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Fluorescein 5′-Isothiocyanate-Modified Na^+,K^+-ATPase, at Lys-501 of the α-Chain, Accepts ATP Independent of Pyridoxal 5′-Diphospho-5′-Adenosine Modification at Lys-480[1]

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The modification of Na^+,K^+-ATPase with increasing pyridoxal 5′-diphospho-5′-adenosine (AP_2PL) concentrations resulted in saturation of the ~0.5 mol AP_2PL probe incorporation into the Lys-480/mol catalytic α-chain and reduced the Na^+,K^+-ATPase activity to around half without affecting the phosphorylation by acetyl phosphate (AcP), and led to increases in the AP_2PL fluorescence caused by ATP and AcP. Further modification with fluorescein 5′-isothiocyanate (FITC) resulted in ~0.9 mol FITC probe incorporation into the Lys-501/mol α-chain and reduced the activity to below 5% without affecting the phosphorylation by AcP and these fluorescence increases. The ATP binding capacity of the AP_2PL-FITC enzyme was shown to be at least 50% of that of the control enzyme (~0.8 mol/mol α-chain). This is the first direct demonstration that Na^+-bound FITC-modified enzymes accept ATP with an affinity for ATP ($K_{1/2} > 150$ μM) reduced by two orders of magnitude. The data also suggest half site reactivity of Lys-480 as to AP_2PL and all site reactivity of Lys-501 as to FITC in the catalytic subunits.

Key words: ATP binding, conformation change, fluorescein, Na^+,K^+-ATPase, pyridoxal.

The transport of sodium and potassium ions coupled with the hydrolysis of ATP is performed by Na^+,K^+-ATPase (*1-4*), which shows high-affinity ATP binding for phosphorylation in the presence of Na^+ and Mg^{2+}, and low-affinity binding for deocclusion of K^+ (*5-8*). To elucidate the mechanism of energy transduction in Na^+,K^+-ATPase, detailed knowledge of conformational changes followed by ATP binding is essential. Several ATP-dependent conformational changes in the reaction cycle have been characterized with an *N*-[*p*-(2-benzimidazolyl)phenyl] maleimide (BIPM) probe at Cys-964 (*9-12*). ATP-protectable modifications of P-type ATPase with pyridoxal compounds (*13-16*) and FITC (*17-20*) reduced the ATPase activity, respectively. Studies on chemical modification of Na^+,K^+-ATPase have been performed using ATP site-directed probes showing ATP-protectable modification and the inhibition of Na^+,K^+-ATPase activity (*21-24*). The occurrence of low-affinity ATP or ADP-analogue binding to the K^+-bound or E_2-form of FITC-modified Na^+,K^+-ATPase has been suggested (*23, 25, 26*). ATP also induces dynamic pyridoxal fluorescence changes of Na^+-bound pyridoxal-modified enzymes at Lys-480 in the presence of Mg^{2+} (*16*). However, detailed studies on the stoichiometries of ATP and probe binding, and the sites of modification have not been carried out. Such information would be very useful for determining whether Na^+,K^+-ATPase functions as a protomer, ($\alpha\beta$), diprotomer, $(\alpha\beta)_2$, or a higher oligomer, and whether high- and low-affinity sites for ATP exist independently or change alternatively during the enzyme cycle (*22, 26-32*).

MATERIALS AND METHODS

Methods have already been reported for the purification of Na^+,K^+-ATPase from pig kidney with sodium deoxycholate followed by NaI (*10*), and from dog kidney by SDS treatment (*29*) with specific activities of 600-1,200 and 2,000-2,400 μmol/mg/h, respectively, as well as the extent of ATP binding to the enzyme (*32*), transient fluorescence measurements, and estimation of the rate and extent of the fluorescence change (*12*). The enzymatic synthesis of [^{32}P]-AcP (*16*) and the synthesis of AP_2PL (*33*) have also been reported.

To determine the amounts of fluorescence probes bound to the α-chains, modified α-chains were isolated with a Superose-12 (Pharmacia) gel filtration column equilibrated with 0.2% SDS, 100 mM NaH_2PO_4-Na_2HPO_4 (pH 6.9), 0.02% NaN_3, and 1 mM β-mercaptoethanol. The amount of α-chain was determined from the absorbance at 280 nm with an extinction coefficient of 109,765 $M^{-1}\cdot cm^{-1}$ (*34*).

