Conformational Changes of Renal Sodium Plus Potassium Ion-transport Adenosine Triphosphatase Labeled with Fluorescein*

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We studied conformational changes of purified renal sodium plus potassium ion-transport adenosine triphosphatase (ATP phosphohydrolase, EC 3.6.1.3) labeled with fluorescein isothiocyanate. Fluorescein covalently binds to the α-subunit of the enzyme and inhibits the ATPase but not the p-nitrophenylphosphatase activity. Four unphosphorylated and three phosphorylated conformations were distinguished by the level of fluorescence and by the rate of its change. The thimerosal-treated fluorescein-enzyme retained fluorescence but the rate of transition from E2-K-Mg to E1-Na became progressively slower with increasing Mg2+ concentration.

Two phosphorylated conformations, (E2-P)•Mg (82%) and (E2-P)•Mg•K (82%) were differentiated by a faster turnover of the latter form. A third conformation, (E2-P)•Mg•ouabain, had the lowest fluorescence (56%) and its formation followed the binding of ouabain to the phosphoenzyme.

Reversible blocking of sulfhydryl groups with thimerosal inhibited the formation of E1•K and (E2-P)•Mg•ouabain but not that of the other conformations of the fluorescein-enzyme. The thimerosal-treated fluorescein-enzyme retained K•-p-nitrophenylphosphatase activity, inhibition of this activity by ouabain and ouabain binding.

The unphosphorylated enzyme had low (K0.5 = 1.2 mm) and the phosphoryzme had high affinity (K0.5 = 0.03 – 0.09 mm) for Mg2+ in the absence of nucleotides. Since low and high affinity for Mg2+ alternates as the enzyme turns over, Mg2+ may be bound and released sequentially during the catalytic cycle.

Several reactive states of (Na,K)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) could be distinguished by kinetic measurements (1) but these measurements did not provide information about the underlying changes in protein structure. Ligand-induced structural differences in the α-subunit (molecular weight, about 100,000) of the enzyme were directly detected by digesting the purified enzyme with trypsin (2-4) or chymotrypsin (5) since different sensitive bonds were exposed as the conformation changed. By analyzing the rate of inactivation and the split products three major conformations of the α-subunit of (Na,K)-ATPase were found: a Na form (E1) (2, 3), a K form (E2) (2, 3), and an ouabain-induced form (5). Phosphorylation from ATP (2, 3) or ouabain-binding (5) induced conformations similar to the K form. Another convenient and sensitive indicator of conformational changes in the protein is fluorescence of intrinsic tryptophan (6, 8) or of extrinsic fluorescent probes (9, 10). An extrinsic probe, fluorescein isothiocyanate binds covalently to the α-subunit at or near the ATP-binding site and, although it prevents reaction of the enzyme with ATP, it does not inhibit conformational changes induced by cations or by phosphorylation (9, 10). Experiments with these intrinsic and extrinsic probes have allowed the estimation of the rate of conformational changes (6, 9, 10).

While the role of Na+ and K+ in the catalytic cycle and in the conformational transitions has been examined in detail, the role of Mg2+ is not clear, mostly because in kinetic studies it is difficult to separate the effects of free Mg2+ from those of the Mg-ATP and Mg-ADP complexes (11-14). The fluorescence technique mentioned earlier allows a study of the interactions of both the dephospho forms and phospho forms of (Na,K)-ATPase with Mg2+ in the absence of nucleotides. It has also become feasible to examine whether the protein conformations of the K-bound form, the phosphoenzyme, and the ouabain-bound form of (Na,K)-ATPase are identical or whether they represent subconformations of the enzyme protein. Another interesting possibility is the use of selective chemical modification to study the underlying chemical mechanism of the transitions in the protein structure.

