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*

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Takeo Tsuda†, Shunji Kaya‡, Takeshi Yokoyama‡, Yutaro Hayashi§, and Kazuya Taniguchi¶

From the ‡Division of Chemistry, Graduate School of Science, Hokkaido University, Sapporo 060 and the §Department of Biochemistry, School of Medicine, Kyorin University, Mitaka, Tokyo 181, Japan

The addition of ATP to Mg\(^2+\)-Na\(^+\)-bound-probe labeled Na\(^+\),K\(^+\)-ATPase preparations containing \(-0.5\) mol of pyridoxal 5\(^'-\)diphospho-5\(^'-\)adenosine (AP\(_2\)PL) probe at Lys-480 and \(-0.9\) mol of fluorescein 5\(^'-\)isothiocyanate (FITC) probe at Lys-501 showed a decrease and an increase in the AP\(_2\)PL fluorescence intensity with neither significant ATP-dependent phosphorylation nor FITC fluorescence change. The rate constants for the fluorescence change increased nearly linearly with increasing ATP concentrations. The substitution of AcP for ATP decreased the FITC fluorescence intensity. Each rate constant for the BIPM fluorescence change was followed by measuring fluorescence changes of the AP\(_2\)PL probe at Lys-480, which reduced the value to \(-0.2\) mol of the FITC probe at Lys-501, which reduced the value to \(-0.03\). The FITC treatment had a negligible effect on the rate and the extent of individual ATP- and AcP-dependent fluorescence changes due to the AP\(_2\)PL chromophore and the amount of AcP-dependent half-site phosphorylation in the presence of Mg\(^2+\) and Na\(^+\) (18, 19). These data are consistent with the hypothesis that phosphorylation is not required for ATP-induced AP\(_2\)PL fluorescence change of the AP\(_2\)PL and AP\(_2\)PL-FITC enzymes and that four ATP binding sites are present in Na\(^+\),K\(^+\)-ATPase. In this paper, ATP- or AcP-induced conformational events were followed by measuring fluorescence changes of the AP\(_2\)PL probe at Lys-480 and the FITC probe at Lys-501 using the AP\(_2\)PL-FITC enzymes. Since the N\(^\text{-(2-benzimidazolyl)phenyl}\)-maleimide (BIPM) treatment had little effect on both Na\(^+\),K\(^+\)-ATPase activity and the phosphorylation capacity (7), ATP-induced BIPM fluorescence changes at Cys-964 of the \(\alpha\)-chain, which is near the transmembrane domain M9 of the enzyme (20), were also followed whether or not ATP induced multiple conformational changes in the BIPM enzyme.

The data indicate the occurrence of four ATP binding induced out of phase fluorescence changes not only near the ATP binding domain, but also in the membrane domain.

MATERIALS AND METHODS

Experimental methods were exactly as described in previous papers unless otherwise indicated (18, 19). Steady-state fluorescence measurements were performed using a Shimadzu RF-503 difference spectrofluorophotometer at 25°C (8) with a sample of 3.2 ml (50 μg of protein/ml) under the same reaction conditions as for the Na\(^+\)-ATPase measurements as described in Fig. 1 legend, except in some experiments, ATP was replaced with AcP. The fluorescence excitation was at 320 (AP\(_2\)PL) and 470 (FITC) nm, and the emission was detected at 390 (AP\(_2\)PL) and 520 (FITC) nm, respectively.

Transient fluorescence measurements were performed using an Ap-

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† To whom correspondence and reprint requests should be addressed. Tel./Fax: 81-11-736-2074.

