Replacement of Several Single Amino Acid Side Chains Exposed to the Inside of the ATP-binding Pocket Induces Different Extents of Affinity Change in the High and Low Affinity ATP-binding Sites of Rat Na/K-ATPase

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To investigate the relationship between the high and the low affinity ATP-binding site, which appears during the Na\(^+/K\(^+\)/ATPase reaction, four amino acids were mutated, the side chains of which are exposed to inside of the ATP-binding pocket. Six mutants, F475Y, K480A, K480E, K501A, K501E, and R544A, where the numbers correspond to the pig Na\(^+/K\(^+\)/ATPase \(\alpha\)-chain, were expressed in HeLa cells. The apparent affinities were determined by high affinity ATP-dependent phosphorylation and by the low affinity activation of Na\(^+/K\(^+\)/ATPase or low affinity ATP inhibition of K\(^-\)-para-nitropheophenylphosphatase (pNPPase). For the mutants K480A and K501A, little affinity change was detected for either the high affinity or the low affinity effect. In contrast, the other four mutants reduced both apparent affinities. Strikingly, R544A had a 30-fold greater effect on the high affinity ATP site than the low affinity site. For the F475Y mutant, it is likely that there was a greater effect on the low affinity site than the high affinity site, but for both F475Y and K480E the affinity for the low affinity ATP effect was reduced so much that it was not possible to estimate a \(K_{a,5}\). However, both the affinities for the K480E were reduced to \(~1/20\). The turnover number of the Na\(^+/K\(^+\)/ATPase and the apparent affinity for Na\(^+\) and pNPP was reduced slightly or not at all for these mutants, but the turnover number of K\(^-\)pNPPase and the apparent affinity for K\(^+\) were increased. These and other data suggest the presence of only one ATP-binding site, which can change its conformation to accept ATP with a high and low affinity. The requirement of Arg-544 and possibly Lys-501 is more important in forming a high affinity ATP-binding conformation, and Phe-475 and possibly Lys-480 are more important in forming the low affinity ATP binding conformation.

Na\(^+/K\(^+\)/ATPase is an integral membrane protein consisting of \(\alpha\) and \(\beta\) subunits and couples ATP hydrolysis to the active transport three Na\(^+\) and two K\(^+\) ions against their electrochemical gradients. Thus it maintains the normal high level of K\(^+\) and low level of Na\(^+\) inside mammalian cells. This enzyme belongs to the P-type ATPase group that includes gastric H\(^+\)\/-K\(^+\)/ATPase and sarcoplasmic Ca\(^2+\)/H\(^+\)/ATPase. The P-type ATPase forms an acid-stable phosphoenzyme (EP)\(^1\) during ATP hydrolysis (1–4). It is generally accepted that at least two different ATP-binding sites are present in the P-type ATPase, namely the \(E_1\) and \(E_2\) sites. The \(E_1\) form has a high affinity ATP-binding site (\(K_{a,5}\) is submicromolar) and is a precursor to ADP-sensitive phosphoenzyme (EP\(^1\)P) formation. The \(E_2\) has a low affinity ATP-binding site (\(K_{a,5}\) is submillimolar), causing the high \(V_{\text{max}}\) and playing a regulatory role in accelerating the partial reactions (1–4). The issue of whether two different ATP-binding sites reside on the same Na\(^+/K\(^+\)/ATPase \(\alpha\) subunit simultaneously or are on a different \(\alpha\) subunit with different conformation states remains controversial (1–7).

The x-ray crystallography (8) revealed that the cytoplasmic head piece of the Ca\(^2+\)/H\(^+\)/ATPase consists of an actuator, a phosphorylation, and a nucleotide domain containing an ATP-binding pocket. The liberation of the actuator domain allows it to induce closure of the gap between the phosphorylation domain containing a phosphorylatable Asp residue and the nucleotide domain containing residues such as Phe-487, Lys-492, Lys-515, and Arg-560 (Phe-475, Lys-480, Lys-501, and Arg-544 in pig Na/K-ATPase). Chemical modification (9–15) or mutagenesis of these residues (16–18) caused a reduction in ATPase activity. However, it has been clearly shown that the high affinity ATP binding to the Na\(^+\)-bound enzyme in the presence of Mg\(^2+\) induced a reversible pyridoxal fluorescence change at position Lys-480 without any ATP-dependent EP formation (10, 12–15). The low affinity ATP binding in the presence of Mg\(^2+\) + Na\(^+\) to the K\(^+\)-bound enzyme also induced a dynamic pyridoxal fluorescence change at Lys-480 and a fluorescein probe at Lys-501 (15). These data can be explained by assuming that the ATP-binding pocket is sufficiently flexible to accept ATP with a high and low affinity despite the presence of these bulky fluorescence probes that are used to sense high and low affinity ATP binding but completely inhibit ATP-dependent phosphorylation. The other possibility is that another different ATP binding in the same \(\alpha\)-chain (19) but not at the ATP-binding pocket (8) induced a conformational change that is detected indirectly by these probes in the ATP-binding pocket. An ATP molecule in the binding pocket appears to be ligated to some of the amino acid side chains exposed to the binding pocket (8, 20). These accumulated data lead us to test, for the first time, whether one single mutation in a side chain