In this study we used purified, membrane-bound renal (Na,K)-ATPase (15) labeled with fluorescein isothiocyanate. The preparation contained exclusively the protein of (Na,K)-ATPase and fluorescein was bound only to the α-subunit (9, 10). The fluorescein-labeled (Na,K)-ATPase was therefore an ideal substrate for a detailed examination of the structural changes in the α-subunit. We have measured the level and the rate of change in fluorescence in response to reaction with specific ligands to monitor differences in protein structure between the K- or Na-bound forms, the phosphorylated forms, and the ouabain-bound forms of (Na,K)-ATPase are identical or whether they represent subconformations of the enzyme protein. The results show that subconformations of the E1 form can be characterized by...
their steady state fluorescence intensity. Our observations also allow a tentative classification of conformational changes by the underlying chemical mechanism since some conformational transitions were blocked by thimerosal, whereas others remained unaffected. The fluorescein-enzyme was also suitable for examining the interconversion of Mg$^{2+}$ with different conformations in the absence of ATP. The phosphoenzyme had high and the unphosphorylated enzyme had low affinity for Mg$^{2+}$. Our results provide evidence for tight binding of Mg$^{2+}$ to the protein during transition between the phosphorylated conformations. Mg$^{2+}$ affected the rate of dissociation of K-bound forms or Rb-bound forms of the protein in a way suggesting that the "occluded" K form of the enzyme also contained magnesium.

**MATERIALS AND METHODS**

(Na,K)-ATPase was purified in membrane-bound form from the red outer medulla of pig kidney as described (15). Specific activity of (Na,K)-ATPase was 28-44 pmol of P/mg of protein/min at 37 °C. The enzyme was stored at -75 °C in 10% (w/v) sucrose, 1 mM EDTA, and 25 mM imidazole-HCl, pH 7.5. Labeling with fluorescein isothiocyanate was done by a slight modification of the method of Karlish (8). Aliquots of (Na,K)-ATPase were sedimented by centrifugation for 10 min at 140,000 × g in a Beckman Airfuge and resuspended in 100 mM NaCl, 1 mM Tris-EDTA, and 50 mM Tris-Cl, pH 9.2, to a final concentration of 1.0-1.4 mg of protein/ml or about 5-6 μM (Na,K)-ATPase. (Molar concentration of the enzyme was estimated by assuming a molecular weight of 280,000 and one binding site of high affinity for ATP and ouabain, respectively.) Fluorescein isothiocyanate was dissolved in dimethyl formamide, to a concentration of 1.5 mM, and was added to the enzyme suspension with continuous stirring to a final concentration of about 20 μM. The final concentration of dimethyl formamide was less than 2% (v/v). After incubation at 20 °C for 45 min, the enzyme was sedimented in a Beckman Airfuge at 145,000 × g for 10 min and was washed four times with 50 mM Tris-Cl and 1 mM Tris-EDTA, pH 8.0 (Buffer E) by centrifugation and resuspension. The final sediment was resuspended in 10% (w/v) sucrose in Buffer E to a final concentration of 1 mg of protein/ml, divided in 100-μl portions, and stored at -75 °C, where it was stable for at least 1 month. The fluorescein-(Na,K)-ATPase could also be stored in Buffer E at 0 °C for 2 weeks without much loss of activity.

To block sulfhydryl groups with thimerosal, fluorescein-(Na,K)-ATPase (0.08 mg of protein/ml) was incubated with 2 mM thimerosal, 1 mM Tris-EDTA, and 25 mM imidazole-HCl, pH 7.5, in an ice bath. After 60 min the enzyme was sedimented at 140,000 × g for 4 °C for 3 h in a Beckman type 65 rotor; the sediment was resuspended in Buffer E and stored at 4 °C. To reverse inhibition by thimerosal, this enzyme was incubated with 3 mM dithiothreitol in Buffer E at room temperature for 60 min and assayed without removing dithiothreitol. For the fluorescein-(Na,K)-ATPase (15), (K)-NPPase (19), and protein (20) were measured by published procedures.

