J. Biochem. **140**, 599–607 (2006)
doi:10.1093/jb/mvj191

New Evidence for ATP Binding Induced Catalytic Subunit Interactions in Pig Kidney Na/K-ATPase

Kan Tanoue[1], Shunji Kaya[1], Yutaro Hayashi[2], Kazuhiro Abe[1], Toshiaki Imagawa[1], Kazuya Taniguchi[1,*] and Kazuyasu Sakaguchi[1]

[1]*Biological Chemistry, Division of Chemistry, Faculty of Science, Hokkaido University, Sapporo 060-0810; and* [2]*Department of Biochemistry, Kyorin University School of Medicine, Mitaka, Tokyo 181-8611*

Received September 4, 2006; accepted September 12, 2006

Pig kidney Na/K-ATPase preparations showed a positive cooperative effect for *p*NPP in Na-*p*NPPase activity. Measurements of the Na-*p*NPPase activity, Na-ATPase activity and the accumulation of phosphoenzyme (EP) under conditions of *p*NPP saturation showed several different ATP affinities. The presence of *p*NPP reduced both the maximum amount of EP and Na-ATPase activity to half showing a value of 4 and a 3,700-fold reduced ATP affinity for EP formation, and a 7 and 1,300-fold reduced affinity for Na-ATPase activity. The presence of low concentrations of ATP in the phosphorylation induced a 2-fold enhancement in Na-*p*NPPase activity despite a reduction in available *p*NPP sites. However, higher concentrations of ATP inhibited the Na-*p*NPPase activity and a much higher concentration of ATP increased both the phosphorylation and Na-ATPase activity to the maximum levels. The maximum Na-*p*NPPase activity was 1.7 and 3.4-fold higher without and with ATP, respectively, than the maximum Na-ATPase activity. These data and the *p*NPP dependent reduction in both Na-ATPase activity and the amount of enzyme bound ATP provide new evidence to show that ATP, *p*NPP and ATP with *p*NPP, respectively, induce different subunit interactions resulting a difference in the maximum Na^+-dependent catalytic activity in tetraprotomeric Na/K-ATPase.

Key words: Na/K-ATPase, membrane bound enzyme, oligomer, P-type ATPase, subunit interactions.

Abbreviations: *p*NPP, *p*-nitrophenyl phosphate; *p*NP, *p*-nitrophenol; AcP, acetyl phosphate; AMP-PCP, adenylyl (β, γ-methylene) diphosphonate; E1P, ADP-sensitive phosphoenzyme; E2P, K^+ sensitive phosphoenzyme; EATP, ATP-Na/K-ATPase complex; E*p*NPP, *p*NPP-Na/K-ATPase complex; EP:EATP, EP:E*p*NPP and EP:EP, oligomeric form of each enzyme state; E2P:EATP, ATP bound K-sensitive phosphoenzyme; TCA, trichloroacetic acid; Na-ATPase, ouabain sensitive Na^+-dependent ATPase; Na-*p*NPPase, ouabain sensitive Na^+-dependent *p*NPPase; $K_{0.5}^{ATP,ATPase}$, $K_{0.5}^{ATP,pNPPase}$ and $K_{0.5}^{ATP,EP}$, ATP concentration giving half maximum Na-ATPase activity, Na-*p*NPPase activity and EP formation, respectively; $K_{i,0.5}^{ATP,pNPPase}$, ATP concentration giving half maximum inhibition of Na-*p*NPPase activity.

Recent progress in the X-ray analysis of monomeric crystal structures of Ca-ATPase from the sarcoplasmic reticulum (*1–4*) and cDNA sequences of ion pumps (*5*, *6*) have contributed greatly to a better understanding of the mechanism of P-type ATPase explained by the Post-Albers scheme (*7–11*) or the E1/E2 model (*12*). In the case of Na/K-ATPase, 1 mol of ATP is hydrolyzed *via* the Na^+-bound enzyme (NaE1) that accepts ATP with a high affinity, the Na^+ occluded ADP-sensitive phosphoenzyme (E1P), the K^+ sensitive phosphoenzyme (E2P) in which a reduced affinity for Na^+ is retained and the K^+-occluded enzyme (KE2) accepts Na^+ and ATP with a low affinity to liberate K^+ to generate NaE1ATP. Thus, the active transport of 3 Na^+ occurs during the transition from E1P to E2P and 2 K^+ are transported during the transition from the KE2 to the NaE1ATP forms with the sequential appearance of the intermediates. Thus the high affinity ATP binding site disappears in the next cycle in the presence of high concentrations of ATP and the monomeric or protomeric forms of the cation pump are assumed to be sufficient for pump function, which has been taken to account in the dynamic atomic structure changes deduced from the monomeric crystal structures of sarcoplasmic reticulum Ca-ATPase (*1–4*). Data have been also accumulated to show that low affinity ATP binding modulates several steps in the catalytic cycle subsequent to phosphorylation in SR-Ca-ATPase (*12*, *13*) and even calcium binding (*14*). However there appear to be no extra low affinity ATP binding to E1P and E2P mimicked crystals (*4*).

Studies of chemical cross-linking (*15*, *16*) and dead end pseudo substrates (*17*), presteady state kinetics (*18*), electron microscopic observations (*19*) and the reactivity of the enzyme and a comparison of the extent of phosphorylation with ligand binding capacities in the presence or absence of ATP hydrolysis and others (see review, *20*) strongly suggest that the functional unit of Na/K-ATPase in the membrane is a diprotomer, $(\alpha\beta\gamma)_2$ or a tetraprotomer, $(\alpha\beta\gamma)_4$ (*15–20*). One of the most compelling pieces of enzymological evidence in favor of the oligomeric nature of Na/K-ATPase has been the finding of the simultaneous presence of EP:EATP during Na^+-dependent ATP hydrolysis with half site phosphorylation (*19*, *20*). Subsequently,

*To whom correspondence should be addressed. Tel: +81-11-706-2721, Fax: +81-11-706-4683, E-mail: ktan40@mvd.biglobe.ne.jp

the presence of EP:EATP in pig stomach H/K-ATPase was demonstrated more clearly (*21*), where l mol of P_i liberation from each 0.5 mol of EP and EATP in EP:EATP was detected in a cold ATP chase experiment. Quite recently, the presence of an active tetraprotomer of H/K-ATPase in solution (*22*, *23*) and the isolation of a soluble tetraprotomer of dog kidney Na/K-ATPase has been reported (*24*). However the role of the enzyme bound ATP in Na/K-ATPase remains to be explained. Mg^{2+} bound Na/K-ATPase catalyzes the hydrolysis of *p*NPP and AcP *via* E1P and E2P in the presence of Na^+ (*25*, *26*) and *via* KE2 in the presence of K^+ (*6*, *8*, *9*). *p*NPP also catalyzes a Na^+/Na^+ exchange in reconstituted liposomes (*27*). These data clearly indicate that Mg^{2+} and Na^+ bound Na/K-ATPase accepts *p*NPP (*27*) and E1P and E2P accumulate, as in the case of ATP (*7*–*10*) and AcP (*26*). If the catalytic unit of the enzyme is a protomer, the addition of increasing concentrations of ATP in the presence of Na^+ under saturated concentrations of *p*NPP would lead to an increase in Na-ATPase activity and a decrease in Na-*p*NPPase activity. If the catalytic unit is an oligomer, the simultaneous presence both ATP and *p*NPP would influence the catalytic activities via subunit interactions in such a manner that could not be explained by simple competition. To investigate this point, we determined Na-ATPase activity and Na-*p*NPPase activity directly in the simultaneous presence of [γ-^{32}P]ATP and *p*NPP by measuring [γ-^{32}P]P_i and *p*NP, respectively. In this study, we provide compelling evidence for the presence of ATP, *p*NPP and ATP with *p*NPP inducing different catalytic subunit interactions in oligomeric Na/K-ATPase. This is based on measurements of the amount of EP and the activities of Na-ATPase and Na-*p*NPPase in the presence of various concentrations of [γ-^{32}P]ATP and 20 mM *p*NPP which fully saturated Na-*p*NPPase activity and the amount of enzyme bound [γ-^{32}P]ATP in the presence of 0.1 mM [γ-^{32}P]ATP and 20 mM *p*NPP. The data show that ATP binding with different affinity induces catalytic subunit interactions, which can not be explained by atomic models that have been presented previously (*1*–*4*) and indicate the need for further studies of membrane bound P-type ATPase, in terms of developing a better understanding of the mechanism of this reaction.