‡ The abbreviations used are: CDTA, 1,2-cyclohexylenedinitrilotetraacetic acid; BIPM, N\(^\text{-(2-benzimidazolyl)phenyl}\)-maleimide; AP\(_2\)PL, pyridoxal 5\(^'-\)diphospho-5\(^'-\)-adenosine; FITC, fluorescein 5\(^'-\)isothiocyanate; AcP, acetyl phosphate.
RESULTS

Steady State Fluorescence Measurement of ATP- and AcP-induced Fluorescence Changes in the Mg\textsuperscript{2+}-Na\textsuperscript{+} -ATPase Activity—Previous stopped flow experiments showed that the addition of ATP and AcP to the Mg\textsuperscript{2+}-Na\textsuperscript{+} -ATPase activity fully accumulated to a level of \( \sim 0.5 \) mol/mol of \( \alpha \)-chain as is shown in the accompanying article (19). The addition of ouabain further increased AP\textsubscript{2PL} fluorescence to accumulate ouabain-bound phosphoenzyme but had little effect on FITC fluorescence (Fig. 1B, top and bottom). Further addition of ATP to ouabain-bound phosphoenzyme had a negligible effect on AP\textsubscript{2PL} fluorescence intensity (Fig. 1B, top).

The AP\textsubscript{2PL} fluorescence intensity at Lys-480, which accompanied the accumulation of ouabain-bound phosphoenzyme was higher than that of K\textsuperscript{+}-sensitive phosphoenzyme from AcP (Fig. 1B, top). Such a conformational difference detected by fluorescence change has not been detected with the BIPM probe at Cys-964 (8) or the FITC probe at Lys-501 (14) to date. The FITC fluorescence change induced by AcP in the presence of Mg\textsuperscript{2+} and Na\textsuperscript{+} is known to be reversible (17), as in the case for AP\textsubscript{2PL} fluorescence change by ATP described above.

Stopped-Flow Measurement of ATP-induced AP\textsubscript{2PL} Fluorescence Changes of the Mg\textsuperscript{2+}-Na\textsuperscript{+} -bound AP\textsubscript{2PL}-FITC Enzyme—To follow the initial ATP-induced phase of the AP\textsubscript{2PL} fluorescence change of the Mg\textsuperscript{2+}-Na\textsuperscript{+} -bound AP\textsubscript{2PL}-FITC enzymes, various concentrations of ATP were added to the AP\textsubscript{2PL}-FITC enzyme (Fig. 2A). The addition of 1 and 3 \( \mu \)M ATP induced a biphasic AP\textsubscript{2PL} fluorescence change similar to that observed for the AP\textsubscript{2PL} enzyme (19), namely a rapid decrease, followed by a slow increase. The rapid decrease apparently disappeared because of an acceleration in the slow increase in the presence of up to 10 \( \mu \)M ATP. However, the extent of the fluorescence increase was near saturation at around 10 \( \mu \)M ATP (Fig. 2B, inset).

The rate constants for the rapid decrease and the slow increase increased in a nearly linear fashion with increasing concentrations of ATP (Fig. 2B), suggesting the participation of at least two different ATP bindings. The slopes of the two straight lines (Fig. 2B) permitted a semiquantitative estimation of the apparent second order rate constants (\( k_{\text{on}} \)) to be \( 1.8 \times 10^6 \) and \( 0.1 \times 10^6 \) mol\(^{-1}\) s\(^{-1}\), respectively, for ATP for the formation of the two different conformational states with negative and positive AP\textsubscript{2PL} fluorescence intensity, compared with the Na\textsuperscript{+}-bound enzyme. The dissociation rate constant (\( k_{\text{off}} \)) and the dissociation constant (\( K_d = k_{\text{off}}/k_{\text{on}} \)) for the latter were estimated from the intercept, 0.82 s\(^{-1}\), and the ratio, 7.5 \( \mu \). These data suggest that at least two different ATP bindings with a \( \sim 20\)-fold difference in \( k_{\text{on}} \) value induced quite different AP\textsubscript{2PL} fluorescence changes in the Mg\textsuperscript{2+}-Na\textsuperscript{+} -bound AP\textsubscript{2PL}-FITC enzymes without significant phosphorylation (18, 19). ATP appears to be a better substrate than AcP in terms of increasing AP\textsubscript{2PL} fluorescence, as evidenced by the fact that each \( k_{\text{on}} \) and \( K_d \) was at least 100-fold and 1/40 of that for AcP (19).