The amounts of PLP and AP_2PL probes were determined using phosphopyridoxyllysine compounds as standards

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Abbreviations: PLP, pyridoxal 5′-phosphate; AP_2PL, pyridoxal 5′-diphospho-5′-adenosine; FITC, fluorescein 5′-isothiocyanate; AcP, acetyl phosphate; CDTA, 1,2-cyclohexylenedinitrilotetraacetic acid; TPCK, tosylphenylalanyl chloromethyl ketone.

(*35*). To estimate the amount of the FITC probe bound to the α-chain, FITC-treated enzymes were (1 mg/ml) incubated with an equal weight of trypsin in the presence of 25 mM sucrose, 10 mM EDTA, and 25 mM imidazole-HCl (pH 7.4) for 12 h at 37°C, which solubilized the FITC fluorescence almost completely. The amounts of the FITC probe bound to the α-chains were estimated from the absorbance of the supernatant with an extinction coefficient of 75,000 $M^{-1}\cdot cm^{-1}$ at 495 nm (*36*), using FITC-bound-$N\alpha$-acetyl-L-lysine as a standard. Other details were essentially the same as already reported (*12, 16*). The experiments were performed using several lots of enzyme preparations and the data shown are typical examples.

RESULTS

Covalent Modification of Na$^+$,K$^+$-ATPase with Pyridoxal Compounds and the Modified Sites—The relationship between Na$^+$,K$^+$-ATPase activity and the amount of the pyridoxal probe bound to the α-chain showed that the activity decreased linearly to ~50% with an increase in the amount of the probe to ~0.5 mol pyridoxal probe/mol α-chain (Fig. 1). Further increases in the amounts of the probe resulted in only a slight decrease in the ATPase activity. The addition of 50 μM AP_2PL to enzyme preparations pretreated with 50 μM AP_2PL, containing ~0.5 mol AP_2PL/mol α-chain, did not result in greater inactivation in spite of the increase in the amount of the probe to 0.8 mol/mol α-chain (not shown).

To investigate the relationship between the inhibition and the sites of these modifications, PLP-labeled α-chains obtained from Na$^+$,K$^+$-ATPase preparations preincubated with various concentrations of PLP were digested with trypsin, which solubilized the fluorescence almost completely (*16*). The solubilized fluorescence peptides were separated by HPLC. A single major fluorescence peak appeared at a retention time of 28 min for the supernatant of samples preincubated with 50 or 20 μM PLP (Fig. 1, insets A and B). This has already been shown to represent a hexapeptide modified with PLP at Lys-480: Asn-Ser-Thr-Asn-Lys^{480}-Tyr (*16*). Several fluorescence peaks appeared, including the major peak at 28 min, for the sample preincubated with 200 μM PLP (Fig. 1, inset C). The data suggested that the PLP probe was preferentially incorporated at Lys-480 until the total incorporation reached ~0.5 mol PLP probe/α-chain, but at other Lys residues with further increases in the total incorporation. The elution profiles of tryptic peptides obtained from a 50 μM AP_2PL-treated preparation also showed a single major fluorescence peak at 30 min, indicating a hexapeptide modified with AP_2PL at Lys-480, as already shown (*16*). These data suggested that pyridoxal modification of half of Lys-480 in the α-chain induced resistance to modification of the other half of Lys-480.

Effect of FITC Modification on AP_2PL-Modified Enzymes—The ATP binding with CDTA and ATP-dependent phosphorylation with Mg^{2+} of Na$^+$-bound AP_2PL-enzymes, containing ~0.5 mol AP_2PL/mol α-chain, were around half of the control enzyme levels in the presence of 10 μM ATP. However, a dynamic AP_2PL fluorescence increase was also observed under the above phosphorylation conditions (Fig. 2A, inset). The data suggested that AP_2PL-bound α-chains might accept ATP in the presence of Mg^{2+} and Na$^+$ independent of ATP-dependent phosphorylation.