To estimate total phosphorylation 1 ml of the reaction mixture contained; 0.048 mg of fluorescein-(Na,K)-ATPase or 0.056 mg of thimerosal/fluorescein (Na,K)-ATPase, 1 μmol of MgCl$_2$, 1 μmol of [γ-32P]ATP (70 cpm/μmol), 0.5 μmol of ouabain, and 40 μg of Tris-Cl (pH 8.0). To estimate nonspecific phosphorylation, MgCl$_2$ was replaced by 1 μmol of Tris-EDTA in an otherwise identical mixture. After incubation at room temperature for 10 min, the reaction was stopped with 5 mM cold 5% (w/v) trichloroacetic acid containing 5% (v/v) phosphoric acid. The precipitated protein was collected by centrifugation at 4,500 × g for 30 min and washed three times with 5 ml of the same acid mixture. The final sediment was dissolved in 1 N NaOH, protein was measured by the Lowry procedure (20), and radioactivity was measured by scintillation counting. Specific phosphorylation of (Na,K)-ATPase is the difference between "total" and "nonspecific" phosphorylation. Nonspecific phosphorylation was less than 20% of the total.

Binding of [H]ouabain in the presence of 5 mM MgCl$_2$, and 5 mM Tris-PO$_4$, was measured and corrected for nonspecific binding as described (21). Dissociation constant and the number of binding sites were calculated from Scatchard plots (22).

Fluorescence (excitation, 495 nm; emission, 519 nm) of fluorescein-labeled enzymes was measured on a Perkin-Elmer MFP 44A spectrophotofluorometer at constant temperature (25 ± 1 °C) in a continuously stirred cuvette. Ligands or reagents were added to the samples in small volumes from Hamilton syringes mounted on pushbutton repetitive dispensers to minimize changes in fluorescence because of dilution. The total additions never increased the original volume by more than 5%. Intensity of fluorescence was expressed in two ways: (a) as a percentage of the fluorescence observed in the absence of cations ("relative fluorescence") or (b) in arbitrary units ("fluorescence change"). To follow rapid changes in fluorescence the fluorimeter was modified and its time constant was lowered to 0.003 s. The fastest rate of change that could be measured depended on the speed of mixing. It was about 5 s$^{-1}$ as estimated by mixing 10 μl of 10% rhodamine B solution into 2.5 ml of water. The rate constants were estimated by graphic analysis of the records as follows. A smooth curve that best approximated the tracings was drawn with a curve ruler. The limiting value of fluorescence that this curve approached asymptotically was estimated and the time necessary to reach half of this value ($t_1/2$) was read from the chart. Since the changes in fluorescence could be described by a single exponential term the rate constant ($k$) was calculated from the first order rate equation $k = 0.693/t_1/2$. For each value of $k$ the mean ± S.E. and the number of the determinations are given. The free Mg$^{2+}$ concentration was calculated on the basis of published equilibrium constants (23).

**RESULTS**

Fluorescein binds at or near the ATP-binding site and prevents reaction with nucleotides but not with phosphatase substrates (9, 10). In four experiments a 4-fold molar excess of fluorescein inhibited 98% of the ATPase but none of the (K)-NPPase activity of the enzyme. We routinely monitored fluorescence at the optimal wavelengths of excitation (495 nm) and emission (519 nm) (see emission spectrum on Fig. 1). Fluorescence intensity was modified by specific cationic ligands, phosphorylation, and the binding of ouabain as shown by typical tracings in Fig. 1. The high fluorescence of ligand-free fluorescein-(Na,K)-ATPase ($E$, conformation, the fluorescence of which was arbitrarily taken as 100%) was quenched by saturating KCl (10 mM) to about 78% (range, 75-81%). A high concentration of NaCl (100 mM) not only reversed quenching by K$^+$ but increased fluorescence to about 103%. Also if added alone, 100 mM NaCl consistently increased fluorescence to 102.3 ± 0.4% (n = 3) (Fig. 1, upper tracing).

![Fig. 1. Emission spectrum of fluorescein-(Na,K)-ATPase](image-url)
indicating that the conformation in Tris buffer was close to that of the Na-bound form (E,E-Na) (cf. Ref. 2). Mg" (1-3 mM), Pi (1-4 mM), or ouabain (0.1 mM) alone did not alter fluorescence of E, or E,E-Na (not shown). When added together to E, (3 mM Pi) plus 2 mM Mg" reduced fluorescence to 82% (Fig. 1, lower tracing). The addition of Pi, Mg" and ouabain quenched fluorescence to 56%, a level which was lower than that obtained with any other combination of ligands (Fig. 1, lower tracing).