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‡The abbreviations used are: EP, phosphoenzyme; pNPP, para-nitrophenyl phosphate.
exposed to the ATP-binding pocket affects both affinity sites for direct ATP binding with a high and low affinity or whether it affects either affinity site. It is important to understand the relationship between high and low affinity ATP binding for developing a better understanding of the mechanism by which Na⁺/K⁺-ATPase operates.

To investigate these points, 10 mutants carrying a single amino acid substitution of the side chain that possibly serve as ligands for ATP in the ATP-binding pocket were constructed. To aid the reader in following these experiments, models of the ATP-binding pocket of the wild type and mutants of Na⁺-ATPase were fitted to Ca⁺²/H⁺-ATPase (8) by Swiss-Model (www.expasy.ch/spdbv/). The Phe-475 (green), Lys-480 (orange), Lys-501 (yellow), and Arg-544 (violet) and mutated residues are shown as a space-filling model, where the number of the residues corresponds to the pig Na⁺/K⁺-ATPase α-chain. In this study, where a single mutation induced an apparent affinity change in both the high and low affinity ATP effects, Na⁺, K⁺, and nPFP were estimated. The data show that each single amino acid substitution, such as F475Y, K480E, K501E, and R544A, reduced the apparent affinity of the high and low affinity ATP effects to different extents. The data suggest that the Arg-544 and possibly Lys-501 are more important for high affinity ATP binding, whereas the Phe-475 and possibly Lys-480 are more important for low affinity ATP binding.

This is the first molecular biological approach to show the importance of an exposed amino acid side chain ligated to ATP for the high and low affinity binding in the pocket. In other words, the ATP-binding pocket changes its conformational state to reflect the high and low affinity ATP binding that accompanies ATP hydrolysis. These data are consistent with the oligomeric nature of the enzyme (5–7), as obtained by molecular weight estimation (21) as well as electron microscopic observation (22), ligand binding stoichiometry during ATP hydrolysis (7, 22), cross-linking experiments (6, 23), the binding of suicide substrates (5), and restraint-based comparative modeling (20).

**Materials and Methods**

**Plasmids**—pGEM-NaK and pCDL-NaK containing the entire coding region of rat Na⁺/K⁺-ATPase α1 cDNA has been described previously (24). The DNA fragments encoding the rat Na⁺/K⁺-ATPase β subunit were amplified by the PCR using a pair of sense and antisense oligonucleotide primers: for sense, 5'-AGCAGCCTGGTTTTCCCTC-3', corresponding to the nucleotides 413–429; for antisense, 5'-GGTCCCAT-ACTGATGAC-3', corresponding to the nucleotides 1455–1471 (25). The PCR products were inserted into a pCR1 vector (Invitrogen) and transferred to the mammalian expression vector pCDL (pCDL-NaKβ). The sequence of the cDNA insert was confirmed by the dyeoxy-mediated chain termination method (26).

**Site-directed Mutagenesis**—Site-directed mutagenesis was performed by the megaprimer PCR method (27) using the following antisense oligonucleotide primers: for F475AF/F475BS/F475SY/F4755Y, 5'-GGT GGA GGA GGT GTG TC (A/C) GGG AAT CTC-3'; for K480AF/K480BS, 5'-GGT GGA GGA GGA GGT GTG TC (A/C) GGG AAT CTC-3'; for K501AR/K501E, 5'-GGT GGA GGA GGA GGT GTG TC (A/C) GGG AAT CTC-3'; for K501AR/K501E, 5'-GGT GGA GGA GGA GGT GTG TC (A/C) GGG AAT CTC-3'; for R544AR/R544E, 5'-GGT GGA GGA GGA GGT GTG TC (A/C) GGG AAT CTC-3'. The amplified cDNA fragment including the region encoding amino acid residues 414–515 (between AarHI and BamHI sites) or 515–684 (between BamHI and KpnI sites) was subcloned back into the wild type rat Na⁺/K⁺-ATPase α1 subunit cDNA in the pGEM vector. The mutations were verified by nucleotide sequencing. The entire coding region of the mutated rat α1 cDNA was transferred to the expression vector pCDL as described previously (24).