To clarify this point, the AP_2PL-enzyme preparation retaining 50% of the Na$^+$,K$^+$-ATPase activity and the phosphorylation capacity of the non-modified enzyme was treated further with increasing concentrations of FITC. The extent of phosphorylation by ATP (Fig. 2A, closed bars) and Na$^+$,K$^+$-ATPase activity (not shown) were reduced to below 5% without any significant influence on either the rate or extent of the ATP-induced AP_2PL fluorescence increase (Fig. 2A and inset), the amount of phosphorylation by AcP, or the rate and extent of the AcP-induced AP_2PL fluorescence increase (Fig. 2B and inset).

These data showed that ATP binding to the Na$^+$-bound AP_2PL-FITC-enzyme in the presence of Mg^{2+} induced the AP_2PL fluorescence increase independent of phosphorylation by ATP. In the following experiments, enzyme preparations treated with 50 μM AP_2PL first and then treated with 15 μM FITC containing 0.41 ± 0.07 mol AP_2PL probe and 0.88 ± 0.14 mol FITC probe/mol α-chain were used as AP_2PL-FITC-enzyme preparations, which retained only slight ATP-dependent phosphorylation capacity (<5%), as described above. The ATP-induced increase in AP_2PL fluorescence suggested that AP_2PL-FITC-enzymes complexed with Mg^{2+}, Na$^+$, and ATP might accumulate, because the fluorescence increase required the simultaneous presence of these three ligands (not shown). However, no significant ^{32}P binding was detected with the centrifugation method in the presence of 4 mM Mg^{2+}, 16 or 160 mM

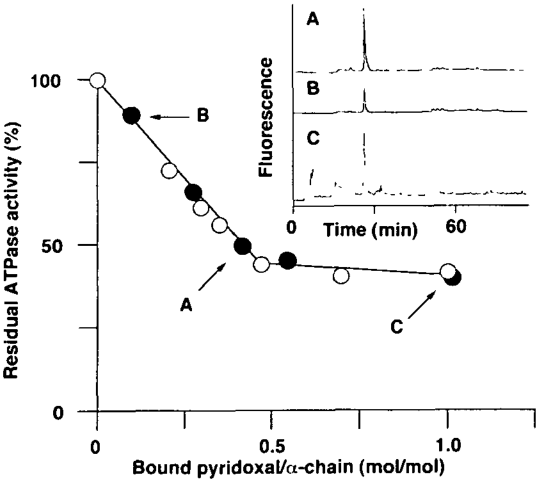


Fig. 1. **Relationship between Na$^+$,K$^+$-ATPase activity and the extent of PLP or AP_2PL probe binding to the α-chain.** A Na$^+$,K$^+$-ATPase preparation (1 mg protein/ml) from pig kidney was incubated with 0, 20, 30, 50, 100, and 200 μM PLP or AP_2PL in a mixture containing 25 mM sucrose, 5 mM $MgCl_2$, and 20 mM Hepes-NaOH, giving 25 mM Na$^+$, pH 7.8, for 10 min at 25°C. After reduction with 4 mM $NaBH_4$, Na$^+$,K$^+$-ATPase activity was measured (*16*). The activity without pyridoxal probe treatment was used as the 100% value. The residual Na$^+$,K$^+$-ATPase activities were plotted against the amount of the PLP (closed circles) or AP_2PL (open circles) probe bound to the α-chain, as described in the text. Insets: Soluble materials in the tryptic digest of PLP-modified α-chains obtained from 50 (A), 20 (B), and 200 μM (C) PLP-treated samples were subjected to HPLC on a C_{18} reversed-phase column (ODS-120T, Tosoh). The bound peptides were eluted, with monitoring of the absorbance at 215 nm (not shown) and the fluorescence of the pyridoxal moiety (excitation at 320 nm and emission at 390 nm), as described (*16*).