EXPERIMENTAL PROCEDURES

The purification of Na/K-ATPase from pig kidney (*28*) with slight modifications and removing the contaminating Na^+ and K^+ in the enzyme preparation (*29*, *30*), the estimation for the amount of acid stable $E^{32}P$ and bound ^{32}P in the presence of [γ-^{32}P]ATP and the liberation of $^{32}P_i$ from [γ-^{32}P]ATP (*20*) and the liberation of *p*-nitrophenol from *p*NPP (*31*) have been described previously. All reactions were carried out at 0°C and were stopped under when the reaction was in a linear phase, where the steady state level of phosphorylation from [γ-^{32}P]ATP was maintained. The reaction mixture used in measuring the activity of Na-ATPase, Na-*p*NPPase and the phosphorylation contained 25 mM Imidazole-HCl (pH 7.4), 16 mM NaCl, 25 mM sucrose, 0.1 mM EDTA-Tris, 0 to 1 mM [γ-^{32}P]ATP-Tris and 0 to 20 mM *p*NPP-Tris unless otherwise stated. The concentration of $MgCl_2$ was always in 1 mM excess to the initial concentration of substrates added. Ouabain sensitive Na-ATPase and Na-*p*NPPase activity were estimated from the difference in activity in the absence and presence of 1 mM ouabain. The reaction mixture used in estimating the amount of ^{32}P bound to the enzyme was essentially the same as described above and contained 0.1 mM [γ-^{32}P]ATP-Tris, 50 mM [^{3}H]-glucose and 20 mM *p*NPP. Other details are described below.

Na^+-Dependent pNPPase Activity—The Na-*p*NPPase reaction was started by the addition of 0.05 ml of a solution containing 0.1 to 2 μmol of both *p*NPP-Tris (pH 7.4) and $MgCl_2$ and choline chloride to maintain a constant ionic strength to 0.05 ml of a reaction mixture containing 2.7 μg protein of the enzyme, 1.6 μmol of NaCl, 0.1 μmol of $MgCl_2$, 2.5 μmol of sucrose, 0.01 μmol of EDTA-Tris and 2.5 μmol of Imidazole-HCl (pH 7.4), respectively, with or without 100 nmol of ouabain. Ouabain sensitive Na-*p*NPPase activity in the presence of 2 μmol of both *p*NPP-Tris (pH 7.4) and $MgCl_2$ with or without 0.05–200 nmol of ATP-Tris, 0.05–50 nmol of ADP-Tris, 0.05–400 nmol of AMP-PCP-Tris, 0.1–1600 nmol of acetyl phosphate and $MgCl_2$ to give a 1 mM excess over the concentrations of each ligand were also measured. The reaction was stopped after 20 min by adding 0.1 ml of a solution containing 5% Na_2CO_3 and 8% SDS.

Sodium-Dependent Phosphoenzyme (EP) Formation from ATP and Na^+-Dependent ATPase Activity—For the Na-ATPase and phosphorylation reactions using [γ-^{32}P]ATP in the absence of *p*NPP, the reaction was initiated by the addition of 0.05 ml of a solution containing 0.005–10 nmol of both [γ-^{32}P]ATP and $MgCl_2$ to 0.05 ml of reaction mixture containing 7.7 μg of SDS-purified enzyme protein, 1.6 μmol of NaCl, 0.1 μmol of $MgCl_2$, 2.5 μmol of sucrose, 0.01 μmol of EDTA-tris and 2.5 μmol of imidazole-HCl (pH 7.4), respectively. The reaction was stopped by the addition of 0.5 ml of 10% TCA with 5 μmol of NaH_2PO_4 and 500 nmol of ATP at 10 s (0.05 to 0.5 μM ATP), 20 s (1 and 2 μM ATP), 1 min (5 μM ATP), 2 min (10 and 20 μM ATP), 3 min (100 μM ATP). The Na-ATPase and the phosphorylation reaction in the presence of *p*NPP was initiated by the addition of 0.02 ml of a solution containing 2 μmol of both *p*NPP and $MgCl_2$ to 0.03 ml of a reaction mixture containing 7.7 to 22 μg of SDS-purified enzyme protein, 0.8 μmol of NaCl, 0.05 μmol of $MgCl_2$, 1.25 μmol of sucrose, 0.05 μmol of EDTA-tris and 1.25 μmol of imidazole-HCl (pH 7.4), respectively. After 1 min, 0.05 ml of a solution containing the same amount of ligands as above and 0.01–100 nmol of both [γ-^{32}P]ATP and $MgCl_2$ was added. The reaction was stopped by the addition of 0.5 ml of a solution containing 10% TCA, 5 μmol of NaH_2PO_4 and 500 nmol of ATP at 0.5 min (0.1 to 0.5 μM ATP), 1 min (1 to 5 μM ATP), 2 min (10 and 20 μM ATP), 3 min (50 and 100 μM ATP) and 10 min (200 to 1000 μM ATP), respectively. The denatured enzyme suspension was centrifuged and the supernatants were treated with charcoal, the amount of P_i liberated was determined and the precipitates were suspended and counted as the amount of EP, as described previously (*21*).

Bound ^{32}P from [γ-^{32}P]ATP—To estimate the amount ^{32}P that binds to the enzyme in the presence of 0.1 mM [γ-^{32}P]ATP, the binding reaction was initiated by adding 18 μl of a solution containing 750 nmol of imidazole-HCl (pH 7.4), 33 nmol of $MgCl_2$, 3 nmol of [γ-^{32}P]ATP and 1,500 nmol of [^{3}H]-glucose with or without 600 nmol of

*p*NPP and $MgCl_2$ to 12 μl of a solution containing 92 μg of enzyme protein, 3,000 nmol of sucrose and 12 nmol of EDTA-Tris and mixed with a pipet tip at 0°C. After approximately 6 s, approximately 18 μl of the reaction mixture with or without the enzyme was applied to a set of two membrane filters (upper: a Bio-Rad nitrocellulose filter with a pore size of 0.45 μm for trapping the enzyme; lower: a type AA Millipore filter with a pore size of 0.8 μm for trapping the filtrate). Approximately 3 μl of the reaction mixture containing little enzyme protein was then trapped in the Millipore filter by aspiration for 6–10 s at 4°C. The Millipore filters were incubated with 1 ml of a solution containing 1 μmol of unlabeled ATP and 50 μmol of glucose for 1 h at room temperature and then removed and 10 ml of scintillation cocktail was added and counted. The amount of ^{32}P bound to the enzyme was calculated from the difference between the ratio of the radioactivity $^{32}P/^{3}H$ of the filtrate with or without the enzyme in the reaction mixture. Na^+-dependent EP formation and Na-ATPase reaction in Table 2 were started and stopped as described in the legend of Fig. 2 except that both 10 nmol of ATP-Tris and $MgCl_2$ with or without both 2,000 nmol of *p*NPP-Tris and $MgCl_2$ were added to the enzyme solution containing 5 μmol of glucose, 10 μmol of sucrose and 40 nmol of EDTA-tris. The Na-*p*NPPase reaction in the Table 2 was started and stopped as described in the legend of Fig. 1 except that 2,000 nmol of *p*NPP-Tris and $MgCl_2$ were added with or without 10 nmol of ATP-tris and $MgCl_2$ to the enzyme solution containing 5 μmol of glucose, 10 μmol of sucrose and 40 nmol of EDTA-tris which had little effect on either of the activities.