Conformational Changes of the Unphosphorylated Fluorescein-(Na,K)-ATPase—To determine the relative affinities of the unphosphorylated conformations for cations the fluorescein-enzyme was titrated with K+, alone or in the presence of Mg" (Fig. 2) or Na+ (Fig. 3). In the absence of other cations quenching of fluorescence by K+ followed a hyperbola (Fig. 2) and the data fitted a single straight line in a double-reciprocal plot (not shown), indicating that K+ was bound to a single site. K0.5 for K+ at this site was 0.07 ± 0.01 mM (n = 5). With increasing Mg" concentration the titration curves with K+ became sigmoid. (In Hill plots of the data the Hill coefficients were 1.2-1.5 (not shown).) The titration curves with K+ became sigmoid also in the presence of Na+ (Hill coefficients varying between 1.3-1.5) and K0.5 for K+ was also a sigmoid function of Na+ concentration (Fig. 3, inset). K+ therefore appeared to act at a single site in the absence of other cations but at multiple interacting sites in the presence of Mg" or Na+

If fluorescence was maximally quenched (to 78%) by 10 mM KCl, the addition of 2 mM MgCl2 increased fluorescence to 90% and slowed the rate of increase in fluorescence induced by 100 mM NaCl (Fig. 4, left panel). In the absence of Mg2+ the rate of the E2,-K to E1,-Na transition was 1.73 ± 0.03 s^-1 (n = 7) and this rate was progressively slowed by increasing concentrations of Mg2+ (Fig. 4, right panel). Mg2+ concentration at half-maximal rate was about 1.2 mM. This value is an estimate of the apparent affinity of the unphosphorylated enzyme for Mg2+. Rb+, a congener of K+, quenched fluorescence to the same extent as K+ but the rate of the E2,-Rb to E1,-Na transition was slower than the rate of the E2,-K to E1,-Na transition, both in the presence and in the absence of Mg2+ (Fig. 4, right panel). High concentrations (more than 3 mM) of ATP slightly enhanced the rate of the E2,-K to E1,-Na transition in proportion to the chelation of Mg". The original rate could be restored by chelating Mg2+ with a 10- to 20-fold excess of EDTA (not shown). The effect of Mg2+ on the level and the rate of change of fluorescence did not depend on the sequence of addition of Mg2+ and K+ or Rb+ to the reaction mixture.

Conformational Changes of the Phosphorylated Fluorescein-(Na,K)-ATPase—We confirmed earlier observations (10, 24) that, in the presence of Mg" and Pi, quenched fluorescence by phosphorylating the enzyme (Fig. 1, lower tracing). We also confirmed the observation of Karlish that the fluorescein-enzyme can be phosphorylated with [32P]Pi. Additions of small amounts of Mg2+ in the presence of a saturating concentration of Pi (3 mM) reduced fluorescence stepwise (Fig. 5) each plateau probably corresponding to a new steady state level of the phosphoenzyme. The minimum level of fluorescence after phosphorylation was 82%, slightly higher than that of the E2,-K form (78%). Quenching by phosphorylation was half-maximal at 0.15 mM Mg2+ in the presence of 3 mM Pi (Fig. 5) and at 0.4 mM Pi in the presence of 1 mM Mg2+. The (E2,-P)-Mg conformation with low fluorescence was formed at a rate of 0.14 ± 0.005 s^-1 (n = 8) as estimated from the time course of quenching following the addition of Pi, plus Mg2+. Na+, added together with Mg2+ but before Pi, prevented quenching just as it prevents phosphorylation from Pi (1, 12). If quenching by Pi,
plus Mg\(^{2+}\) had reached completion, the addition of 100 mM Na\(^{+}\) reversed fluorescence to the original level at a rate of 0.20 ± 0.05 s\(^{-1}\) (n = 8) presumably by allowing spontaneous dephosphorylation and by blocking further phosphorylation.