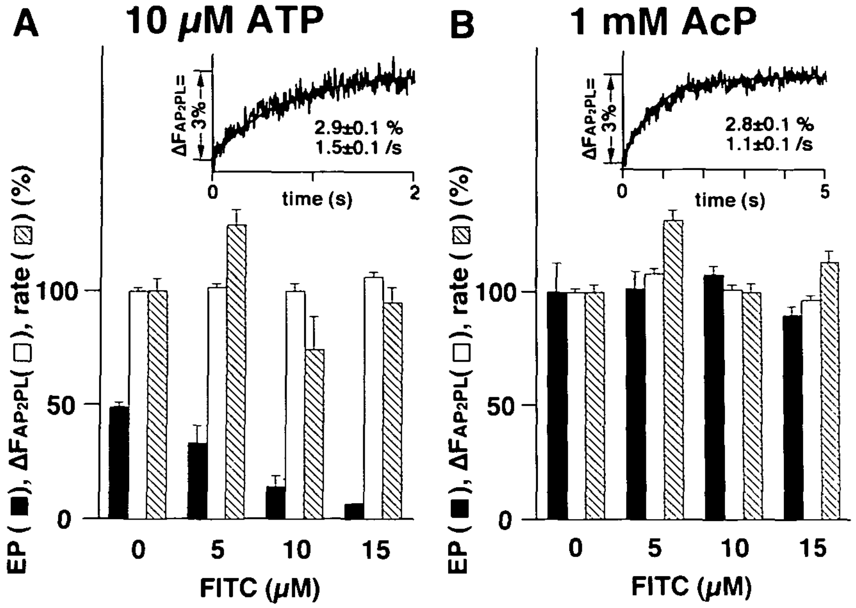
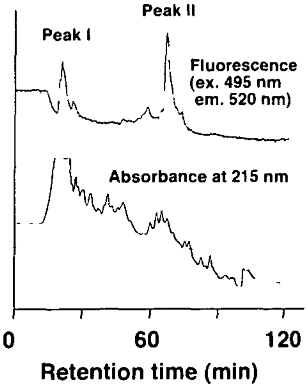


Fig. 2. **Effect of FITC treatment of the AP_2PL-modified enzyme on ATP and AcP-induced phosphorylation and the AP_2PL fluorescence change.** An AP_2PL-modified Na$^+$,K$^+$-ATPase preparation from pig kidney containing ~0.5 mol AP_2PL /mol α-chain was further treated with 5, 10, or 15 μM FITC in a mixture containing 50 mM sucrose, 2 mM EDTA, and 100 mM Tris-HCl, pH 9.2, for 30 min at 25°C. The phosphorylation reaction, and the rate and extent of the fluorescence increase (excitation at 320 nm and emission at 390 nm) were followed in a reaction mixture containing 25 mM imidazole-HCl (pH 7.4), 25 mM sucrose, 0.1 mM EDTA-Tris, 16 mM NaCl, and 4 mM $MgCl_2$ with 10 μM ATP and 1 mM AcP, with 1 mg and 0.03 mg protein/ml, respectively. The amounts of phosphoenzyme (closed bars) formed from 10 μM [γ-^{32}P]ATP (Amersham) in 10 s (A), and from 1 mM [^{32}P]AcP in 30 s (B), both at 0°C, were measured as described (*32*). The rate (stripe bars) and extent (open bars) of the fluorescence increase at 25°C induced by 10 μM ATP (A) or 1 mM AcP (B) were estimated by single exponential curve fitting (*12*). The 100% values of the amounts of phosphoenzyme of the non-AP_2PL-treated enzyme formed from 10 μM ATP and 1 mM AcP were 1.3±0.1 (A) and 1.1±0.1 nmol/mg protein (B), respectively. The extent and rate of the AP_2PL fluorescence increase of the AP_2PL-modified enzyme are shown in the insets. The data shown are the means±SD for 4 samples in phosphorylation experiments, and 7 to 10 accumulated data in stopped flow experiments.

Na$^+$ and 10 μM [α-^{32}P]ATP or [γ-^{32}P]ATP without or with both 1 mM phosphoenolpyruvate and 10 units/ml pyruvate kinase to keep the ATP concentration constant in the presence of 160 mM Na$^+$ (*37*). However, the centrifugation method permitted the detection of not only ATP binding to Na$^+$,K$^+$-ATPases in the presence of CDTA, as described later (Fig. 4A), but also [^{32}P] bound to non-modified control enzymes in the presence of 4 mM Mg^{2+}, 16 mM Na$^+$, and 100 μM [γ-^{32}P]ATP, which was around 70% of the radioactivity of the acid-stable phosphoenzyme. The data suggested that the enzyme-form exhibiting high AP_2PL fluorescence intensity accumulated on the addition of 10 μM ATP in the presence of Mg^{2+} and Na$^+$ had already liberated ATP or ADP and P_i.