Curve Fitting of the Data—The experimental values shown in Figs 1 and 2 represent the mean ± S.D. from duplicate experiments that were performed several times. Error bars within each symbol are not shown. Data were subjected to curve fitting using the Hill equations (Graph Pad Prism; Graph Pad Software Inc.). The one site model equation, $V = V_{max}[L]^n/(K_{0.5}{}^n + [L]^n)$ was used for the activation of Na-*p*NPPase versus *p*NPP concentration, the Na-ATPase and the amount of EP versus ATP concentration in the absence of *p*NPP. The two site model equation, $V = V_{max1}[L]^{n1}/(K_{0.5}{}^{n1} + [L]^{n1}) + V_{max2}[L]^{n2}/(K_{0.5}{}^{n2} + [L]^{n2})$ was used for the activation of Na-ATPase activity versus ATP concentration in the presence of *p*NPP. The one site inhibition model, $V = V_{max}[1 - [L]^n/([L]^n + K_{i,0.5}{}^n)]$ was used for the inhibition of Na-*p*NPPase activity versus various concentrations of ligands. V_{max} (or EP_{max}), $K_{0.5}$ and n are the maximum velocity (or the amount of EP), the concentration of ligand for the half maximum velocity (or the half maximum amount of EP) and Hill coefficient, respectively. The correlation coefficient, r, was also obtained.

RESULTS

Effect of ATP, Acetyl Phosphate, ADP and AMP-PCP on Na^+-Dependent p-Nitrophenyl Phosphatase Activity of Pig Kidney Na/K-ATPase—The reactivity of the EP of Na/K-ATPase formed in the presence of Mg^{2+} and Na^+ from ATP, AcP and *p*NPP, and that of EP formed in the presence of Mg^{2+} from P_i (*7–10*, *15*, *25*, *26*, *32*, *33*) has been studied extensively. However, the role of enzyme bound ATP preceding the formation of EP (*34*) and in EP:EATP

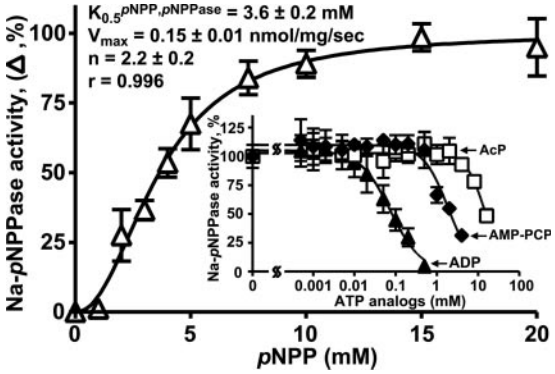

Fig. 1. ***p*NPP concentration dependence of Na-*p*NPPase activity and the effect of ADP, AMP-PCP and acetyl phosphate on the activity.** Na^+-dependent *p*NPPase activity of SDS-purified enzyme was measured at 0°C in a reaction medium containing 2.7 μg of enzyme protein, 16 mM NaCl, 1 mM $MgCl_2$, 25 mM Sucrose, 0.1 mM EDTA-tris, 25 mM imidazole-HCl (pH 7.4), 0–20 mM of both *p*NPP and $MgCl_2$ in the presence or absence of 1 mM ouabain. Data for Na-*p*NPPase activity, namely the difference in activity with and without ouabain, *versus* *p*NPP concentration (open triangle) were subjected to curve fitting using the Hill equation for the one site activation model to be $K_{0.5}{}^{pNPP,pNPPase}$ = 3.6 mM and the Hill coefficient, n = 2.2 and correlation coefficient r = 0.996. inset, Na-*p*NPPase activity of SDS-purified enzyme was measured under the same condition as described above except that the reaction mixture contained 20 mM of both *p*NPP and $MgCl_2$ and 0–0.5 mM of both ADP and $MgCl_2$ (closed triangle) or 0–4 mM of both AMP-PCP and $MgCl_2$ (closed diamond) or 0–16 mM of both acetyl phosphate and $MgCl_2$ (open square). The Hill plot of the inhibition model was used for activity versus ADP, AMP-PCP, and AcP. Parameters are in the text. One hundred percent of the activity was the Na-*p*NPPase activity in the absence of ATP analogs.

accompanying half site phosphorylation during the Na-ATPase reaction (*19*) is not well understood. The reason for this may be that the bound ATP preceding the formation of EP appears transiently. The EATP in EP:EATP is labile in cold ATP chase experiments compared with the case of H/K-ATPase (*21*). The possibility that enzyme bound *p*NPP is formed during the Na-*p*NPPase reaction as in the case of ATP (*20*, *34*) can not be excluded, because ATP is replaceable by *p*NPP in Na^+-dependent reactions, as described above. In such a case, the addition of low concentrations of [γ-^{32}P]ATP may induce the formation of reaction intermediates from *p*NPP and $AT^{32}P$, such as EP:E*p*NPP, EP:E$AT^{32}P$, $E^{32}P$:E*p*NPP or $E^{32}P$:EP. The liberation of *p*NP and $^{32}P_i$ which accompany the formation of such complexes, would be influenced by subunit interactions (*15–20*, *32*, *35*, *36*), because 1 mol of the phosphorylation site or ATP binding site/mol of protomer is widely accepted in Na/K-ATPase (*9*, *10*, *16*, *17*, *20*, *37*).

To investigate this point, the concentration dependence of *p*NPP on ouabain sensitive Na-*p*NPPase activity was measured first, while keeping the ionic strength constant by adding choline chloride. A Hill plot of the data (Fig. 1) showed that the Na^+-dependent hydrolysis of *p*NPP occurs with a strong positive cooperative effect by *p*NPP ($K_{0.5}{}^{pNPP,pNPPase}$ = 3.6 ± 0.2 mM with n = 2.2 ± 0.2).

The effect of increasing concentrations of ATP on Na-*p*NPPase activity in the presence of 20 mM *p*NPP was then investigated (Fig. 2, triangles). ATP showed

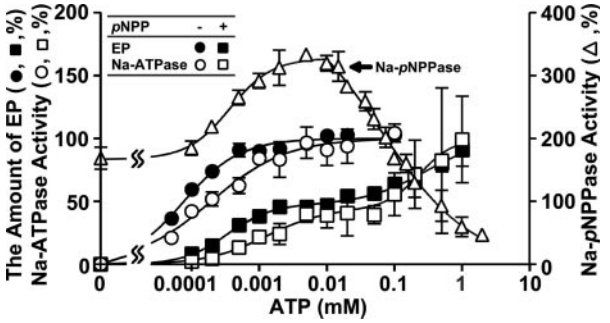

Fig. 2. **ATP concentration dependence of Na-ATPase activity and Na^+-dependent EP formation in the presence or absence of 20 mM *p*NPP**. Na-*p*NPPase activity versus the concentration of ATP-Tris (open triangles) were subjected to a Hill plot for the activation model and the inhibition model, respectively, in the ATP concentration range of 0 to 10 μM and 10 to 2,000 μM. The values for Na-*p*NPPase activity shown are relative to the activity of the V_{max} of ouabain sensitive Na-ATPase activity, estimated as described below. The Na-ATPase activity and the amount of EP versus the concentration of ATP were subjected to the Hill plot for the activation model. The maximum Na-ATPase activity (open circles, 0.14 nmol/mg of protein/s) and the maximum amount of EP (closed circles, 2.3 nmol/mg of protein) were set at 100%, respectively. Data for Na-ATPase activity (open squares) and the amount of EP (closed squares) in the presence of *p*NPP were subjected a Hill plot of the two site model $[V = V_{max1}[L]^{n1}/(K_{0.5}{}^{n1} + [L]^{n1}] + V_{max2}[L]^{n2}/(K_{0.5}{}^{n2} + [L]^{n2})]$. Where the maximum Na-ATPase activity or the maximum amount of EP, V_{max1} with high affinity and V_{max2} with low affinity for ATP was set, respectively, to 46% estimated from the ATP concentration dependence of the activity or the amount of EP in the presence of 0 to 10 μM ATP with 20 mM *p*NPP and 53 (= 100–47) % from the difference in activity with the amount of EP in the presence of 0 to 100 μM ATP without *p*NPP (100%) and the value 47%. When each value was set to the theoretical value of 50% due to half site phosphorylation, each $K_{0.5}$ value increased within 10%, except for $K_{0.5}{}^{ATP,ATPase}$ for the high affinity, which increased from 1.3 to 1.9 μM, an increase that had negligible influence on the present results.