When the concentration of added ATP was reduced to 0.3 \( \mu \)M ATP, the concentration of which was \( \sim 3 \) fold larger than the concentration of the \( \alpha \)-chain present (Fig. 2A, inset), the rate...
The time course for the ATP-induced AP2PL fluorescence change was measured after the addition of various concentrations of ATP under the same conditions as described in Fig. 1 except that the protein concentration was 250 μg/ml. A, each ratio of data for the AP2PL-FITC enzyme, which accumulated after the addition of 0.3, 1, 3, 10, and 50 μM ATP, to that of the corresponding concentration of Tris-HCl (pH 7.4) is shown. The starting positions for the entire curve for fluorescence change were offset. The smooth lines in the figure were obtained by fitting to single (10 and 50 μM) or double exponential functions (0.3, 1, and 3 μM). B, The rate constants for the rapid decrease (closed circles) and slow increase (open circles) with the extents (inset, closed and open circles) of the fluorescence changes obtained from single or double exponential fitting are plotted against the initial added ATP concentrations. Each data set of closed, and open circles were fitted to a single straight line, respectively, which represents the equation Y = 18 × 10^6 × [ATP] and Y = 0.11 × 10^6 × [ATP] + 0.82 where Y is the apparent first order rate constant, (1/s), for the initial fluorescence decrease and increase, respectively, and [ATP] is the initial ATP concentration added (in μM). The data shown are from the same enzyme preparations as in Fig. 1.

Stop-flow Measurement of ATP- and Na’-induced Fluorescence Changes of K’-bound AP2PL-FITC Enzyme—High concentrations of AP2PL are known to accelerate the transition of K’-bound enzyme to Na’-bound enzyme (21). The ATP binding capacity of the AP2PL-FITC enzyme was already shown to be at least 50% of that of the control enzyme (18). To investigate whether ATP accelerated the transition of K’-bound AP2PL-FITC enzyme to the Na’-bound enzyme, both AP2PL and FITC fluorescence changes were followed immediately after the addition of ATP and/or Na’ (22). The addition of 5 mM ATP induced fluorescence changes in either AP2PL (Fig. 3A, second trace) or FITC (Fig. 3B, third trace). The addition of 160 mM Na’ induced a decrease in the AP2PL fluorescence (Fig. 3A, third trace), 3.6%/μM, and a biphasic increase in FITC fluorescence (Fig. 3B, second trace), 4.4 and 0.8%/μM. The addition of 160 mM Na’ with 0.5 mM ATP induced a biphasic AP2PL change (Fig. 3A, bottom), a rapid decrease (21%/μM), followed by a slow increase (0.7%/μM) with only slight effect of ATP to increase in both the extent and the rate of the FITC fluorescence (trace not shown). The extent of the initial rapid decrease in AP2PL fluorescence with increasing the concentrations of ATP could not be detected accurately due to the subsequent increase (Fig. 4B, closed circles).

The addition of 160 mM sodium with 5 mM ATP induced a monophasic increase in AP2PL fluorescence (Fig. 3A, top), 6.4%/s, with a biphasic increase in FITC fluorescence (Fig. 3B, top), 7.0 and 1.2%/s, with slight increase in the extent. Although the addition of 5 mM Na’ induced slight increases in FITC fluorescence (Fig. 3B, bottom), the simultaneous addition of both 5 mM Na’ and 5 mM ATP induced larger biphasic increases in FITC fluorescence (Fig. 3B, fourth trace), 5.8 and 1.8%/s, which showed more clearly the effects of ATP on the extent of FITC fluorescence change.