**HeLa Cells Expressed Rat Na⁺/K⁺-ATPase β Subunit**—HeLa cells were cotransfected with plasmid DNAs containing a 10:1 molar ratio of pCDL-NaKβ to the pKNH vector (28) carrying neomycin-resistant gene by the calcium phosphate precipitation method (29) with an CellPfect Transfection Kit (Amersham Biosciences), selected in 500 µg/ml G418, and several cell lines and tissue isolates were isolated. The total RNAs were isolated from each cell lines and treated with DNase I. A pair of PCR primers, 5'-GGT AGA AAT TCA TCT GG-3' and 5'-GGTCCCATACCTGATGAC-3' corresponding to the nucleotides 494–510 and 1455–1471 of rat β subunit cDNA were designed (24). These regions were the same DNA sequences found in both the rat and human β subunits (30). By using the ³²P-labeled sense primer, the 975- or 982-bp DNA fragment from rat or human cDNA was amplified and was found to contain three or four recognition sites for restriction enzyme PstI, respectively. After PstI treatment, the 692- or 415-bp DNA fragment derived from rat or human cDNA was radioactive. The ratio of the radioactivity of the 692-bp fragment to that of the 415-bp fragment indicates the expression level of rat Na⁺/K⁺-ATPase β subunit to the human one. The selected cell line (A6 cell line; the ratio was 1.23) expressed similar amounts of mRNA for the Na⁺/K⁺-ATPase β subunit to that for the endogenous β subunit.

**Transfection of HeLa Cells**—Transfection of HeLa cells expressing rat Na⁺/K⁺-ATPase α1 subunit—HeLa cells expressing the rat β subunit (A6 cell line) were cultured in a 35-mm dish in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Cells were transfected with 2 µg of plasmid DNA containing a wild type or mutant Na⁺/K⁺-ATPase α1 subunit cDNA with the GenePORTER transfection reagent (Gene Therpay Systems) and subjected to selection in 10 µg/ml ouabain. Ouabain-resistant cells were expanded into stable cell lines.

**Preparation of SDS-purified Membrane Vesicles**—Crude plasma membranes were prepared from HeLa cells (24) or pig kidney microsomes as described previously (31). Crude plasma membranes at 1 mg/ml were incubated with 0.15 mg/ml of SDS in a buffer containing 0.25 M sucrose, 10 mM dithiothreitol, and 10 mM Tris-HCl, pH 7.4, for 5 min at room temperature. The sample was loaded onto a stepwise sucrose density gradients consisting of 10 and 40% sucrose layers and centrifuged at 350,000 × g in a Beckman TLA-100.3 rotor for 20 min at 4 °C. The fractions at the 10.4% interface were collected and pooled. The pooled sample was diluted with 3 volumes of the sucrose buffer and centrifuged at 350,000 × g in a Beckman TLA-100.3 rotor for 10 min. The pellet was suspended in the sucrose buffer, and the protein concentration was estimated (32) with bovine serum albumin as a standard.

**Phosphoenzyme (EP) Formed from ATP**—SDS-treated membrane vesicles (20 µg) were incubated at 0 °C for 10 s in 100 µl of 16 mM NaCl, 0.43 mM MgCl₂, 25 mM imidazole HCl, pH 7.2, and various concentrations of [γ-³²P]-ATP. The reaction was stopped by adding 500 µl of an ice-cold 10% trichloroacetic acid solution containing 10 mM inorganic phosphate and 1 mM ATP. The samples were centrifuged at 15,000 × g for 10 min at 4 °C. The precipitates were washed with ice-cold 10% trichloroacetic acid solution by centrifugation. The resulting precipitates were washed with ice-cold water by centrifugation. The precipitates were dissolved in the sample buffer containing a trace amount of a bromphenol blue, 10% glycerol, 1% SDS, 5% β-mercaptoethanol, and 10 mM sodium phosphate buffer, pH 6.0, and subjected to SDS-PAGE at pH 6.0 (33). After drying the gels, the radioactivity of [γ-³²P] incorporated into the Na⁺/K⁺-ATPase α subunit was detected and quantitated with a BAS 2000 system (Fuji). In order to correct for losses due to hydrolysis...
of EP during the above procedure, the SDS-treated Na⁺/K⁺-ATPase preparation from pig kidney (20 μg) was incubated under the same conditions as described above. The final precipitates were dissolved in the sample buffer in the absence of bromphenol blue, and half of the aliquots was taken for estimation of the amount of EP by liquid scintillation counting, and the other aliquot (0.75 μg of protein) was subjected to SDS-PAGE and quantitated with a BAS system. The data were analyzed using nonlinear least squares regression (GraphPad Prism; GraphPad Software Inc.) by using the Michaelis-Menten equation: EP (pmol/mg) = EP\text{max} \left( ATP\left/K_{\text{M}}(\text{ATP}) \right) + \left( ATP\right) \right) in which ET\text{max} is the maximum amount of EP formed, and K_{\text{M}} is the concentration of ATP giving the half-maximum formation of EP.