clear dual effects, activation followed by inhibition with increasing concentrations of ATP. In the presence of less than 10 μM ATP, ATP induced a nearly 2 fold increase ($K_{0.5}{}^{ATP,pNPPase} = 0.37 \pm 0.05$ μM with $n = 1.4 \pm 0.2$) in Na-*p*NPPase activity, as shown by the activation model of the Hill plot. A higher concentration of ATP induced a negative cooperative inhibition ($K_{i,0.5}{}^{ATP,pNPPase} = 63 \pm 17$ μM and $n = 0.6 \pm 0.1$) as shown by the inhibition model of the Hill plot (Table 1, *p*NPPase 2nd, and 3rd columns).

Although little activation of Na-*p*NPPase activity was detected with the following the substrates, a Na^+-dependent phosphorylating substrate, AcP (open squares, $K_{i,0.5}{}^{AcP,pNPPase} = 15 \pm 1.2$ mM with $n = 1.9 \pm 0.3$) or a pseudo substrate, AMP-PCP (closed diamonds, $K_{i,0.5}{}^{AMP\text{-}PCP,pNPPase} = 1.8 \pm 0.2$ mM with $n = 1.6 \pm 0.4$) or ADP (closed triangles, $K_{i,0.5}{}^{ADP,pNPPase} = 86 \pm 10$ μM with $n = 1.3 \pm 0.2$) inhibited Na-*p*NPPase activity as shown (Fig. 1, inset).

These data suggest that ATP binding triggers the activation of Na-*p*NPPase activity. EP formation itself appeared to be either insufficient or not required for activation because neither of the Na^+-dependent phosphorylating substrates AcP nor *p*NPP induced any additional activation, where both E1P and E2P accumulate (*25*, *26*) as in the case of ATP.

The data suggest the presence of at least two different ATP effects on Na-*p*NPPase activity. (i) A high affinity ATP binding which induces conformation change preceding EP formation to affect neighboring catalytic unit(s) such as activating Na-*p*NPPase activity in spite of reducing the available sites for *p*NPP. (ii) A low affinity ATP binding which further expels *p*NPP from the enzyme to inhibit Na-*p*NPPase activity. The ATP induced activation with positive coopreravitity and inhibition with negative cooperativity may indicate the presence of more than two ATP binding sites (Table 1, *p*NPPase, 2nd and 3rd columns).

Comparison of Concentration Dependence of ATP on the Na-pNPPase Activity with the Na-ATPase Activity and the Amount of EP—Figure 2 (closed and open circles) show the ATP concentration dependence of the amount of EP and the Na-ATPase activity in the absence of *p*NPP. The Hill plot of these data (closed and open circles in Fig. 2) show that the apparent $K_{0.5}{}^{ATP,EP}$ for phosphorylation and the $K_{0.5}{}^{ATP,ATPase}$ for the Na-ATPase activity was 0.076 ± 0.007 and 0.19 ± 0.03 μM, respectively, where each n value was close to 1. The estimated EP_{max} and the $V_{max}{}^{ATPase}$ were assumed to be 100% (all parameters estimated by the Hill plot from Fig. 2 are shown in Table 1).

The ATP concentration dependence for the amount of EP and Na-ATPase activity in the presence of 20 mM *p*NPP (Fig. 2, closed and open squares) showed biphasic increases approaching the maximum values detected without *p*NPP. The large scattered values for ouabain sensitive Na-ATPase activity in the presence of more than 0.2 mM ATP was due to an increase in the amount of $^{32}P_i$ in the [γ-^{32}P]ATP solution compared with the liberation of $^{32}P_i$ by the enzyme. The Hill plot assuming a two site model shows the presence of high and low ATP affinity sites, each fraction of which was around half, for both phosphorylation and Na-ATPase activity. The value of $K_{0.5}{}^{ATP,EP}$ for high and low affinity sites was, 0.33 ± 0.03 μM with $n = 1.4 \pm 0.2$ and 280 ± 28 μM with $n = 1.0 \pm 0.1$, respectively. That of $K_{0.5}{}^{ATP,ATPase}$ was 1.3 ± 0.2 μM with $n = 1.0 \pm 0.2$ and 238 ± 29 μM with $n = 1.7 \pm 0.3$, respectively.

The data clearly show that *p*NPP not only reduces the ATP affinity but also splits the two apparently different ATP effects observed in the absence of *p*NPP into two with an almost equal fraction of each. The presence of *p*NPP reduced both the maximum amount of EP and Na-ATPase activity to half showing a value of 4 (= 0.33/0.076) and a 3,700 (= 280/0.076) fold reduction in ATP affinity for EP formation, and a 7 (=1.3/0.19) and 1,300 (= 238/0.19) fold reduction in affinity for Na-ATPase activity (Table 1, EP and ATPase). The difference in ATP affinity for phosphorylation and Na-ATPase activity in between each fraction, was around 850 (=280/0.33) and 180 (=238/1.3) fold, respectively. The increase in the amount of EP and Na-ATPase activity in the presence of *p*NPP with increasing concentrations of more than 0.1 mM ATP (Fig. 2, closed and open squares) show that ATP bound to the low affinity sites, possibly expelling bound *p*NPP, was also broken down to ADP + P_i, not to accumulate a dead end complex. Another important finding was that the value of the half maximum effect of ATP for high affinity phosphorylation ($K_{0.5}{}^{ATP,EP} = 0.33 \pm 0.03$ μM) was almost the same as the value for the ATP induced Na-*p*NPPase activation ($K_{0.5}{}^{ATP,pNPPase} = 0.37 \pm 0.05$ μM) but significantly different from the value for Na-ATPase activation ($K_{0.5}{}^{ATP,ATPase} = 1.3 \pm 0.2$ μM).

Table 1. **Parameters estimated from the Hill plot**. Each parameter was obtained from the data shown in Fig. 2 except for the case of the maximum ouabain sensitive Na-*p*NPPase activity, which was obtained from Fig. 1. The maximum ouabain sensitive Na-ATPase activity was set to 100%, and the relative hydrolytic activity of Na-*p*NPPase in the presence and absence of ATP became 339 and 173% as shown, respectively, $V_{max}^{\text{with ATP}}$, and $V_{max}^{\text{without ATP}}$ in the table. All correlation coefficients (r) were ranged from 0.993 to 0.998, and are not included in the table.