We could detect another conformation of the phosphorylated fluorescein-(Na,K)-ATPase, (E\(_2\)-P)-Mg-K, by measuring the rate of quenching by P, and Mg\(^{2+}\) and the rate of Na\(^{+}\)-induced reversal in the presence of K\(^{+}\). Since both phosphorylation and K\(^{+}\) reduced fluorescence, we chose conditions in which quenching by K\(^{+}\) was excluded. First we added 2 mM MgCl\(_2\) to fluorescein-(Na,K)-ATPase then 1–2 mM K\(^{+}\). This much Mg\(^{2+}\) prevented quenching by K\(^{+}\) and quenching started only after the addition of 3 mM P. K\(^{+}\) had two effects; first, it increased the rate of quenching by P, plus Mg\(^{2+}\) from 0.14 ± 0.005 s\(^{-1}\) (n = 8) to 1.60 ± 0.23 s\(^{-1}\) (n = 5). Second, K\(^{+}\) increased the rate of reversal of this quenching after addition of 100 mM Na\(^{+}\) from 0.20 ± 0.05 s\(^{-1}\) (n = 8) to 0.82 ± 0.20 s\(^{-1}\) (n = 5). Similar effects of K\(^{+}\) on the rate of phosphorylation were observed previously (1, 12). The turnover rate for phosphate of the (E\(_2\)-P)-Mg-K conformation seems therefore to be faster than that of the (E\(_2\)-P)-Mg form.

The large quenching by P, Mg, and ouabain (Fig. 1, lower tracing) probably accompanied formation of the phosphoenzyme-ouabain complex, the (E\(_2\)-P)-Mg-ouabain conformation. This conformation differed from (E\(_2\)-P)-Mg in three ways. The fluorescence intensity of (E\(_2\)-P)-Mg-ouabain was lower (56 versus 78%), it was formed more slowly (k = 0.058 ± 0.005 s\(^{-1}\) (n = 4) versus 0.14 ± 0.005 s\(^{-1}\), and it did not revert to the E\(_2\)-Na conformation after the addition of 100 mM Na\(^{+}\), but 20 mM EDTA partially restored fluorescence at a slow rate (k = 0.042 ± 0.001 s\(^{-1}\) (n = 5)).

If the rate of phosphorylation is slower than the rate of ouabain-binding (e.g. at low Mg\(^{2+}\) concentration), ouabain can "trap" the phosphoenzyme, so that dephosphorylation is negligible relative to phosphorylation. Under these conditions it is possible to determine the initial rate of formation of the (E\(_2\)-P)-Mg-ouabain conformation and the affinity of this form for Mg\(^{2+}\). In the presence of 4 mM P, and 0.1 mM ouabain the rate of formation of (E\(_2\)-P)-Mg-ouabain could be measured after the addition of as little as 0.01 mM Mg\(^{2+}\) (Fig. 6). Mg\(^{2+}\) concentration at half-maximal rate, measured on two fluorescein-(Na,K)-ATPase preparations, was between 0.03 and 0.09 mM. These changes in fluorescence were not due to the presence of ouabain, because ouabain does not absorb light at 495 nm nor emits light at 519 nm.

Conformational Changes of Fluorescein-(Na,K)-ATPase after Treatment with Thimerosal—Thimerosal reversibly blocks some sulfhydryl groups of (Na,K)-ATPase and inhibits the ATPase but not the (K)-NPPase activity of the enzyme (16–18). If the reaction with thimerosal took place at 0 °C, thimerosal actually increased the hydrolysis of p-nitrophenylphosphate by both the native (18) and the fluorescein-enzyme (Fig. 7), not only in the presence but also in the absence of K\(^{+}\). The maximal (K)-NPPase activity (at 40 mM K\(^{+}\)) of both the fluorescein-enzyme and the thimerosal/fluorescein-enzyme was completely inhibited by 0.1 mM ouabain (not shown). The basic reaction medium did not contain any K\(^{+}\) or its congeners and in this medium the native or fluorescein-enzyme had no ouabain-sensitive nitrophenylphosphatase activity.