Site of FITC Modification in the AP_2PL-FITC Enzyme—To determine whether AP_2PL modification at Lys-480 changed the modification site of the FITC probe at Lys-501, fluorescein peptides derived from the AP_2PL-FITC enzymes solubilized with TPCK trypsin were purified as shown (Fig. 3). The sequence of the purified peptides was determined to be Val^{499}-Met-*X*-Gly-Ala-Pro-Glu-Arg^{506}, where *X* corresponds to Lys-501 in the sequence of Na$^+$,K$^+$-ATPase (*38*), as already reported (*20, 39*). The data showed that FITC modification at Lys-501 occurred independently of AP_2PL modification at Lys-480.

ATP Binding to the AP_2PL-FITC-Enzyme—The data given above clearly showed that ATP could bind to the Na$^+$-bound AP_2PL-FITC-enzyme in the presence of Mg^{2+} to induce an AP_2PL fluorescence increase at Lys-480, abolishing subsequent phosphorylation. These data also suggested that ATP binding to the Na$^+$-bound AP_2PL-FITC enzyme required Mg^{2+} absolutely or that the enzyme had reduced affinity for ATP in the absence of Mg^{2+}. To investigate this point, the extents of ATP binding in the presence of CDTA were measured with increasing concentrations of ATP. The maximum extent of ATP binding to



Cycle	Amino acid		α-chain amino acid residue
		pmol	
1	Val	(126)	499
2	Met	(73)	500
3	*X*		501
4	Gly	(28)	502
5	Ala	(22)	503
6	Pro	(13)	504
7	Glu	(9)	505
8	Arg	(5)	506
9	End		

Fig. 3. **Elution profile of FITC-labeled peptides on reverse-phase HPLC and the amino acid sequence.** The AP_2PL-FITC enzyme preparation (5 mg protein/ml) from pig kidney was digested with 0.1 weight of TPCK-trypsin (Sigma) in 25 mM imidazole-HCl (pH 7.4) at 37°C for 3 h. Digestion was terminated by the addition of formic acid to give 2.7% and then the sample was centrifuged at 70,000 rpm for 10 min at 2°C (Optima, TLA 100.3; Beckman). Up to 90% of the FITC fluorescence became solubilized. The soluble material was subjected to HPLC on a 3 ml Resource™ RPC column (Pharmacia). The column was washed with 0.1% trifluoroacetic acid for 10 min, and then the bound peptides were eluted with a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid (0-5 min, 0-7%; 5-120 min, 7-42%; 120-130 min, 42-70%) at the flow rate of 0.5 ml/min. The relative FITC fluorescence intensity and absorption *versus* retention time were followed (left). A peak at 28 min (Peak I) exhibited no FITC fluorescence when measured with a fluorescence spectrophotometer. The main FITC-labeled peptide containing ~90% of the total FITC fluorescence at a retention time of 66~74 min (Peak II) was subjected to HPLC on a 1 ml Resource™ PHE, column (Pharmacia) equilibrated with 0.1% trifluoroacetic acid, and the bound peptides were eluted with the same gradient system as described above. A single fluorescent peak appeared (not shown) and an aliquot of the sample was sequenced (right).