Fig. 4 (A and B, respectively) shows the dependence of ATP concentration on the rate constants and the corresponding extent of both fluorescence changes in the presence of 160 mM Na’. Each rate constant for the decrease and the increase of AP2PL fluorescence increased with increasing concentrations of ATP accompanied by some deviation from linearity (Fig. 4A, closed and open circles). The concentration dependence of ATP on FITC fluorescence changes that accompanied the transition from the K’-form to the Na’-form also suggest two different extents of ATP-induced FITC fluorescence increases with only a small increase in the rate constants (Fig. 4, A and B, open and closed triangles). These data showed that level of ATP up to submillimolar levels exerted two different actions on each of the AP2PL and FITC fluorescence changes for the K’-bound AP2PL-FITC enzymes, namely that the same concentrations of ATP resulted in four different fluorescence changes with different rate constant and/or extents. However, approximately micromolar ATP resulted in two actions on the AP2PL fluorescence change of the Na’-bound AP2PL-FITC enzymes (Fig. 2B) with little FITC fluorescence change.

AcP-induced Fluorescence Changes and Phosphorylation of Mg’-Na’-bound AP2PL-FITC Enzyme—To investigate the relationship between the AcP-dependent phosphorylation and fluorescence changes, 1 mM AcP was added, the concentration of which is sufficient to saturate phosphorylation (19). This caused a decrease in the FITC fluorescence with rate constants of 8.5 and 1.2/s, and a decrease of 5.5 and 0.8%/s respectively (Fig. 5, second trace). The data could be also fitted to a single exponential curve with 7.1/s and 5.7%. Phosphorylation occurred with a rapid (7.2/s) and slow (4.6/s)
Concentration dependence of ATP on the rate and extent of fluorescence change of Mg\(^{2+}\)-K\(^{+}\)-bound AP\(_{2}\)PL-FITC enzyme. Data for the rate constant (A) and extent (B) of Fig. 3 and similar experiments using the same enzyme preparation with different concentrations of ATP were plotted against initial added ATP concentrations. AP\(_{2}\)PL decrease (closed circles) and increase (open circles), respectively, indicate the rate and extent of rapid decrease and slow increase of AP\(_{2}\)PL fluorescence; FITC rapid (closed triangles) and FITC slow (open triangles), respectively, indicates the rate and extent of rapid and slow increases of FITC fluorescence. Lines shown are drawn by visual fitting.

**Fig. 4**

AcP-induced FITC fluorescence changes of AP\(_{2}\)PL-FITC, FITC-AP\(_{2}\)PL, and FITC enzymes. Stopped-flow experiments were performed under the same conditions as in Figs. 2 and 3. Time courses for 1 mM AcP-induced FITC fluorescence changes of the Mg\(^{2+}\)-Na\(^{+}\)-bound AP\(_{2}\)PL-FITC enzymes, the FITC enzymes (the Na\(^{+}\),K\(^{+}\)-ATPase preparations treated with 15 \(\mu\)M FITC), and FITC-AP\(_{2}\)PL enzymes (the FITC enzymes treated with 50 \(\mu\)M AP\(_{2}\)PL) (19) are shown. Ten \(\mu\)M ATP-induced FITC fluorescence change in the AP\(_{2}\)PL-FITC enzyme is also shown. The starting positions of the entire curve for fluorescence change were offset. The rate constants (1/s) and the corresponding extent of fluorescence changes (%) are given in parentheses.

**Fig. 5**

Comparison of time course of AcP-dependent phosphorylation with AP\(_{2}\)PL fluorescence changes in AP\(_{2}\)PL-FITC enzyme. Stopped-flow and rapid-quenching experiments were performed as described in Fig. 5 and under “Materials and Methods.” Time courses of 1 mM AcP-induced AP\(_{2}\)PL fluorescence changes and phosphorylation of the Mg\(^{2+}\)-Na\(^{+}\)-bound AP\(_{2}\)PL-FITC enzymes are shown. AcP- and ATP-induced AP\(_{2}\)PL fluorescence changes in the FITC-AP\(_{2}\)PL enzyme are also shown. The starting positions for the entire curve for fluorescence change were offset.

