 mM TES−Tris, pH 7.5, and protease inhibitors (*7*). Phosphorylation was started by addition of 10 μL of [γ-^{32}P]ATP to final concentrations from 1 nM to 5 μM, and the phospho-enzyme was isolated and counted as described before (*7*).

RESULTS

Expression in Yeast of Mutations of Arg^{544}, Cys^{549}, Asp^{555}, Glu^{556}, Asp^{565}, and Asp^{567} in the α-Subunit of Na,K-ATPase. The conservative mutations are listed in Table 1, and in Table 2 the expression levels are estimated by ouabain binding at equilibrium, since it is known that one αβ unit of yeast Na,K-ATPase binds one molecule of ouabain at saturation (*17*).

Table 2: Capacities and Affinities for Ouabain Binding and Na,K-ATPase Activities after Substitutions of R544, C549, D555, E556, D565, and D567[a]

	ouabain (pmol/mg of protein)	ouabain K_d (nM)	Na,K-ATPase activity (%)
R544K	5.0 ± 0.1	6.0 ± 0.6	30 ± 8
R544Q	10.4 ± 0.5	8 ± 2	0
C549A	4.5 ± 0.5	4 ± 2	49 ± 3
C549S	9.3 ± 1	8 ± 2	84 ± 10
D555N	9.1 ± 0.7	8 ± 2	38
D555E	8.3 ± 0.2	6.6 ± 0.5	84
E556D	8 ± 1	8 ± 4	72
E556Q	3.7 ± 0.2	6 ± 1	45
D565E	10 ± 1	4 ± 1	84
D565N	5.4 ± 0.6	7 ± 3	38
D567E	13.8 ± 0.6	11 ± 2	75
D567N	7.3 ± 0.6	6 ± 2	43
WT	8.4 ± 0.5	5 ± 1	100 ± 17

[a] [^{3}H]Ouabain binding was performed as described in Experimental Procedures using aliquots of yeast membranes containing 200 μg of protein. Maximal capacities and dissociation constants were calculated as described in the legend to Figure 2. Measurements of Na,K-ATPase activities were made on 20 μg of yeast membranes incubated with or without 1 mM ouabain, and specific activity was determined as ouabain-inhibitable release of P_i as described before (*7*). Activities of R544K, R544Q, C549A, C549S, and WT are given as the mean ± SEM for n = 4−6, while the remaining activities are average values of two independent assays.

The extrapolated capacities for ouabain binding varied from 4 to14 pmol/mg of protein, in the same range as the concentrations of α-subunit protein as estimated from western blotting (not shown). The affinities for ouabain (4−11 nM) in the presence of vanadate and Mg^{2+} were close to the range of wild-type Na,K-ATPase from yeast (5 ± 1 nM). As previously observed, expression of these mutations to residues in the cytoplasmic protrusion at 30 °C caused folding problems and elicited the unfolded protein response and proteolytic degradation, while the protein accumulation approached wild-type levels at 15 °C (*18*). The properties of wild-type pig Na,K-ATPase from yeast cells were the same whether the fermentation was processed at 30 or at 15 °C (*18*). As an intrinsic control of this procedure of protein expression, data in Table 2 show that the temperature-sensitive mutations Cys[549]Ser or Cys[549]Ala were expressed at high levels at 15 °C with wild-type affinities for binding of ouabain or ATP and Na,K-ATPase activities.

Substitution of Arg[544] with Lys reduced Na,K-ATPase activity to 30%, and all Na,K-ATPase activity was lost after substitution for Gln. Substitution of carboxylate-containing residues in the segment Arg[544]−Asp[567] caused more moderate reduction of Na,K-ATPase activity to 38−84% of wild-type levels (Table 2).

Consequences for Free ATP Binding of Single or Double Mutations of Arg[544] and Asp[369]. Substitution of Arg[544] with lysine did not interfere significantly with the binding of free ATP (Figure 2 and Table 4). As estimated in terms of ATP:ouabain binding ratio, the data for Arg[544]Lys are close to those of wild type (Figure 2 and Table 4). In contrast, replacement of Arg[544] with Gln without a basic group abolished ATP binding in the range up to 200 nM free ATP, whereas the affinity for ouabain binding remained close to that of wild type.