Na⁺/K⁺-ATPase Activity—The ATPase activity of SDS-treated membrane vesicles from HeLa cells was measured at 37 °C for 30 min in a reaction medium containing 0.5–2 μg of enzyme protein, various concentrations of ATP, 40 mM NaCl, 16 mM KCl, 5 mM MgCl₂, 125 mM sucrose, 0.5 mM EDTA, and 20 mM Tris-HCl, pH 7.4, in the presence of 5 μM or 5 mM ouabain. The reactions were stopped by adding an equal volume of 12% SDS. The colorimetric determination of inorganic phosphate with ammonium molybdate complexes was performed (34). Rat Na⁺/K⁺-ATPase activity was determined as the difference between the activities in the presence of 5 μM and 5 mM ouabain. The activity was divided by the amount of EP\text{max} estimated as described above and expressed as 1. All determinations were performed in duplicate. The data were analyzed essentially the same manner as described above, where EP and EP\text{max} were, respectively, replaced with ATPase activity (a⁻¹) and V\text{max} (the maximum turnover number). Dependence on Na⁺ or K⁺ concentration of Na⁺/K⁺-ATPase activity was measured with 10 μM or 5 mM ouabain at 37 °C for 30 min in the presence of 5 mM MgCl₂, 4 mM ATP, and various concentrations of NaCl or KCl. The data were analyzed by nonlinear least squares regression by using the Hill equation: ATPase activity (a⁻¹) = V\text{max} \left( [Na]^{C} / K_{\text{Na}}^{C} + [Na]^{C} \right) + \left( [K]^{C} / K_{\text{K}}^{C} + [K]^{C} \right), in which V\text{max} is the maximum activity, n_{Hill} is the Hill coefficient, and K_{Hill} is the concentration of ion giving half-maximum ATPase activity.

Potassium-dependent pNPPase Activity — The pNPPase activity of the SDS-treated membrane vesicles was measured in duplicate in the presence or absence of 16 mM KCl at 37 °C for 30 min containing 1–2 μg of protein and various concentrations of pNPP. 6 mM MgCl₂, 125 mM sucrose, 0.5 mM EDTA, and 20 mM imidazole HCl (pH 7.2). The K⁺-pNPPase activity was the difference between the activity with and without KCl. The K⁺-pNPPase activity was also measured in the presence of 5 mM pNPP and various concentrations of ATP to estimate the K₉.₀.₅ for ATP by nonlinear least squares regression.

RESULTS

Construction of Stable Cell Lines Expressing the Rat Na⁺/K⁺-ATPase a Subunit Variant in HeLa Cells — The 10 expression plasmid vectors (pCDL-NaKs) carrying a single amino acid substitution at Phe-475, Lys-480, Lys-501, or Arg-544, namely substitution at Phe-475, Lys-480, Lys-501, or Arg-544, namely F475A/F475S/F475D/F475Y, K480A/K480E, K501A/K501E, K501A/K501E, and R544A/R544E, were constructed and transfected to HeLa cells expressing the rat wild type Na⁺/K⁺-ATPase. The amount of steady state ATPase activity, the amount of steady state of Na⁺/K⁺-ATPase in wild-type HeLa cells, was measured at 37 °C for 10 s in 100 μl of 0.43 mM MgCl₂, 25 mM imidazole HCl, pH 7.2, and 2 μM of [γ-³²P]ATP with (+Na⁺) or without (-Na⁺) 16 mM NaCl. The radioactivity of ³²P incorporated into the Na⁺/K⁺-ATPase α subunit was detected as described under “Materials and Methods.” TCA, trichloroacetic acid. The trichloroacetic acid solution was added before the addition of [γ-³²P]ATP. The radioactivity of ³²P incorporated into the Na⁺/K⁺-ATPase α subunit was detected and quantitated, and the data were subjected to curve fitting using a nonlinear least square regression. Details are described under “Materials and Methods” unless otherwise stated.

Effect of Each Substitution of Phe-475, Lys-480, Lys-501, and Arg-544 on the Apparent High Affinity ATP Binding Registered by EP Forming Activity. To estimate the apparent affinity for the high affinity ATP effect, Na⁺/K⁺-ATPase activity of the rat wild-type Na⁺/K⁺-ATPase was measured with increasing ATP concentrations up to 4 mM in the presence of 40 mM NaCl, 16 mM KCl, and 5 mM MgCl₂ and either 5 μM or 5 mM ouabain. The difference between these activities was assumed to be the Na⁺/K⁺-ATPase activity (V) of phosphorylated enzyme. The data were fitted to the Michaelis-Menten equation (Fig. 2), which gave the maximum amount of phosphoenzyme (EP\text{max}) and a half-maximum concentration of ATP (K_{0.₅}). The K_{0.₅} of the wild-type and the K480A were similar and low in value, a mean value ~0.026 μM, and that of K480E, K501A, and K501E increased, respectively, to 19-, 2-, and 9-fold. The value of F475Y and K544A increased to 5- and 59-fold (Table 1, line a, and Scheme 1), respectively.