**Fig. 6**

AcP-induced FITC fluorescence changes of AP\(_{2}\)PL-FITC, FITC-AP\(_{2}\)PL, and FITC enzymes. Stopped-flow experiments were performed under the same conditions as in Figs. 2 and 3. Time courses for 1 mM AcP-induced FITC fluorescence changes of the Mg\(^{2+}\)-Na\(^{+}\)-bound AP\(_{2}\)PL-FITC enzymes, the FITC enzymes (the Na\(^{+}\),K\(^{+}\)-ATPase preparations treated with 15 \(\mu\)M FITC), and FITC-AP\(_{2}\)PL enzymes (the FITC enzymes treated with 50 \(\mu\)M AP\(_{2}\)PL) (19) are shown. Ten \(\mu\)M ATP-induced FITC fluorescence change in the AP\(_{2}\)PL-FITC enzyme is also shown. The starting positions of the entire curve for fluorescence change were offset. The rate constants (1/s) and the corresponding extent of fluorescence changes (%) are given in parentheses.

phases, and showed nearly the same amount of components, namely 24 and 26%, to give half-site phosphorylation (Fig. 6, open circles), followed by two different AP\(_{2}\)PL fluorescence increases with rate constants of 1.4 and 0.2/s and an extent of 2.4 and 0.7%, respectively (Fig. 6, top trace). Such double exponential AP\(_{2}\)PL fluorescence changes cannot be obtained for the case of AcP-induced AP\(_{2}\)PL fluorescence of AP\(_{2}\)PL enzyme (19). The addition of 10 \(\mu\)M ATP induced neither FITC fluorescence change (Fig. 5, top) nor significant phosphorylation (18, 19) despite an increase in AP\(_{2}\)PL fluorescence (Fig. 2). However, the less bulky substrate, AcP, induced both AP\(_{2}\)PL (13, 18) and FITC (10, 11, 14) fluorescence change and phosphorylation as was described above.

The extent of FITC fluorescence decrease of the AP\(_{2}\)PL-FITC enzyme induced by 1 mM AcP was 6.3 (= 5.5 + 0.8) % (Fig. 5, second trace), while that of the FITC enzyme, which was not treated with AP\(_{2}\)PL, was 10.5 (= 10.1 + 0.4) % (Fig. 5, fourth trace), with similar rate constants. The relative FITC fluorescence changes accompanying the transition from the K\(^{+}\) form AP\(_{2}\)PL-FITC enzyme to the Na\(^{+}\) form enzyme with or without ATP were also approximately half of those of the FITC enzyme (16). These data suggest that the half-site modification of Lys-480 with AP\(_{2}\)PL reduced the extent of the FITC fluorescence change at Lys-501 by nearly 50%.

When the FITC enzyme, which contained ~0.9 mol of FITC probe at Lys-501, was treated with AP\(_{2}\)PL, the resulting FITC-AP\(_{2}\)PL enzyme showed neither an AcP- nor an ATP-induced AP\(_{2}\)PL fluorescence change (Figs. 6, second and third trace) but the FITC fluorescence changes were retained, similar to those of the FITC enzyme (Fig. 5, third and bottom traces). The data show that it is not possible to modify Lys-480 with AP\(_{2}\)PL when Lys-501 is already modified with FITC.

Stopped-flow Measurement of ATP-induced Fluorescence Changes of the Mg\(^{2+}\)-Na\(^{+}\)-bound BIPM Enzyme—The ATP-induced biphasic AP\(_{2}\)PL and FITC fluorescence changes (Figs. 2–4) and other experiments (14, 16, 18, 19) strongly suggest that the enzyme is oligomeric in nature. However, one may ask whether the complexity observed above is due to the results of chemical modification in the vicinity of the ATP protectable Lys residues. To investigate this point, ATP-induced conformational changes using the BIPM-modified enzyme containing ~1 mol of BIPM probe at Cys-964/\(\alpha\)-chain, which retained the full, original Na\(^{+}\),K\(^{+}\)-ATPase activity (7), were followed.