To estimate the contribution of Arg[544] to the change in Gibbs free energy of ATP binding, the substitution with Gln was combined with mutation of Asp[369]Ala. Replacement

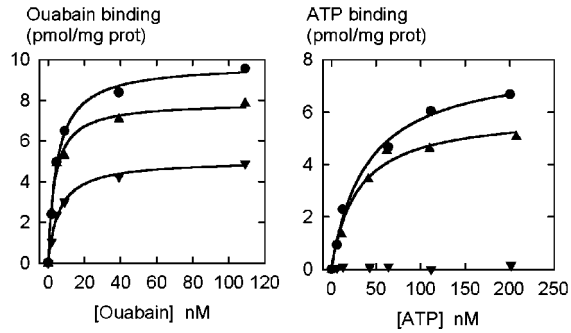

FIGURE 2: Effect of substitutions R544K (▲) and R544Q (▼) on the concentration dependence of ouabain binding (left panel) and ATP binding (right panel) compared to wild type (●). For ouabain binding aliquots containing 200 μg of yeast membrane protein were incubated for 60 min at 37 °C with 3 mM $MgSO_4$, 3 mM Tris−vanadate, 1 mM EGTA, and 10 mM MOPS−Tris, pH 7.2, in the presence of [^{3}H]ouabain and cold ouabain to final concentrations of 1−100 nM before separation of bound and unbound ouabain by centrifugation. Curves were fitted by a nonlinear least-squares regression using the equation: ouabain binding (pmol/mg) = a[oua]/(c + [oua]) in which a is the maximal capacity for binding and c is the dissociation constant, K_D. For ATP-binding aliquots of 300−500 μg of yeast membrane protein were incubated for 30 min on ice with 10 mM EDTA, 5 mM MOPS−Tris, pH 7.2, [^{3}H]ATP (Amersham), and Tris−ATP to final concentrations of 6−200 nM in the presence of either 10 mM NaCl or 10 mM KCl before separation of bound and unbound ATP as described in Experimental Procedures. Specific binding was calculated as the difference between binding in NaCl and KCl. Curves were fitted using a nonlinear least-squares regression by using the equation: specific ATP binding (pmol/mg of protein) = a[ATP]/(c + [ATP]) in which a is the maximum ATP binding and c is the dissociation constant, K_D. The estimated binding capacities and dissociation constants are shown in Tables 2 and 4.

Table 3: Consequences of Mutations of C549, D555, E556, D565, and D567 for Maximal Capacities and Dissociation Constants for ATP[a]

	ATP binding (pmol/mg of protein)	K_D(ATP) (nM)	$\Delta\Delta G_b$ (kJ/mol)
C549A	3.3 ± 0.3	27 ± 8	0.04
C549S	6.8 ± 0.5	37 ± 8	0.76
D555N	8.9 ± 1.4	138 ± 40	3.1
D555E	5.1 ± 0.4	55 ± 12	2.1
E556D	5.4 ± 0.8	79 ± 25	2.7
E556Q	1.7 ± 0.1	21 ± 6	0.5
D565E	5.4 ± 0.2	73 ± 6	3.0
D565N	3.4 ± 0.1	52 ± 6	1.9
D567E	9.3 ± 0.6	49 ± 8	1.6
D567N	7.0 ± 0.6	95 ± 17	2.3
WT	7.4 ± 0.6	32 ± 5	0

[a] [^{3}H]ATP binding assays were performed as described in Experimental Procedures. Alterations in Gibbs free energy ($\Delta\Delta G_b$) were calculated from the K_D(ATP) values using the equation of Fersht et al. (*26*): $\Delta\Delta G_b = -RT \ln[(\text{ATP:ouabain ratio}/K_{0.5})\text{mut}/(\text{ATP:ouabain ratio}/K_{0.5})\text{WT}]$. The ATP:ouabain ratio is the capacity for binding of ATP divided by the capacity for binding of ouabain.

of the transiently phosphorylated Asp[369] with Ala is known to increase ATP binding affinity 20−30-fold (*7*, *19*). This is also apparent in the experiment in Figure 3, where the K_D for ATP binding was 1.5 ± 0.2 nM for Asp[369]Ala as compared with 39 ± 7 nM for wild type. The K_D for ATP binding for the double mutation of Arg[544]Gln plus Asp[369]Ala was 28 ± 6 nM or about 19-fold higher than for Asp[369]Ala (Table 4). From these data the contribution of the basic groups of Arg[544] to the binding of free ATP was estimated to be $\Delta\Delta G_b$ = 6.85 kJ/mol (Table 4).