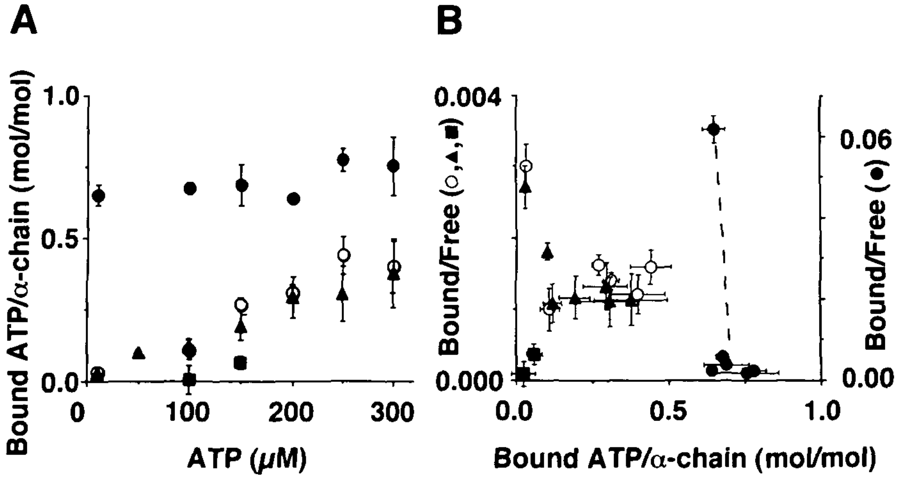


Fig. 4. **ATP binding to AP$_2$PL-FITC-, FITC-modified, and control non modified Na$^+$,K$^+$-ATPase.** A: The reaction mixtures (100 μl) containing 30 μg of protein of the AP$_2$PL-FITC-modified (open circles and closed squares), FITC-modified (closed triangles), and control non-modified (closed circles) Na$^+$,K$^+$-ATPase preparations from pig kidney were incubated with the buffer described in Fig. 2 except that ATP and AcP were replaced with 10-300 μM [α-^{32}P]ATP and 4 mM Mg^{2+} was replaced with 10 mM CDTA-Tris (pH 7.4), without or with 50 mM nonradioactive ATP, for 1 min at 2°C. In some experiments 16 mM NaCl was replaced with 1.6 mM KCl (closed squares). The samples were centrifuged at 100,000 rpm for 10 min at 2°C. The precipitates were resuspended and then counted. The differences between the counts without and with nonradioactive ATP were taken as the extents of ATP binding (*32*). The data points represent the means for triplicate samples with SD. The data obtained (nmol ATP bound/mg protein) were converted to mol ATP bound/α-chain to permit direct comparison with the stoichiometry of probes bound to the enzymes and to exclude ambiguity due to the difference in the specific activities of the enzyme preparations. The α-chain/mg protein contents were estimated from the maximum amount of enzyme-ouabain complex/mg protein obtained in the presence of 5 mM P$_i$, 5 mM Mg^{2+}, and 10 μM [^{3}H]ouabain, as already reported (*32*), because the maximum amount of enzyme-ouabain complex was shown to be 1 mol/mol α-chain (*49, 50*), and the ratio of the maximum amount of enzyme-ouabain complex/the maximum amount of phosphoenzyme was ~2, independent of the specific activity of the enzyme from pig or dog kidney (not shown), as already reported (*32*). B: Scatchard plot of the data obtained. The symbols are the same as in A. Because of the rather large scattering of data points obtained in the presence of higher concentrations of free ATP, only a dotted line for the control enzyme (closed circles) was drawn by eye fitting. The K_d value was calculated from the reciprocal of the slope to be 0.9 μM.

the non-modified Na$^+$-bound enzymes (Fig. 4, A and B, closed circles) was 0.7-0.8 mol/mol α-chain, with a K_d value ~0.9 μM. ATP binding to the Na$^+$-bound AP$_2$PL-FITC-enzyme (Fig. 4A, open circles) increased with increasing concentrations of ATP. When 16 mM Na$^+$ was replaced with 1.6 mM K$^+$, the ATP binding (Fig. 4A, closed squares) decreased to near the basal level and ~1/4 level in the presence of 100 and 150 μM ATP, respectively. The decrease in ATP binding caused by K$^+$ is consistent with the data obtained in flow dialysis experiments using nonmodified enzyme preparations (*31, 40*). Na$^+$-bound FITC-enzymes (Fig. 4A, closed triangles) containing 0.9 mol FITC probe/mol α-chain showed similar ATP binding. The data clearly showed that FITC modification reduced the affinity for ATP, retaining K$^+$-sensitivity to reduce the affinity further without blocking of the binding site for ATP but with abolition of the ATP-dependent phosphorylation.

The amounts of phosphoenzyme in the AP$_2$PL-FITC-enzyme preparations in the presence of 10 to 200 μM ATP, Na$^+$ and Mg^{2+} were nearly constant, ~0.03 mol/mol α-chain, due to the phosphorylation of the residual non-modified enzyme, which was around 10% of the maximum ATP binding detected for the AP$_2$PL-FITC- and FITC-enzymes (Fig. 4, A and B).