		20 mM *p*NPP			
		–	+	+	+
EP:	$K_{0.5}^{ATP,EP}$, μM	0.076 ± 0.007	0.33 ± 0.03		280 ± 28
	EP_{max}, %	100 ± 1.8	47		53
	n	1.1 ± 0.1	1.4 ± 0.2		1.0 ± 0.1
ATPase:	$K_{0.5}^{ATP,ATPase}$, μM	0.19 ± 0.03	1.3 ± 0.2		238 ± 29
	V_{max}^{ATPase}, %	100 ± 3.6	44		56
	n	0.8 ± 0.1	1.0 ± 0.2		1.7 ± 0.3
*p*NPPase:	$K_{0.5}^{ATP,pNPPase}$, μM		0.37 ± 0.05	–	
	$K_{i,0.5}^{ATP,pNPPase}$, μM		–	63 ± 17	
	$V_{max}^{\text{with ATP}}$, %		339 ± 17	–	
	$V_{max}^{\text{without ATP}}$, %		173 ± 7	–	
	n		1.4 ± 0.2	0.6 ± 0.1	

Table 2. **Comparison of the amount of EP, ^{32}P binding and Na-ATPase and Na-*p*NPPase activities in the presence of 100 μM [γ-^{32}P]ATP with or without 20 mM *p*NPP.**

20 mM *p*NPP	–	+
100 μM [γ-^{32}P]ATP	+	+, (–)
Capacity, %		
$E^{32}P$:$EAT^{32}P$	134 ± 19	69 ± 20
$E^{32}P$	100 ± 7	60 ± 3
Activity, %		
Na-ATPase	100 ± 13	34 ± 14
Na-*p*NPPase	–	81 ± 11, (100 ± 4)

Effect of pNPP on the Amount of EP:EATP and EP, and Activities of Na-pNPPase and Na-ATPase—To further investigate the effect of *p*NPP on the ATP dependent reactions, the amount of both ^{32}P binding (*21*) to the enzyme ($E^{32}P$:$EAT^{32}P$) and $E^{32}P$ accumulated, and the liberation of both $^{32}P_i$ and *p*NP in the presence of 16 mM Na^+ with both 0.1 mM [γ-^{32}P]ATP and 20 mM *p*NPP or the presence of either were measured. Table 2 shows that the amount of EATP in EP:EATP was 34 (=134–100) and 9 (=69–60) % of the amount of EP in the absence and presence of *p*NPP, respectively. The presence of *p*NPP reduced the amount of EATP and EP to 26 (=9×100/34) and 60% level, respectively, accompanied by a reduction in Na-ATPase activity to 34% with the appearance of 81% of Na-*p*NPPase activity. The data suggest that the major effect of *p*NPP was to expel the enzyme bound ATP in EP:EATP, thus inducing the inhibition of Na-ATPase activity. While the major effect of low affinity ATP binding in the presence of 20 mM *p*NPP was to expel enzyme bound *p*NPP to increase both the amount of EP and Na-ATPase activity (Fig. 2, closed and open squares, more than 0.1 mM ATP). The reactivity of EP in the presence of 20 mM *p*NPP, possibly present as $E^{32}P$:EATP, $E^{32}P$:EP or $E^{32}P$:E*p*NPP, was shown to be K^+ sensitive with little sensitivity to ADP (data not shown), indicating the accumulated phosphoenzyme to be E2P.

DISCUSSION

The present data demonstrate the presence of two different very high affinity ATP effects in the absence of *p*NPP and two different high and low affinity ATP effects each in the presence of *p*NPP, during the Na-ATPase reaction (Table 1, EP and ATPase, 1st, 2nd and 4th column). The value for the very high ATP affinity effects and the high affinity ATP effects detected as $K_{0.5}^{ATP,EP}$ and $K_{0.5}^{ATP,ATPase}$ without and with *p*NPP, respectively, are significantly different from each other. Each ATP binding appeared to require the maximum Na-ATPase activity (Fig. 2, open and closed symbols, below 0.1 mM ATP). A report of the presence of two ATP effects on Na-ATPase (*38*) has been questioned (*7*) because of the underestimation of Na-ATPase activity, possibly, due to the decrease in ATP concentration to as little as a 50% level compared to the level for the amount of estimated EP. The present data exclude such a possibility because phosphorylation and Na-ATPase activity, respectively, were estimated in the supernatant and precipitates from the same acid-terminated reaction mixture as described in the Experimental procedures section. The data are consistent with an oligomeric nature of the enzyme such as the presence of enzyme bound ATP (EATP) and EP in different catalytic units (*19*), the stoichiometry of ouabain binding to each protomer, diprotomer and tetraprotomer to be close to 1:1:0.75, respectively (*35*), and others (*15–20*, *24*, *29*, *31*, *35*, *36*, *39*, *40*). The partial reactions of the electric organ Na/K-ATPase (*18*) seems to be different from the reactivity of pig and dog enzymes as described above. The results of homology modeling of Na/K-ATPase (*41*) using the atomic structure of Ca-ATPase (*1–3*) and others (*10*, *11*, *16*, *17*, *19*, *20*, *24*, *29*, *31*, *36*, *37*, *39*, *40*, *42*) suggest the presence of one ATP binding domain/protomer without an extra ATP binding domain except that E1P accepts ADP to synthesize ATP (*8*). The presence of a single ATP binding site with a different conformational state in Na/K-ATPase has been also suggested, based on studies using single amino acid mutated enzymes inside the ATP binding pocket (*31*) and in the conserved 442GDSE446 sequence (*43*).

High affinity ATP effects detected in the presence of *p*NPP with Mg^{2+} and Na^+ appeared to occur in the same ATP concentration range, because value of $K_{0.5}^{ATP,EP}$ = 0.33 ± 0.03 μM and $K_{0.5}^{ATP,pNPPase}$ = 0.37 ± 0.05 μM was essentially the same, within experimental error. The

enzyme bound ^{32}P detected was only $E^{32}P$ without $EAT^{32}P$ (not shown) suggesting that the same ATP molecule induced not only a quarter site phosphorylation in one catalytic unit but also a 2 fold increase in Na-*p*NPPase activity in neighboring catalytic unit(s), which would have accepted *p*NPP to form E2P from *p*NPP (*25*) and/or the E*p*NPP complex. Neither *p*NPP nor AcP (Fig. 1 and inset), both of which form E2P (*25*, *26*) as does ATP, replace the role of ATP. Thus, ATP binding rather than the subsequent formation of EP appears to lead to the activation of Na-*p*NPPase activity. The reason for why two different ATP affinities, one for $K_{0.5}^{ATP,EP}$ and $K_{0.5}^{ATP,pNPPase}$ and another for $K_{0.5}^{ATP,ATPase}$ were detected in spite of the quarter site phosphorylation from ATP may be that the bound ATP induced dephosphorylation immediately because of EP, already formed from *p*NPP (*25*) in neighboring subunits. The enzyme, possibly, retained half site phosphorylation (*19*, *20*) or prohibited more than half site phosphorylation (Fig. 3B′).

Table 2 shows that the enzyme bound ATP (*19*) with a low affinity for EP:EATP protected Na-ATPase activity from the *p*NPP induced inhibition. It has previously been shown that high concentrations of ATP (*10*) deocclude K^+ from KE2 where Na^+ is possibly required (*19*), and the deocclusion has been assumed to be the rate determining step in the Na/K-ATPase reaction in the sequential scheme of the Post-Albers mechanism in the protomeric enzyme form (*7*–*10*). KE2 has been reported to hydrolyze more than 10 molecules of *p*NPP for each molecule of ATP hydrolyzed (*7*) in the presence of both 4 mM Mg^{2+} and *p*NPP, 10 mM Na^+, and 0.5 mM K^+ and 67 μM ATP, where ATP induced a 4 fold activation of *p*NPPase activity. However, whether K^+ occluded catalytic units, namely KE2, and/or the neighboring catalytic units hydrolyze *p*NPP remains to be determined. These data clearly show the ATP induced activation of *p*NPPase activity occurs via two different mechanisms, one involving the formation of EATP preceding phosphorylation in the presence of Na^+ as shown in this work and another involving the accumulation of KE2 *via* E2P (*7*, *10*) and possibly that of EATP in the presence of Na^+ with K^+. The presence of a modifying site for the action of ATP to reject the participation of phosphoenzyme has been reported (*44*) to activate *p*NPPase activity in the presence of Na^+ with K^+. The EP:EATP detected during the Na-ATPase reaction (*19* and Table 2) accepted K^+ to liberate P_i immediately. This might result in an accumulation of KE2:EATP which then accepts Na^+ to liberate K^+ to form EP:EATP during the Na/K-ATPase reaction.