Table 4: Dissociation Constants for ATP and ADP for Mutations R544K and D369A/R544Q Compared to Wild Type and D369A[a]

	ATP:ouabain ratio	K_D(ATP) (nM)	$\Delta\Delta G_b$ (kJ/mol)	$K_{0.5}$(ADP) (μM)	K_D(ADP) (μM)
WT	0.88 ± 0.06	32 ± 5	0	0.10 ± 0.02	0.071
R544K	0.75 ± 0.04	29 ± 7	0.14	1.7 ± 0.5	1.26
R544Q	0.02	nd	nd	nd	nd
D369A	0.9 ± 0.1	1.5 ± 0.2	0	0.94 ± 0.08	0.12
D369A/ R544Q	0.86 ± 0.06	28 ± 6	6.85	1.0 ± 0.3	0.74

[a] Values are calculated from curves in Figures 3−5 as described in the legends. Alterations in Gibbs free energy ($\Delta\Delta G_b$) were calculated from the K_D(ATP) values as in Table 3. Values of K_D(ADP) were estimated as before (*19*) from the equation: $K_{ADP} = K_{1/2}(ADP)K_{ATP}/(K_{ATP} + [ATP])$.

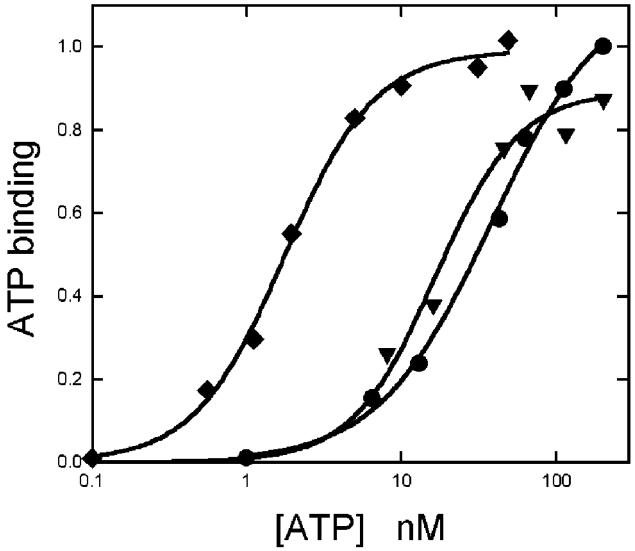

FIGURE 3: Effect of single and double mutation D369A (◆) or D369A/R544Q (▼) on the concentration dependence of ATP binding compared to wild type (●). Binding assays were performed as described in the legend to Figure 2, and estimated binding data are given in Table 4.

Consequences of Substitutions of Arg^{544} for Binding of MgATP and Phosphorylation. Binding of Mg^{2+} to Asp^{710} (*7*) and other negatively charged residues on the surface of the P-domain is expected to reduce electrostatic repulsion of the phosphate groups and thus facilitate binding of MgATP. Binding of MgATP cannot be determined in equilibrium conditions since the γ-phosphate is rapidly transferred to the Asp^{369} residue at high rates, e.g., 190 s^{-1} at 21 °C (*25*) in the phosphorylation reaction:

$$MgATP + E \leftrightarrow MgEATP \rightarrow MgE_1P + ADP$$

To monitor binding, the phosphorylated intermediate can be trapped in the MgE_1P[3Na] complex with oligomycin, which prevents transition to the E_2P conformation. From Figure 4 it is seen that $K_{1/2}$ values as low as 12 nM ATP in medium with 3 mM Mg^{2+} can be obtained when the protein concentrations of yeast or pig kidney wild-type Na,K-ATPase are kept at 3−5 nM, as estimated from the ouabain-binding capacity (*17*). The $K_{1/2}$ value of 12 nM ATP is at least 3-fold lower than the dissociation constant, $K_D = 38 \pm 5$ nM (Figure 4) for binding of free ATP at equilibrium in absence of Mg^{2+}. Binding of Mg^{2+} may therefore increase the apparent affinity of wild-type Na,K-ATPase for ATP. The