DISCUSSION

Due to the decreased affinity for ATP binding of the AP$_2$PL-FITC- and FITC-enzymes, accurate binding measurements with saturating concentrations of ATP were hindered. However, it could be safely concluded that the Na$^+$-bound AP$_2$PL-FITC- and FITC-enzymes retained ATP binding capacity at least ~0.4 mol/mol α-chain on the order of $K_{1/2}>$~150 μM in the presence of CDTA, with much higher apparent affinity in the presence of Mg^{2+} because 10 μM ATP was sufficient to saturate the AP$_2$PL fluorescence increase of the Na$^+$-bound AP$_2$PL-FITC-enzyme in its presence (not shown). The reason for the higher affinity in the presence of Mg^{2+} would be that the MgATP complex could bind to the ATP binding sites more tightly or Mg^{2+} induced a conformational change of the enzyme such that it tightly accepted MgATP and/or ATP. However, no significant ^{32}P binding to the AP$_2$PL-FITC-enzyme was detected in the presence of Mg^{2+}, Na$^+$, phosphoenolpyruvate, and pyruvate kinase, as described. These data suggested that these modifications changed the reactivity of the γ-phosphoryl group of the enzyme bound-ATP from the carboxyl group at Asp-369 to H_2O without changing the reactivity of the phosphoryl group of AcP to the carboxyl group. Although Lys-480 was reported to be replaceable with Arg, Ala, and Glu without a significant change in the Na$^+$,K$^+$-ATPase (*41*), further studies are needed to clarify the roles of Lys-480 and 501 in the enzyme.

After modification of ~0.5 mol AP$_2$PL at the Lys-480/mol α-chain, the other half became resistant to AP$_2$PL modification (Fig. 1). However, FITC modification occurred in all sites at Lys-501, reducing both the ATPase activity and ATP-dependent phosphorylation to below 5%. It seems unlikely that ~50% of the pig kidney Na$^+$,K$^+$-ATPase catalytic subunit with no Lys-480 was present in the preparations, because both Lys-480 and Lys-501 are conserved in various Na$^+$,K$^+$-ATPases (*15, 38, 39*). The resistance might have been due to the AP$_2$PL probe binding to half of Lys-480 in an α-chain in such a way as to inhibit the reaction of the remaining Lys-480 in another α-chain with AP$_2$PL due to some conformational change or simple steric hindrance. The present data might indicate that the Lys-480 residues were close enough to sense either modification by AP$_2$PL. The ratio of the maximum amount of the ouabain-enzyme complex to that of phosphoenzyme formed from ATP in Na^+,K^+-ATPase under steady-state conditions was shown to be ~2 (*32*), which indicated that only half sites and full sites were available for the phosphorylation and ouabain binding, respectively. The simultaneous presence of high- and low-affinity ATP-binding sites has also been reported (*22-24*).

If we assume that Na^+,K^+-ATPase acts as a diprotomer because of the half-site reactivities described above, ATP binding to the AP_2PL modified subunit followed by hydrolysis without phosphorylation would seem to sustain Na^+,K^+-ATPase activity of the non-modified subunit. However, the situation may be more complicated, because the ratio, ~2, given above suggested the phosphorylation capacities of the control enzyme and AP_2PL-enzyme preparation/α-chain to be ~1/2 and ~1/4, respectively. To clarify the oligomeric nature of the enzyme and whether ATP sites change alternatively requires further experiments such as following of the ATP- and AcP-induced molecular events in each catalytic subunit in Na^+,K^+-ATPase, monitoring the AP_2PL probe at Lys-480 and the FITC probe at Lys-501, respectively. Similar half- and full-site reactivities have been reported for H^+,K^+-ATPase (*44*) and Ca^{2+},Mg^{2+}-ATPase (*45*).

To our knowledge, direct ATP binding to AP_2PL-FITC treated enzymes in P-type ATPases has not been demonstrated except in the present study. H^+,K^+- (*42, 43*) and Ca^{2+},Mg^{2+}-ATPases (*14, 18*) have similar but two different Lys residues reactive to pyridoxal and FITC, respectively. It has been reported that FITC-treated sarcoplasmic reticulum Ca^{2+},Mg^{2+}-ATPase vesicles had ~1/5 of the ATP binding sites of the control vesicles with reduced affinity for ATP (*46*). X-ray crystallographic data showed that diiodofluorescein binds to the AMP- and ADP-binding domain of hexokinase (*47*), and to the same location as the adenosine ring of NAD in lactate dehydrogenase (*48*). These data suggest that the ATP-protectable FITC binding domain is not always a nucleotide binding domain and/or that the ligand-dependent conformational state of proteins in solution is more flexible than one might imagine.

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