Although more quantitative data will need to be collected, the data presented here can be explained by assuming a hypothetical tetraprotomer model (Fig. 3 and its legend; also see Table 1). Upper pathway (A→B→C) and lower pathway (A′→B′→C), respectively, designates the intermediates accumulated with increasing concentrations of ATP in the absence (upper pathway) and presence of *p*NPP (lower pathway). Both enzymes form B and C, and B′ and C′, respectively, assumed to become A and A′ after stoichiometric P_i liberation from EP and EP:EATP. Evidence in support of EATP as an active intermediate and not a dead end complex in EP:EATP has been obtained as described below. ATP reduced the rate of EP breakdown of Na/K-ATPase (*32*, *33*). Bound ATP in EP:EATP and ATP added to EP:E of H/K-ATPase (*21*) also reduced the rate of EP breakdown, in which K^+ accelerated the breakdown remarkably in the presence of high concentrations of ATP (*45*), as is well known in the case of the activation of Na/K-ATPase and H/K-ATPase. The rate constant for EP breakdown of pig stomach H/K-ATPase was almost equal to the value of *v*/(EP + EATP) under accumulation conditions of EP:EATP (*21*) and the liberation of 2 mol of P_i from 1 mol of EP and 1mol of EATP was directly demonstrated without (*21*) and with K^+ (*46*). However, bound ATP in EP:EATP of Na/K-ATPase appeared to immediately undergo exchange with added cold ATP in the chase experiment, which prohibited measuring P_i liberation from EATP, directly. These data indicate that ATP hydrolysis occurs via two parallel pathways: one mole of P_i liberation occurs from the EP formed as the result of high-affinity ATP binding in one catalytic unit, and a second mole from the EATP formed with low affinity ATP binding in another catalytic unit.

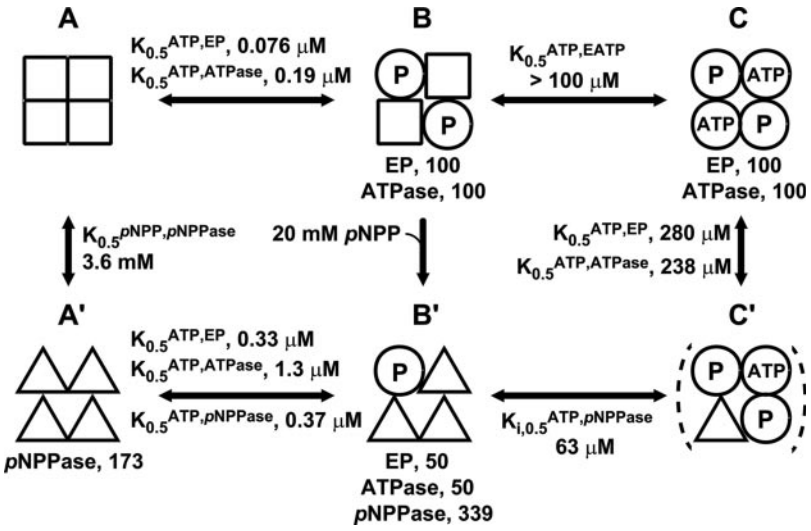

Fig. 3. **Hypothetical model of four different ATP effects on Na/K-ATPase.** The Na/K-ATPase is assumed to be a tetraprotomer (*19*, *20*) and each square indicates a Mg^{2+} and Na^+ bound enzyme. Each triangle indicates an EP from *p*NPP (*25*) or the E*p*NPP complex. Each circle indicates EP from ATP and the ATP bound enzyme, respectively, as shown in the circles P and ATP. Each value of $K_{0.5}^{ATP,ATPase}$ and $K_{0.5}^{ATP,pNPPase}$ and percent value for the phosphorylation (EP) to the maximum amount of EP and that of the hydrolytic activity (ATPase or *p*NPPase) to the maximum Na-ATPase activity are from Table 1, except that the amount of EP and Na-ATPase activity in B′ was set to 50%, respectively, not 47 and 44% for simplicity and $K_{0.5}^{ATP,EATP}$ was assumed to be 0.1 mM<, because the amount of EATP in the presence of 0.1 mM ATP was below half the amount of EP (Table 2). The amount of EATP in each enzyme state remains to be determined, except the amount of EATP, possibly, in the mixture of C′ and B′ was estimated (Table 2).

Two very high affinity ATP bindings to the Na^+-bound enzyme (A), one for EP formation ($K_{0.5}^{ATP,EP}$ = 0.076 μM) and the other for Na-ATPase activation ($K_{0.5}^{ATP,ATPase}$ = 0.19 μM) induce the formation of half site phosphorylated enzymes (B). Both forms A and B, respectively, become A′ and B′ in the presence of 20 mM *p*NPP. A′ hydrolyzes *p*NPP 1.7 (= 173/100)-fold that of the ATP hydrolyzed in the B, under each saturated substrate concentration (see percent value in Table 1, first column, ATPase and second column *p*NPPase). A′ is converted to B′ by binding ATP with high affinity at possibly two different sites ($K_{0.5}^{ATP,EP}$ = 0.33 μM and $K_{0.5}^{ATP,ATPase}$ = 1.3 μM), where both phosphorylation and Na-ATPase activity become half of that of B in the upper pathway. Thus in B′ one-fourth of the catalytic units are phosphorylated (Fig. 2 closed squares in the presence of around 0.01 to 0.1 mM ATP) compared to one-half in B (Fig. 2 closed circles) without any detectable enzyme bound ATP. While the Na-ATPase activity in B′ (Fig. 2, open squares in the same ATP concentration range as described above) is roughly half that in B (Fig. 2, open circles) but the total hydrolytic activity is almost 4 [= (44 + 339)/100]-fold of B or 2 (= 339/173)-fold of the Na-*p*NPPase activity of A′ (see percent value in Table 1. first column, ATPase without *p*NPP and second column, ATPase and *p*NPPase with *p*NPP). The similar values for $K_{0.5}^{ATP,EP}$ and $K_{0.5}^{ATP,pNPPase}$ (Table 1, second column, EP and *p*NPPase) show that the ATP participating in phosphorylation also induces an extra activation of Na-*p*NPPase. The reason for the reduction in Na-ATPase activity in B′ to half would be related to the accumulation of EP from *p*NPP or the E*p*NPP complex (*25*). B′ accepts ATP with a moderate ATP affinity to become C′, showing the cooperative inhibition of Na-*p*NPPase activity ($K_{i,0.5}^{ATP,pNPPase}$ = 63 μM) accompanied by an increase in the amount of EP from ATP and possibly enzyme bound ATP (Table 1, third column and Fig. 2), as shown in the dotted parenthesis in Fig. 3. The last, and possibly fourth, ATP binding to C′ induces the maximum phosphorylation ($K_{0.5}^{ATP,EP}$ =280 μM) and Na-ATPase activity ($K_{0.5}^{ATP,ATPase}$ = 238 μM) with the nearly complete removal of Na-*p*NPPase activity to form enzyme form C, the half site phosphorylated and half site ATP bound tetraprotomeric Na/K-ATPase (*20*).