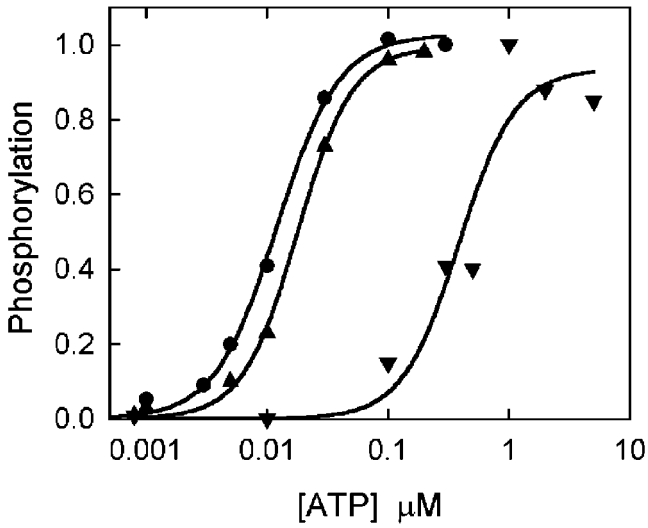

FIGURE 4: Effect of mutations of Arg^{544} on MgATP dependence of phosphorylation in the presence of oligomycin to stabilize the E_1P form. Phosphorylation was done as described in Experimental Procedures, and the phosphoenzyme was isolated and counted as described before (*7*). The lines were fitted, and $[ATP]_{1/2}$ values were estimated from the equation: phosphorylation = $a[ATP]^n/(c^n + [ATP]^n)$ in which a is the maximum phosphorylation level, c is $K_{0.5(ATP)}$, and n is the Hill coefficient. The $[ATP]_{1/2}$ value for wild type was 12.2 ± 0.7 nM (●); for Arg^{544}Lys (▲), 18.0 ± 0.8 nM; and for Arg^{544}Gln(▼), 389 ± 105 nM.

$K_{1/2}$ value of 12 nM ATP for wild type is much lower than previous estimates of the apparent affinities for MgATP for phosphorylation (*25*), but these assays were conducted at much higher concentrations (>1 μM) of Na,K-ATPase protein. The substitution of Arg^{544} by lysine had only minor effects on the apparent affinity for MgATP, whereas substitution by glutamine caused a 30-fold increase of the $K_{1/2}$ value for binding of MgATP. This corresponds to a change in free energy of binding of MgATP of 7.8 kJ/mol. This value is close to that (6.85 kJ/mol) obtained from the double mutant analysis in Table 4, suggesting that Arg^{544} has as important function in high-affinity binding of ATP in the Na^+-bound E_1 form of Na,K-ATPase.

Consequences for ADP Binding of Single or Double Mutations of Arg^{544} and Asp^{369}. One approach to examine if Arg^{544} interacts with the γ-phosphate or with α- or β-phosphate moieties of ATP is to determine the binding constants for ADP. From the curves of ADP displacement of ATP in Figure 5, the binding constants for ADP (K_{ADP}) were estimated as in Table 4.

As previously observed (*19*), the Asp^{369}Ala mutation caused only a minor change of K_{ADP} while the double mutation with Arg^{544}Gln increased K_{ADP} 6-fold. The Arg^{544}-Lys mutation consistently had a larger effect on K_{ADP} than on K_{ATP}, suggesting that the lysine provides sufficient binding energy for the interaction between enzyme and ATP, while it may not be capable of interacting with ADP.

Consequences for ATP Binding of Conservative Mutations of Cys^{549}, Asp^{555}, Glu^{556}, Asp^{565}, and Asp^{567}. In the segment Cys^{549}−Asp^{567}, the mutations of Cys^{549} by Ala or Ser had properties similar to those of wild type. Significant reductions of ATP affinities by 3−4-fold were seen after conservative substitutions of negatively charged groups, Asp^{555} or Asp^{565}, with Asn or Glu. As seen from Table 2, the affinities for ouabain binding of these mutations were close to those for