Effect of Mutagenesis of ATP-binding Pocket of Na⁺,K⁺-ATPase

FIG. 1. ATP concentration dependence of Na⁺-dependent EP formation in wild type, F475Y, K480A, K480E, K501A, K501E, and R544A. A, SDS-treated membrane vesicles (20 μg) from pig kidney microsomes or the HeLa cell stably expressing the rat wild type Na⁺/K⁺-ATPase were incubated at 0 °C for 10 s in 100 μl of 0.43 mM MgCl₂, 25 mM imidazole HCl, pH 7.2, and 2 μM of [γ-³²P]ATP with (+Na⁺) or without (-Na⁺) 16 mM NaCl. The radioactivity of ³²P incorporated into the Na⁺/K⁺-ATPase α subunit was detected as described under “Materials and Methods.” TCA, trichloroacetic acid. The trichloroacetic acid solution was added before the addition of [γ-³²P]ATP. B and C, SDS-treated membrane vesicles from HeLa cells expressing the rat wild type (X), F475Y (), K480A (.), K480E (A), K501A (C), K501E (B), and R544A (W) were incubated at 0 °C for 10 s in the presence of various concentrations of [γ-³²P]ATP. The radioactivity of ³²P incorporated into the Na⁺/K⁺-ATPase α subunit was detected and quantitated, and the data were subjected to curve fitting using a nonlinear least square regression. Details are described under “Materials and Methods” unless otherwise stated.
Each parameter of the wild type enzyme obtained as described in Figs. 1 to 6 was taken as 1. The values shown in parentheses represent the original parameters obtained.

| Wild type | Relative values |
|-----------|-----------------|
| a $K_{0.5}$ (0.026 μM) | F475Y | K480A | K480E | K501A | K501E | R544A |
| b $K_{0.5}$ (0.69 mM) | 4.8 | 1.0 | 19 | 2.0 | 8.5 | 58.9 |
| c $V_{\text{max}}$ (241 s⁻¹) | 1.0 | 1.0 | 0.5 | 6.7 | 4.0 |
| d $E_{\text{max}}$ (0.36 mU) | 8.4 | 1.5 | 0.9 | 1.1 |
| e $K_{0.5}$ (1.9 mM) | 1.0 | 1.6 | 1.4 |
| f $V_{\text{max}}$ (108 s⁻¹) | 0.9 | 1.4 |
| g $K_{0.5}$ (7.5 mM) | 0.8 | 0.7 |
| h $K_{0.5}$ (0.67 mM) | 0.8 |
| i $K_{0.5}$ (0.089) | 1 |
| j $K_{0.5}$ (2.7 × 10⁻⁴) | 1.2 |
| k $K_{0.5}$ (1.3 × 10⁻⁴) | 1.7 |
| l $K_{0.5}$ (3.36) | 0.73 |
| m $K_{0.5}$ (0.19) | 0.97 |
| n $K_{0.5}$ (1.4 × 10⁻⁴) | 0.66 |

Table I
Comparison of the relative affinity and activity of mutants with those of wild type enzyme

Fig. 2. ATP concentration dependence of Na⁺/K⁺-ATPase activity. The ATPase activity of SDS-treated membrane vesicles of the rat wild type (×), F475Y (♦), K480A (○), K480E (▲), K501A (□), K501E (■), and R544A (▲) was measured at 37 °C for 30 min in a reaction medium containing 0.5–2 μg of enzyme protein, various concentrations of ATP, 16 mM NaCl, 5 mM MgCl₂, 125 mM sucrose, 0.5 mM EDTA, and 20 mM Tris-HCl, pH 7.4, in the presence of 5 μM or 5 mM ouabain, and the data were subjected to curve fitting, where 100% of Na/K-ATPase activity was the value of $V_{\text{max}}$ estimated from the fitted data. To compare the activity between the wild type and mutants, the ratio (V to P) estimated as described in Fig. 1 was taken, and the $P_{\text{max}}$ and $V_{\text{max}}$ values are shown with the $K_{0.5}$. $P_{\text{max}}$ represents the turnover number, which is shown in Table I.