Although EP breakdown appeared to be rate limiting under saturated levels of each substrate in the presence of Mg^{2+} and Na^+ (*7*, *9*, *10*, *25*), A′ and B′, respectively, showed a 1.7- and a 3.8 [(= 339 + 44)/100]-fold increased hydrolytic activity compared to B or C. If each protomer independently hydrolyzed each substrate, the maximum activity would be the same, which was not the case. The difference in catalytic subunit interactions induced by ATP (B), *p*NPP (A′) and ATP + *p*NPP (B′) might be related to the difference in maximum activity. ATP was required for the activation of Na-*p*NPPase (Figs 1 and inset and 2) and neither Na^+-dependent phosphorylation by *p*NPP or AcP nor the binding of the pseudo substrate, AMP-PCP or ADP, induced any activation. Those data exclude the possibility that an increase in the amount of E2, accumulated after dephosphorylation of EP formed by ATP, activated *p*NPP hydrolysis as in the well known case of KE2 (*7*).

Available data suggest that high affinity ATP binding without and with *p*NPP, respectively, triggered a conformational change preceding EP formation, as has been previously shown without *p*NPP (*34*). Such a conformation change would result in subunit interactions with neighboring subunits to accept ATP with a slightly reduced affinity to activate Na-ATPase activity. An explanation for the little difference in the low affinity ATP effect compared with a clear difference in the high affinity ATP effect, detected as $K_{0.5}^{ATP,EP}$ and $K_{0.5}^{ATP,ATPase}$ in the presence of *p*NPP, is not possible because of the inevitable large scattering of the Na-ATPase activity data.

The increase in protomer-protomer interaction accompanying EP formation has previously been demonstrated by gel chromatography using octaethyleneglycol n-dodecyl ether solubilized dog kidney Na/K-ATPase (*47*). Direct chemical evidence for subunit interactions has already been shown by the presence of a C-terminal 64-kDa homodimer using chemical cross-linking and controlled trypsin digestion at Arg438-Ala439 (*35*). The atomic structure of Ca-ATPase indicates that ATP binds to the N domain and approaches domain P for phosphorylation (1) in P-type ATPase (*1–6*, *48*), the movement of which might be related to catalytic subunit interactions. Further studies will be required to confirm the above hypothesis.

The possible existence of four different ATP effects and changes in different maximum catalytic activity by ATP, *p*NPP and ATP with *p*NPP were observed without any chemical modification of the enzyme. These findings suggest a new strong enzymatic basis for different catalytic subunit interactions, induced by ATP binding with different affinities with or without *p*NPP, in tetoraprotomeric Na/K-ATPase for the first time, to our knowledge. They may be useful for developing a better understanding of the mechanism of P-type pump ATPase in general.

This work was supported, in part, by Grants-in-Aid for Scientific Research (13142201 to S.K.) and from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

REFERENCES

1. Toyoshima, C., Nakasako, M., Nomura, H., and Ogawa, H. (2000) Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 Å resolution. *Nature* **405**, 647–655
2. Toyoshima, C. and Inesi, G. (2004) Structural basis of ion pumping by Ca^{2+}-ATPase of the sarcoplasmic reticulum. *Annu. Rev. Biochem.* **73**, 269–292
3. Olesen, C., Sørensen, T.L., Nielsen, R.C., Møller, J.V., and Nissen, P. (2004) Dephosphorylation of the calcium pump coupled to counterion occlusion. *Science* **306**, 2251–2255
4. Toyoshima, C., Nomura, H., and Tsuda, T. (2004) Lumenal gating mechanism revealed in calcium pump crystal structures with phosphate analogues. *Nature* **432**, 361–368
5. Shull, G.E., Schwartz, A., and Lingrel, J.B. (1985) Amino-acid sequence of the catalytic subunit of the (Na^+ + K^+)ATPase deduced from a complementary DNA. *Nature* **316**, 691–695
6. MacLennan D.H., Brandl, C.J., Korczak, B., and Green, N.M. (1985) Amino-acid sequence of a Ca^{2+} + Mg^{2+}-dependent ATPase from rabbit muscle sarcoplasmic reticulum, deduced from its complementary DNA sequence. *Nature* **316**, 696–700
7. Post, R.L., Hegyvary, C., and Kume, S. (1972) Activation by adenosine triphosphate in the phosphorylation kinetics of sodium and potassium ion transport adenosine triphosphatase. *J. Biol. Chem.* **247**, 6530–6540
8. Taniguchi, K. and Post, R.L. (1975) Synthesis of adenosine triphosphate and exchange between inorganic phosphate