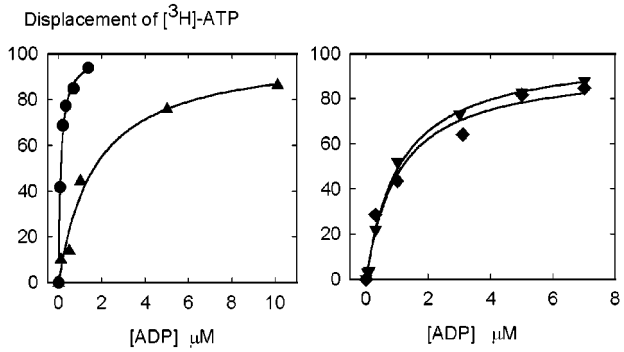

FIGURE 5: ADP displacement of [^{3}H]ATP binding at equilibrium to membranes of wild type (●) and R544K(▲), D369A (◆), and D369A/R544Q (▼). The displacement assay was performed as described in Experimental Procedures using 300−400 μg of yeast membrane protein and a [^{3}H]ATP concentration of 13 nM. Lines were fitted, and the dissociation constant for ADP, K_{ADP}, was estimated by nonlinear least-squares analysis using the equation: $K_{ADP} = [ADP]_{1/2}(K_{ATP}/(K_{ATP} + [ATP]))$, where $[ADP]_{1/2}$ is the concentration of ADP needed to displace half of the initially bound ATP, K_{ATP} is the dissociation constant for ATP (Table 4), and [ATP] is the concentration of ATP used during the assay. Estimated values of K_{ADP} are shown in Table 4.

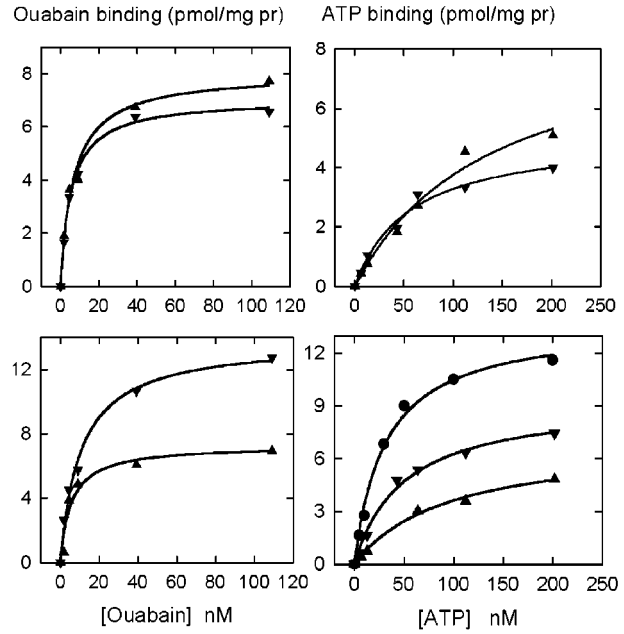

FIGURE 6: Effect of substitutions D555N (▲) and D555E (▼) (upper panels) or D567N (▲) and D567E (▼) (lower panels) on the concentration dependence of ouabain binding (left panels) or ATP binding (right panels) compared to wild type (●). The same procedure as in Figure 2 was used. Estimated values are given in Tables 2 and 3.

wild type. The largest change of ATP affinity was seen for the Asp[555]Asn mutation with K_D for ATP = 138 nM corresponding to an increase of 3.1 kJ/mol of the Gibbs free energy of ATP binding (Table 3). This residue is positioned 11 residues downward from Arg[544]. Mutation of the carboxylate groups of Asp[565] and Asp[567] also caused only moderate changes of K_D for ATP corresponding to contributions of 2−3 kJ/mol to the free energy of free ATP binding (Table 3).

DISCUSSION

The substitution of Arg[544] with Gln in the α-subunit abolished high-affinity binding of free ATP and the Na,K-ATPase activity. The ability to transfer phosphate from ATP to the protein was preserved but with 30-fold reduction of the apparent affinity for MgATP. This can explain the loss of Na,K-ATPase activity as the large reduction of the affinity may prevent ATP binding in the presence of K^+ ions. These defects were not due to inadequate expression of the protein in yeast, since both western blot analysis and ouabain binding showed that the α-subunit protein of the Arg[544]Gln mutation was expressed at levels similar to those of wild type. The preservation of high-affinity ouabain binding also shows that the mutant protein interacts with Mg^{2+} and vanadate. These substitutions thus interfere selectively with the binding of ATP, and Arg[544] is essential for high-affinity ATP binding in the range up to 200 nM ATP and for completion of the Na,K-ATPase reaction cycle. The free energy of ATP binding as estimated from the binding affinity of wild type (K_D = 39 nM) is 39 kJ/mol, suggesting that a number of residues contribute to coordination of ATP in the high-affinity site of the E_1 conformation of the protein.

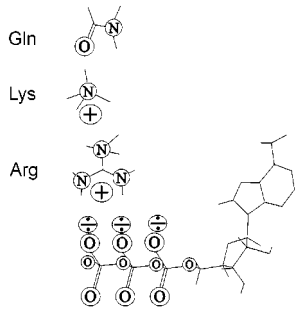

FIGURE 7: Potential interactions of the β- and γ-phosphates of ATP with the guanido group of wild-type Arg[544], the amino group of mutant Lys[544], or the carboxamide group of mutant Gln[544]. Only the parts of the amino acid side chains that are relevant for interactions with ATP are shown.