Registered by $K^+$-pNPPase activity—The hydrolysis of pNPP in the presence of $K^+$ and $Mg^{2+}$ of Na⁺/K⁺-ATPase was assumed to occur in the $K^+$-occluded enzyme form (36) without the formation of $E_0P$ and $E_2P$. Mutants such as F475Y, K480E, K501E, and R544A showed a reduced affinity in both or either ATP binding (Table I, lines a–c). To test the relationship between a mutation in the $K^+$-binding pocket and $K^+$-pNPP hydrolysis, $p$NPPase activity was measured in the presence of 6 mM MgCl₂ and various concentrations of pNPP with and without 16 mM KCl. The $K^+$-pNPP activity (V) represents the difference in the activity in the presence and absence, respectively, of 16 mM KCl. The addition of 1 μM ouabain reduced the activity to the level found in the absence of K⁺ and suggests that the K⁺-pNPPase activity is due to a property unique to Na⁺/K⁺-ATPase. The data were fitted to Michaelis-Menten kinetics. Fig. 4 shows the plots of V against the concentration of pNPP, where $V_{\text{max}}$ as 100% and the turnover number, $V_{\text{max}}/P_{\text{max}}$, are also shown (Fig. 4). In this case, the value of $P_{\text{max}}$ was taken from data in Fig. 1. Both the turnover number, $V_{\text{max}}/P_{\text{max}}$, and the $K_{0.5}$...
were altered only slightly as a result of these mutations, i.e. the maximum changes were ~2-fold increases in R544A (Table I, lines e and f).

Effect of the Substitution on the Apparent Affinity for Na⁺ and K⁺ Regulated by Na⁺/K⁺-ATPase Activity.—To investigate the relationship between these mutations and the apparent affinity change for Na⁺ or K⁺, the Na⁺/K⁺-ATPase activity was measured in the presence of different concentrations of Na⁺ or K⁺ as described above except that 4 mM ATP was used. The values of $K_{0.5}$ for Na⁺ ($K_{0.5}^{Na}$) and the Hill coefficient ($n_H$) were estimated using nonlinear least square regression (Fig. 5). The $K_{0.5}^{Na}$ for the wild type was 7.5 mM, and the maximum decrease and increase were, respectively, observed in K480A (5.5 mM) and both K501E and R544A (10.5 mM). The data show that a change in the apparent affinity for Na⁺, if present, is rather small compared with the affinity change for the high and low affinity ATP-binding sites (Table I, compare lines a, b, and d). The $n_H$ of the wild type was 1.8 and that of K480A and R544A was 1.3 and 2.3, respectively, which represents the minimum and the maximum value (dotted lines in Fig. 5). The reasons for the decrease or the increase of $n_H$ in K480A or R544A, respectively, were not clear.

The dependence of ATPase activity on K⁺ concentration (Fig. 6 and Table I, line h) showed that the $K_{0.5}^{K⁺}$ for the wild type was 0.67 mM, which was the maximum, and all mutants had lower values. These data indicate that these mutants had a slightly increased apparent affinity for K⁺, i.e. the maximum increase was observed in R544A, namely 2.5 (1/0.4)-fold. The $n_H$ of wild type and mutants was ~1.5 except for the value of 1.9 for R544A.

**DISCUSSION**

Flexibility of the ATP-binding Pocket.—Each mutant, F475Y, K480A, K501E, and R544A, respectively, showed different extents of apparent decreased affinity between the high and low affinity ATP effects. Two mutants in which the cationic Lys side chain was replaced by a less bulky methyl group (K480A and K501A) had only a slight affinity changes for both ATP-binding sites (Scheme I).

The replacement of Phe-475 with Tyr, namely the addition of a phenolic hydroxyl group, reduced the apparent affinity for the high and low affinity ATP effects, respectively, to 1/5 and <1/6 or 1/8 without affecting the binding of the less bulky pNPP (Scheme I). Stable cell lines could not be obtained when Phe was replaced with Ala, Ser, and Asp, respectively.

Each replacement of a cationic side chain with a less bulky anionic side chain (K480E and K501E) showed a decreased affinity for both ATP-binding sites. Such a reduction did not occur in the case of replacement with a methyl side chain (K501A).

The replacement of a guanidinium moiety with a methyl side chain (R544A) showed a very strong reduction in affinity (1/59) for the high affinity ATP effect with a slight reduction in affinity for the low affinity ATP effect as well as pNPP binding (1/2) (Table I, lines a, b, and e). The replacement of the Arg with Glu side chain resulted in no appearance of the HeLa cells surviving in the presence of 10 μM ouabain. The importance of Arg-544 was also reflected by the fact that R544K and R544Q, respectively, showed only a small effect on the apparent affin-
it for MgATP with a 30% of Na+/K+-ATPase activity and a 1/30 reduction in the binding of MgATP with little activity (37). Thus the requirement of the Arg side chain (Scheme I) for the high affinity site is considerably more strict than the low affinity site (Table I, lines a, b, and d).

The F475Y, K480A/K480E, K501A/K501E, or R544A showed a slight increase in affinity for K+ with a small decrease in affinity for pNPP and Na+ (Table I, lines h, e, and g). When the ratios, K_0.5/K_Na^2, were calculated, the increase in K_0.5 affinity becomes more clear (Table I, line i) such as the case of K480E, K501E, and R544A, which showed reduced affinity for both ATP effects (Table I, lines a, b, and d). Such an apparent antagonism between ATP binding and K+ binding has been also reported for mutations in the cation-binding pocket of Na+/K+-ATPase which showed a decreased affinity for K+ and an increase in the apparent affinity for ATP (38–40).