The addition of 0.01 and 0.03 \(\mu\)M ATP to the BIPM enzyme in the presence of Na\(^{+}\) and Mg\(^{2+}\) induced a single exponential increase in BIPM fluorescence (Fig. 7A). However, with increasing concentrations of added ATP, the BIPM fluorescence change deviated from a single exponential curve and could be fit to a double exponential curve. The extent of BIPM fluorescence changes increased to give maximum values, ~4 and ~3% with \(K_{d}\) values for ATP in the submicromolar range and then decreased with increasing concentrations of ATP, respectively (Fig. 7A, B, and inset). The apparent rate constant for the high fluorescence intensity was approximately 1 order of magnitude larger than that of the low intensity at the same ATP concentrations (Fig. 7B, open and closed circles). These apparent rate constants were significantly larger than the turnover number of Na\(^{+}\)-ATPase for the unmodified control enzyme, 0.35/s (19) indicating that these conformational changes were sufficiently
rapid to be candidates for reaction intermediates. Each plot of the apparent rate constant versus ATP concentrations gave two intersecting straight lines (Fig. 7B). These data suggest that each of the two different ATP bindings to the enzyme in the presence of low and high concentrations of ATP induced, respectively, the formation of two different conformational states that can be associated with the high and low BIPM fluorescence. The extents of both the high and low BIPM fluorescence increases with increasing concentrations of up to micromolar ATP (Fig. 7B, inset) which was also detected under steady-state fluorescence measurement using a difference spectrophotometer (not shown). The \( k_{-1} \) values for ATP were semiquantitatively estimated, assuming that these plots gave straight lines (Fig. 7B). The \( k_{-1} \) values for the formation of the conformation with higher BIPM fluorescence in the presence of low and high ATP concentrations were 18 \( \times 10^{6} \) and 4.2 \( \times 10^{6} \) M\(^{-1}\) s\(^{-1}\), the corresponding \( k_{-1} \) was 9.4 and 77 s\(^{-1}\), and the \( k_{-1} \) was 0.52 and 19 \( \mu \)M, respectively. The \( k_{+1} \) values of the conformation with lower BIPM fluorescence in the presence of low and high concentrations of ATP was 3.9 \( \times 10^{6} \) and 0.69 \( \times 10^{6} \) M\(^{-1}\) s\(^{-1}\), and the corresponding \( k_{+1} \) was 2.7 and 5.1 s\(^{-1}\) and \( k_{+1} \) was 0.69 and 7.5 s\(^{-1}\), respectively. These data suggest the occurrence of at least two different ATP bindings, respectively, which induce different BIPM fluorescence changes at Cys964 in the presence of low and high concentrations of ATP. It has already been shown that a dynamic BIPM fluorescence change at Cys964 requires the simultaneous presence of ATP, Na\(^{+}\), and Mg\(^{2+}\) (9) accompanying or followed by half-site phosphorylation (19), respectively. ATP can be replaced by neither AMP-PNP nor ADP (9).
clear evidence in favor of the oligomeric nature of the enzyme. Rather lower concentrations of ATP induced two different effects on the rate and extent of not only AP$_2$PL fluorescence of the Mg$^{2+}$-Na$^+$-bound AP$_2$PL-FITC enzymes (Fig. 2) but also BIPM fluorescence changes in the Mg$^{2+}$-Na$^+$-bound BIPM enzyme (Fig. 7). Plots of apparent rate constants against concentrations of ATP gave two straight lines for AP$_2$PL fluorescence (Fig. 2B) and two intersecting straight lines in the presence of low and high concentrations of ATP for the BIPM fluorescence (Fig. 7B). The linear increase in the apparent rate constant with increasing ATP concentrations has been explained to be the result of a large dissociation constant for ATP of a nonfluorescent precursor of fluorescent enzyme form, wherein the conformation (fluorescence) change is slow (23, 24). However, the data obtained favor another possibility that the ATP affects other ATP-induced fluorescence changes in the same molecule of a different subunit such as observed in the Mg$^{2+}$-Na$^+$-bound AP$_2$PL enzyme and the Mg$^{2+}$-Na$^+$-bound BIPM enzyme. More direct evidence for four different effects of ATP were detected with respect to both the rate and extent of AP$_2$PL and FITC fluorescence changes which accompanied the transition of the K$^+$-bound form to the Na$^+$-bound form (Figs. 3 and 4) of the AP$_2$PL-FITC enzyme. In actual fact, the enzyme preparations were shown to retain up to 50% of the ATP binding capacity with affinity reduced by 2 orders of magnitude (18). The decrease in the extents of BIPM fluorescence at the same Cys-964 with increasing concentrations of ATP (Fig. 7B, inset) also reflect some cooperative interaction between the a-chains which reduce the BIPM fluorescence intensity. These data are consistent with a hypothesis that all a-chains are capable of accepting ATP and interacting with adjacent a-chains, thus accelerating ATP-induced conformational changes. A similar decrease in Trp fluorescence with the absence of K$^+$ was 50% (Fig. 5, bottom traces) of the AP$_2$PL-FITC enzyme. In actually, it was 95% (Fig. 5, second and bottom traces). Further experiments will be required to explain the subsequent slow increases (1.4 and 0.2 s$^{-1}$) in AP$_2$PL fluorescence that occur for the case of two AP$_2$PL-FITC labeled a-chains, and ATP induced a quarter-site phosphorylation followed by biphasic AP$_2$PL fluorescence change in the AP$_2$PL enzyme (19). However, the present experiments demonstrate that sequential molecular events in each subunit occur out of phase in real time not only in the vicinity of the ATP binding domain, Lys-480 and Lys-501 (18, 22), but also in the vicinity of the transmembrane segment, Cys-964 (7, 20). Such dynamic conformational differences would not have been detected by proteolytic digestion patterns, which showed clear differences between the Na$^+$-bound enzyme and the K$^+$-bound or phosphorylated enzyme form (27).