and adenosine triphosphate in sodium and potassium ion transport adenosine triphosphatase. *J. Biol. Chem.* **250**, 3010–3018
9. Albers, R.W. (1976) The (sodium plus potassium)-transport ATPase in *The Enzymes of Biological Membranes* (Martonossi, A., ed.) Vol. 3, pp. 283–301, Plenum Publishing Corp., New York
10. Glynn, I.M. (1985) The Na^+,K^+-transporting adenosine triphosphatase in *The Enzymes of Biological Membranes* (Martonossi, A., ed.) Vol. 3, pp. 35–114, Plenum Publishing Corp., New York
11. Jørgensen, P.L., Hakansson, K.O., and Karlish, S.J. (2003) Structure and mechanism of Na,K-ATPase: functional sites and their interactions. *Annu. Rev. Physiol.* **65**, 817–849
12. Møller, J.V., Juul, B., and le Maire, M. (1996) Structural organization, ion transport, and energy transduction of P-type ATPases. *Biochim. Biophys. Acta* **1286**, 1–51
13. McIntosh, D.B. (2000) Portrait of a P-type pump. *Nat. Struct. Biol.* **7**, 532–535
14. Nakamura, J., Tajima, G., Sato, C., Furukohri, T., and Konishi, K. (2002) Substrate regulation of calcium binding in Ca^{2+}-ATPase molecules of the sarcoplasmic reticulum. I. Effect of ATP. *J. Biol. Chem.* **277**, 24180–24190
15. Askari, A., Huang, W., and Antieau, J.M. (1980) Na^+,K^+-ATPase: ligand-induced conformational transitions and alterations in subunit interactions evidenced by cross-linking studies. *Biochemistry* **19**, 1132–1140
16. Askari, A. (2000) Significance of protein-protein interactions to Na^+/K^+-ATPase functions in *Na/K-ATPase and Related ATPases* (Taniguchi, K. and Kaya, S., eds.) pp. 17–26, Elsevier Science, Amsterdam
17. Schoner, W., Thönges, D., Hamer, E., Antolovic, R., Buxbaum, E., Willeke, M., Serpersu, E.H., and Scheiner-Bobis, G. (1994) Is the Sodium pump a functional dimer? in *The Sodium Pump. Structure Mechanism, Hormonal Control and its Role in Disease* (Bamberg, E. and Schoner, W., eds.) pp. 332–341, Dietrich Steinkopff Verlag GmbH & Co. KG, Darmstadt
18. Froehlich, J.P., Bamberg, E., Kane, D.J., Mahaney, J.E., and Albers, R.W. (2000) Contribution of quaternary protein interactions to the mechanism of energy transduction in Na^+/K^+-ATPase in *Na/K-ATPase and Related ATPases* (Taniguchi, K. and Kaya, S., eds.) pp. 349–356, Elsevier Science, Amsterdam
19. Yokoyama, T., Kaya, S., Abe, K., Taniguchi, K., Katoh, T., Yazawa, M., Hayashi, Y., and Mårdh, S. (1999) Acid-labile ATP and/or ADP/P_i binding to the tetraprotomeric form of Na/K-ATPase accompanying catalytic phosphorylation-dephosphorylation cycle. *J. Biol. Chem.* **274**, 31792–31796
20. Taniguchi, K., Kaya, S., Abe, K., and Mårdh, S. (2001) The oligomeric nature of Na/K-transport ATPase. *J. Biochem.* **129**, 335–342
21. Abe, K., Kaya, S., Imagawa, T., and Taniguchi, K. (2002) Gastric H/K-ATPase liberates two moles of P_i from one mole of phosphoenzyme formed from a high-affinity ATP binding site and one mole of enzyme-bound ATP at the low-affinity site during cross-talk between catalytic subunits. *Biochemistry* **41**, 2438–2445
22. Abe, K., Kaya, S., Hayashi, Y., Imagawa, T., Kikumoto, M., Oiwa, K., Katoh, T., Yazawa, M., and Taniguchi, K. (2003) Correlation between the activities and the oligomeric forms of pig gastric H/K-ATPase. *Biochemistry* **42**, 15132–15138
23. Abe, K., Kaya, S., Taniguchi, K., Hayashi, Y., Imagawa, T., Kikumoto, M., Oiwa, K., and Sakaguchi, K. (2005) Evidence for a relationship between activity and the tetraprotomeric assembly of solubilized pig gastric H/K-ATPase. *J. Biochem.* **138**, 293–301
24. Hayashi, Y., Shinji, N., Tahara, Y., Hagiwara, E., and Takenaka, H. (2003) Isolation of $(\alpha\beta)_4$-tetraprotomer having half-of-sites ATP binding from solubilized dog kidney Na^+/K^+-ATPase. *Ann. N.Y. Acad. Sci.* **986**, 232–234
25. Yamazaki, A., Kaya, S., Tsuda, T., Araki, Y., Hayashi, Y., and Taniguchi, K. (1994) An extra phosphorylation of Na^+,K^+-ATPase by paranitrophenylphosphate (pNPP): evidence for the oligomeric nature of the enzyme. *J. Biochem.* **116**, 1360–1369
26. Taniguchi, K., Tosa, H., Suzuki, K., Kamo, Y. (1988) Microenvironment of two different extrinsic fluorescence probes in Na^+,K^+-ATPase changes out of phase during sequential appearance of reaction intermediates. *J. Biol. Chem.* **263**, 12943–12947
27. Guerra, M., Robinson, J.D., and Steinberg, M. (1990) Differential effects of substrates on three transport modes of the Na^+/K^+-ATPase. *Biochim. Biophys. Acta* **1023**, 73–80
28. Jørgensen, P.L. (1974) Isolation of (Na^+ plus K^+)-ATPase. *Methods Enzymol.* **32**, 277–290
29. Hayashi, Y., Mimura, K., Matsui, H., and Takagi, T. (1989) Minimum enzyme unit for Na^+/K^+-ATPase is the αβ-protomer. Determination by low-angle laser light scattering photometry coupled with high-performance gel chromatography for substantially simultaneous measurement of ATPase activity and molecular weight. *Biochim. Biophys. Acta* **983**, 217–229
30. Hayashi, Y., Takagi, T., Maezawa, S., and Matsui, H. (1983) Molecular weights of αβ-protomeric and oligomeric units of soluble (Na^+, K^+)-ATPase determined by low-angle laser light scattering after high-performance gel chromatography. *Biochim. Biophys. Acta* **748**, 153–167
31. Teramachi, S., Imagawa, T., Kaya, S., and Taniguchi, K. (2002) 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. *J. Biol. Chem.* **277**, 37394–37400
32. Askari, A. and Huang, W. (1982) Na^+, K^+-ATPase: evidence for the binding of ATP to the phosphoenzyme. *Biochem. Biophys. Res. Commun.* **104**, 1447–1453
33. Fukushima, Y., Yamada, S., and Nakao, M. (1984) ATP inactivates hydrolysis of the K^+-sensitive phosphoenzyme of kidney Na^+,K^+-transport ATPase and activates that of muscle sarcoplasmic reticulum Ca^{2+}-transport ATPase. *J. Biochem.* **95**, 359–368
34. Taniguchi, K., Suzuki, K., Kai, D., Matsuoka, I., Tomita, K., and Iida, S. (1984) Conformational change of sodium- and potassium-dependent adenosine triphosphatase. Conformational evidence for the Post-Albers mechanism in Na^+- and K^+-dependent hydrolysis of ATP. *J. Biol. Chem.* **259**, 15228–15233
35. Ganjeizadeh, M., Zolotarjova, N., Huang, W.H., and Askari, A. (1995) Interactions of phosphorylation and dimerizing domains of the α-subunits of Na^+/K^+-ATPase. *J. Biol. Chem.* **270**, 15707–15710
36. Hayashi, Y., Shinji, N., Tahara, Y., Hagiwara, E. and Takenaka, H. (2005) Comparison of ouabain binding amount among oligomeric protein components constituting Pig Kidney Na^+/K^+-ATPase. *J. Gen. Physiol.* **126**, 36a
37. Martin, D.W. and Sachs, J.R. (2000) Ligands presumed to label high affinity and low affinity ATP binding sites do not interact in an $(\alpha\beta)_2$ diprotomer in duck nasal gland Na^+,K^+-ATPase, nor Do the sites coexist in native enzyme. *J. Biol. Chem.* **275**, 24512–24517
38. Kanazawa, T., Saito, M., and Tonomura, Y. (1967) Formation and decomposition of a phosphorylated intermediate in the reaction of Na plus-K plus dependent ATPase. *J. Biochem.* **67**, 693–711
39. Tsuda, T., Kaya, S., Yokoyama, T., Hayashi, Y., and Taniguchi, K. (1998) Half-site modification of Lys-480 of the Na^+,K^+-ATPase α-chain with pyridoxal 5′-diphospho-5′-adenosine reduces ATP-dependent phosphorylation stoichiometry from half to a quarter. *J. Biol. Chem.* **273**, 24334–24338

40. Tsuda, T., Kaya, S., Yokoyama, T., Hayashi, Y., and Taniguchi, K. (1998) ATP and acetyl phosphate induces molecular events near the ATP binding site and the membrane domain of Na^+,K^+-ATPase. The tetrameric nature of the enzyme. *J. Biol. Chem.* **273**, 24339–24345
41. Sweadner, K.J. and Donnet, C. (2001) Structural similarities of Na,K-ATPase and SERCA, the Ca^{2+}-ATPase of the sarcoplasmic reticulum. *Biochem. J.* **356**, 685–704
42. Pedemonte, C.H. and Kaplan, J.H. (1990) Chemical modification as an approach to elucidation of sodium pump structure-function relations. *Am. J. Physiol.* **258**, C1–C23
43. Imagawa, T., Kaya, S., and Taniguchi, K. (2003) The amino acid sequence 442GDASE446 in Na/K-ATPase is an important motif in forming the high and low affinity ATP binding pockets. *J. Biol. Chem.* **278**, 50283–50292
44. Askari, A. and Koyal, D. (1968) Different oligomycin sensitivities of the Na^++K^+-activated adenosinetriphosphatase and itspartial reactions. *Biochem. Biophys. Res. Commun.* **32**, 227–232
45. Mårdh, S. (1975) Bovine brain Na^+, K^+-stimulated ATP phosphohydrolase studied by a rapid-mixing technique. Detection of a transient [^{32}P]phosphoenzyme formed in the presence of potassium ions. *Biochim. Biophys. Acta* **391**, 464–473
46. Abe, K., Kaya, S., Imagawa, T., and Taniguchi, K. (2003) K^+ induced simultaneous liberation of two moles of P_i, one from one mole of EP and the other from EATP, of oligomeric H/K-ATPase from pig stomach. *Ann. N.Y. Acad. Sci.* **986**, 281–282
47. Hayashi, Y., Kobayashi, T., Nakajima, T., and Matsui, H. (1994) Protomer association of solubilized Na^+/K^+-ATPase induced by ATP in *The Sodium Pump. Structure, Mechanism, Hormonal Control and its Role in Disease* (Bamberg, E. and Schoner, W., eds.) pp. 453–456, Dietrich Steinkopff Verlag GmbH & Co. KG, Darmstadt
48. Rice, W.J., Young, H.S., Martin, D.W., Sachs, J.R., and Stokes, D.L. (2001) Structure of Na^+,K^+-ATPase at 11-Å resolution: comparison with Ca^{2+}-ATPase in E1 and E2 states. *Biophys. J.* **80**, 2187–2197