The present data suggest the importance of the Phe-475, Lys-480, Lys-501, and Arg-544 in the construction of a flexible K501E, and R544A, which showed reduced affinity for both ATP binding. When the affinity ratio for the high affinity ATP binding showed a larger reduction in the apparent low affinity ATP both affinities, the extent of the high affinity effect decreased becomes the wild type enzyme was assumed to be 1, and the relative Ki affinity for MgATP with a 30% of Na+ 1/30 reduction in the binding of MgATP with little activity (37). These data suggest that the contribution of each side chain in the pocket also reported for mutations in the cation-binding pocket of Na+/K+-ATPase (8), has been also reported. It was also shown unequivocally that a similar reduction in ATP binding capacity occurred during the turnover of pig gastric H/K-ATPase, from 1 mol/mol of α-chain to the half to form EP-EATP and the resulting liberation of each 0.5 mol of P_i (45). In other words, ATP binding capacity decreases accompanying the turnover in both Na/K- and H/K-ATPase (22, 45).

Each single mutation, such as F475Y, K480E, K501E, and R544A, strongly reduced both the apparent ATP affinities as in the case of a single mutation of Phe-475 (corresponding to Phe-475 of the pig kidney enzyme), Arg-489, and Lys-492 in Ca^{2+}/H^{+}-ATPase, which has an ATP-binding pocket similar to Na+/K+-ATPase (18). It had been already reported that the modification of Lys-501 of Na+/K+-ATPase with N-(2-nitro-4-isothiocyanophenyl)-imidazole affected both the high and low affinity ATP effects (46).

These considerations suggest that each α-chain contains only one ATP-binding pocket. One accepts ATP with a high affinity to form EP and the other, in the adjacent α-chain, accepts ATP with a low affinity to accelerate Na+/K+-ATPase activity. The present finding is consistent with the view that Na+/K+-ATPase functions out of phase (5–7, 21, 23, 47, 48) as a dipo- tomer, (αβ)2, or a much higher oligomer, (αβ)3, as in the case of cross-talking gastric H+/K+-ATPase (45) and possibly Ca^{2+}/H^{+}-ATPase (49, 50).