The large changes in affinity for ADP after substitutions with Lys or Gln exclude the possibility that Arg[544] interacts exclusively with the γ-phosphate of ATP as observed for Arg[182] in the β-subunit of F_1-ATPase of *Escherichia coli* (*27*). Instead, the relative changes of the affinities for ADP and ATP (Table 4) suggest that the single positive charge of the guanido group of Arg[544] is shared between the negative charges at the γ- and β-phosphates. The 15-fold decrease in ADP affinity of the Arg[544]Lys mutation without changes of the ATP affinity (Table 4) could reflect that substitution of arginine with lysine localizes the positive charge to the ϵ-amino group and restricts the interactions of this amino group to a single phosphate, the γ-phosphate. In contrast, introduction of a glutamine residue removes the positive charge and introduces an amino group and a carbonyl group. Hydrogen bonding between the amino group and the β-phosphate and repulsion between the carbonyl group and the γ-phosphate could explain the observed 19-fold reduction in the affinity for ATP and the more modest 6-fold reduction in ADP affinity of the Arg[544]Gln mutation. From these considerations, the mutagenesis data suggest that Arg[544] interacts with the γ- and β-phosphates of ATP and with the β-phosphate of ADP.

The identification of Arg[544] as important for ATP binding agrees with previous chemical labeling data demonstrating that Na,K-ATPase (*28*) and H,K-ATPase (*29*) are inactivated by specific modification of arginine residues. In adenylate

kinase, the guanido group of Arg^{107} is engaged in stabilization of the negative charges of the α- and β-phosphates of ATP (*20*). In ADP/ATP translocase several arginine residues are essential for oxidative phosphorylation in yeast (*30*).

The N-domain is separated from the P-domain by about 20 Å in the high-resolution structure of Ca-ATPase (*6*), while there is evidence that the gap between the N-domain and P-domain is closed in the ATP−E_1[3Na] form of the α-subunit of Na,K-ATPase, even in the absence of Mg^{2+} (*7*, *19*). In Na,K-ATPase, binding of free ATP in the absence of Mg^{2+} elicits strong electrostatic repulsion between the γ-phosphate and the negative charges at the surface of the P-domain (*7*, *19*). Removal of the charge of the carboxylate groups in the Asp^{369}Ala mutation increases the affinity for ATP 30−40-fold (*19*), and substitution of Asp^{710} for Ala in the P-domain of Na,K-ATPase causes a more moderate 2−3-fold increase of the ATP affinity (*7*). The strong electrostatic repulsion indicates that the γ-phosphate of the tightly bound free ATP approaches the surface of the P-domain in Na,K-ATPase as discussed before (*7*). Considering the positions of Arg^{544}, Asp^{369}, and Asp^{710} in the structure model of Figure 1, it is therefore reasonable to assume that the interactions of Arg^{544} with the phosphate moieties of ATP can be important for aligning the γ-phosphate for interaction with the phosphorylated residue, Asp^{369}. The phosphorylation experiments suggest that the apparent affinity for MgATP may be higher than for free ATP. Coordination of Mg^{2+} to the phosphate groups of ATP and to Asp^{710} (*7*) and other negatively charged groups in the surface of the P-domain may therefore reduce the electrostatic repulsion and thus facilitate ATP binding and phosphoryl transfer.

Mutation of the negatively charged carboxylates further down the sequence, Asp^{555}, Glu^{556}, Asp^{565}, and Asp^{567}, revealed more moderate contributions of 0.5−3 kJ/mol to the change in free energy of binding of free ATP. Mutations of Cys^{549} did not interfere with high-affinity ATP binding, and the substitution for Ala caused only a moderate depression of Na,K-ATPase activity, in agreement with previous data (*31*). It is therefore unlikely that Cys^{549} contributes directly to coordination of high-affinity ATP binding. Cys^{549} has been identified as the site of covalent attachment of erythrosin isothiocyanate at neutral pH (*32*), and insertion of this compound interferes with ATP binding. These data are not in conflict with our observations since it is probable that insertion of a large fluorophore group at Cys^{549} may interfere with the folding of this amino acid segment or with the function of the neighboring basic guanido group of Arg^{544} in coordination of ATP.

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