Recently, a considerable body of experimental evidence that is consistent with multiple active sites has been reported, as was described in the accompanying article (19). The present data are consistent with the same concentrations of submillimolar ATP inducing different fluorescence changes that accompany the transition from the K$^+$ form to the Na$^+$ form of the AP$_2$PL-FITC enzyme (Fig. 4) and that up to 1 order of magnitude lower concentrations of ATP induce four different conformational changes of Mg$^{2+}$-Na$^+$-bound BIPM enzyme in the absence of K$^+$ with lower K$_D$ values, $0.5–1.9 \mu M$ as detected by the BIPM fluorescence change at Cys-964 (Fig. 7). One may ask if there is some heterogeneity in the enzyme preparations such as due to the presence of denatured enzyme, isozymes, and heterogeneous labeling other than Lys-480, Lys-501, and Cys-964. The former two possibilities have been shown in the accompanying article to be unlikely (19). The present data could not exclude some possibility of heterogeneous labeled enzymes as minor components to give a small amplitude of some of the ATP effect. However, the following data of ours also strongly suggest such a conclusion independent of the presence of such heterogeneity: (a) quarter-site reactivities of the phosphoenzyme (10, 14) and a quarter-site phosphorylation from P, in the presence of Mg$^{2+}$, Na$^+$, ADP, and ouabain (8) and a reduction of phosphorylation stoichiometry from half to a quarter after half-site modification of AP$_2$PL at Lys-480 (19); (b) half-site phosphorylation capacity (19) with showing nearly full-site ATP binding capacity (14, 18) and half-site modification of AP$_2$PL at Lys-480 (18); (c) full-site ouabain binding capacity (14) and full-site reactivity of FITC at Lys-501 (18).

In conclusion, the present data represent the first direct conformational evidence to support the hypothesis that the functional unit of membrane-bound Na$^+$,K$^+$-ATPase is a tetramotroter, composed of $\alpha\beta$ protomers (Fig. 5B).

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