REFERENCES
1. Glynn, I. M. (1985) in The Enzymes of Biological Membranes (Martonassi, A., ed.) Vol. pp. 35–114, Plenum Publishing Corp., New York.
2. Glynn, I. M., and Karlsh, S. J. D. (1990) Annu. Rev. Biochem. 59, 171–205.
3. Møller, J. V., Juun, B., and Maire, M. (1996) Biochim. Biophys. Acta 1268, 1–5.
4. McIntosh, D. H. (1998) Adv. Mol. Cell. Biol. 32A, 33–99.
5. Schöner, W., Thøges, D., Hamer, E., Antolovic, R., Buschau, E., Willeke, M., Serpsera, E. H., and Schéiner-Bois, G. (1994) in The Sodium Pump, Structure, Mechanism, Control and Its Role in Disease (Bamber, E., and Schöner, W., eds) pp. 332–341, Eisevier Science Publishers B.V., Amsterdam.
6. Taniguchi, K., Kaya, S., Abe, K., and Måråd, S. (2002) J. Biochem. (Tokyo) 129, 335–342.
7. Toyoshima, C., Nakasaki, M., Nemura, H., and Ogawa, H. (2000) Nature 405, 647–655.
8. Pedemonti, G. H., and Kaplan, J. H. (1990) Am. J. Physiol. 258, C1–C23.
9. Tran, C. M., Schinehmer-Bohs, G., Schöner, W., and Farley, R. A. (1993) Biochemistry 33, 4140–4147.
10. Kaya, S., Tsuda, T., Hagiwara, K., Fukui, T., and Taniguchi, K. (1994) J. Biol. Chem. 269, 7419–7422.
13. Tsuda, T., Kaya, S., Funatsu, H., Hayashi, Y., and Taniguchi, K. (1998) J. Biochem. (Tokyo) 124, 169–174
14. Tsuda, T., Kaya, S., Yokoyama, T., Hayashi, Y., and Taniguchi, K. (1998) J. Biol. Chem. 273, 24334–24338
15. Tsuda, T., Kaya, S., Yokoyama, T., Hayashi, Y., and Taniguchi, K. (1998) J. Biol. Chem. 273, 24339–24345
16. Wang, K., and Farley, R. A. (1992) J. Biol. Chem. 267, 3577–3580
17. Farley, R. A., Heart, E., Kabalin, M., Putnam, D., Wang, K., Kasho, V. N., and Faller, L. D. (1997) Biochemistry 36, 941–951
18. McIntosh, D. B., Woolley, D. G., Vilsen, B., and Andersen, J. P. (1996) J. Biol. Chem. 271, 25778–25789
19. Faller, L. D., Kasho, V. N., Smirnova, I., Lin, S.-H., and Farley, R. A., (2000) in Na/K-ATPase and Related ATPases (Taniguchi, K., and Kaya, S., eds) pp. 389–396, Elsevier Science Publishers B.V., Amsterdam, The Netherlands
20. Ettrich, R., Melicherek, M., Teisinger, J., Ettrichova, O., Schoner, W., and Amler, E. (2001) J. Mol. Model. 7, 184–192
21. Hayashi, Y., Kameyama, K., Kobayashi, T., Hagiwara, E., Shinji, N., and Takagi, T. (1997) Ann. N. Y. Acad. Sci. 834, 19–28
22. Yokoyama, T., Kaya, S., Abe, K., Taniguchi, K., Kato, T., Yazawa, M., Hayashi, Y., and Märdh, S. (1999) J. Biol. Chem. 274, 31792–31796
23. Ivanov, A. V., Modyanov, N. N., and Askari, A. (2002) Biochem. J. 364, 283–299
24. Imagawa, T., Shida, M., Matsuzawa, K., Kaya, S., and Taniguchi, K. (1998) Jpn. J. Pharmacol. 76, 415–423
25. Young, B. M., Shull, G. E., and Lingrel, J. B. (1987) J. Biol. Chem. 262, 4905–4910
26. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
27. Tao, B. Y., and Lee, R. C. P. (1994) in PCR Technology Current Innovations (Griffin, H. G., and Griffin, A. M., eds) pp. 69–83, CRC Press, Inc., Boca Raton, FL
28. Fukuda, K., Higashida, H., Kubo, T., Maeda, A., Akiba, I., Boje, H., Mishina, M., and Numa, S. (1988) Nature 335, 355–358
29. Graham, F. L., van der Eb, A. J. (1973) Virology 52, 456–467
30. Kawakami, K., Nojima, H., Ohta, T., and Nagano, K. (1986) Nucleic Acids Res. 14, 2635–26446
31. Hayashi, Y., Kimimura, M., Homareda, H., and Matsu, H. (1977) Biochim. Biophys. Acta 482, 185–196
32. Bradford, M (1976) Anal. Biochem. 72, 248–254
33. Weber, K., and Osborn, M. (1961) J. Biol. Chem. 244, 4406–4412
34. Chiflet, S., Torriglia, A., Chiesa, R., and Tolosa, S. (1988) Anal. Biochem. 168, 1–4
35. Taniguchi, K., Suzuki, K., and Iida, S. (1982) J. Biol. Chem. 257, 10159–10167
36. Post., R. L., Hegyvary, C., and Kume, S. (1972) J. Biol. Chem. 247, 6530–6540
37. Jacobsen, M. D., Pedersen, P. A., and Jørgensen, P. L. (2002) Biochemistry 41, 1451–1456
38. Arguello, J. M., and Lingrel, J. B (1995) J. Biol. Chem. 270, 22764–22771
39. Koster, J. C., Blanco, G., Mills, P. B., and Mercer, R. W. (1996) J. Biol. Chem. 271, 2413–2421
40. Blistern, R., Wilczynska, A., Karlis, S. J. D., Arguello, J. M., and Lingrel, J. B. (1997) J. Biol. Chem. 272, 2487–2493
41. Taniguchi, K., Tosa, H., Suzuki, K., and Kamo, Y. (1988) J. Biol. Chem. 263, 29343–29347
42. Yamazaki, A., Kaya, S., Tsuda, T., Araki, Y., Hayashi, Y., and Taniguchi, K. (1994) J. Biochem. (Tokyo) 116, 1360–1369
43. Taniguchi, K., and Märdh, S. (1983) J. Biol. Chem. 268, 15588–15594
44. Ward, D. G., and Caviers, J. D. (1996) J. Biol. Chem. 271, 12317–12321
45. Abe, K., Kaya, S., Imagawa, T., and Taniguchi, K. (2002) Biochemistry 41, 2438–2445
46. Ellis-Davies, G. C., and Kaplan, J. H. (1993) J. Biol. Chem. 268, 11622–11627
47. Linnertz, H., Urbanova, P., Obsil, T., Herman, P., Almer, E., and Schoner, W. (1998) J. Biol. Chem. 273, 28813–28821
48. Donnet, C., Arystarkhova, E., and Sweadner, K. (2001) J. Biol. Chem. 276, 7357–7365
49. Nakamura, S., Suzuki, H., and Kanazawa, T. (1997) J. Biol. Chem. 272, 6232–6237
50. Nakamura, J., Taji, G. A., Sato, C., Furukohri, T., and Konishi, K. (2002) J. Biol. Chem. 277, 24180–24190