Fluorescence measurements showed that thimerosal selectively inhibited some conformational changes of both the unphosphorylated and the phosphorylated enzyme. Thimerosal actually increased the hydrolysis of p-nitrophenylphosphate by both the native (18) and the fluorescein-enzyme (Fig. 7), not only in the presence but also in the absence of K\(^{+}\). The maximal (K)-NPPase activity (at 40 mM K\(^{+}\)) of both the fluorescein-enzyme and the thimerosal/fluorescein-enzyme was completely inhibited by 0.1 mM ouabain (not shown).
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osal inhibited quenching by K⁺ (i.e., the E₁ to E₂-K transition), but not the Na⁺-induced increase in fluorescence (i.e., the E₁ to E₂-Na transition). (Fig. 8, upper tracing). Incubation of the thimerosal/fluorescein-enzyme with 3 mM dithiothreitol, as described under "Materials and Methods," restored quenching by K⁺ (Fig. 8, lower tracing).

Fig. 9 shows the effects of thimerosal on the phosphorylated forms. Thimerosal did not alter, or in some experiments slightly reduced, quenching by P_i plus Mg²⁺ and did not affect reversal of this quenching by Na⁺. When present, the slight reduction of quenching could be reversed with dithiothreitol. (Fig. 9, tracings T-F-E and T-F-E + DTT). The thimerosal/fluorescein-enzyme could be phosphorylated with [³²P]P_i (not shown). In contrast to its small effect on the (E₂-P)·Mg form, thimerosal completely prevented formation of the (E₂-P)·Mg-ouabain form. After thimerosal treatment the rate and the extent of quenching by P_i, Mg²⁺ and ouabain were the same as those by P, and Mg²⁺ (Fig. 9, tracings T-F-E and T-F-E + 0.1 mM ouabain). Though ouabain failed to induce the (E₂-P)·Mg·ouabain conformation, the thimerosal/fluorescein-enzyme did bind ouabain at a site of high affinity. (Estimated from Scatchard plots (22), the dissociation constant of the enzyme-ouabain complex was 21.7 nM for the thimerosal/fluorescein-enzyme and 18.8 nM for the parent fluorescein-enzyme.) Also ouabain could inhibit (K)-NPPase activity of the thimerosal/fluorescein-enzyme (discussed earlier) and the Na⁺-induced transition from the (E₂-P)·Mg to the E₁-Na form (Fig. 9).

An equimolar mixture of p-nitrophenylphosphate and Mg²⁺ (final concentration, 1.56 mM) reduced fluorescence of the thimerosal/fluorescein-(Na,K)-ATPase to 83% but that of the fluorescein-(Na,K)-ATPase only to 96% (Fig. 10). This decrease in fluorescence was probably related to the hydrolysis of p-nitrophenylphosphate because fluorescence of the fluorescein-enzyme, which does not hydrolyze p-nitrophenylphosphate in the absence of K⁺, was only slightly reduced (upper tracing on Fig. 10). Mg²⁺ plus p-nitrophenylphosphate

![Fig. 8. Reversible inhibition by thimerosal of the K⁺-induced quenching of fluorescence. The samples (2.5 ml) contained: 3.8 µg/ml of enzyme protein, 0.1 mM Tris-EDTA, and 50 mM Tris-C1 (pH 8.0). At the arrows KCl and NaCl were added to the final concentration indicated. Upper tracing, change in the fluorescence of thimerosal/fluorescein-(Na,K)-ATPase; lower tracing, change in that of the same enzyme after reversal of the thimerosal effect with dithiothreitol as described under "Materials and Methods." ——, response of fluorescein-(Na,K)-ATPase (4.3 µg of protein/ml) before treatment with thimerosal.](image)

![Fig. 9. Reversible effects of thimerosal on the quenching of fluorescence by Mg²⁺ and P, or by Mg²⁺, P, and ouabain. The samples (2.5 ml) contained: 4 mM Tris-P, 0.1 mM Tris-EDTA, 50 mM Tris-C1 (pH 8.0), with or without 0.1 mM ouabain and 3.8 µg/ml of thimerosal/fluorescein-(Na,K)-ATPase protein before (T-F-E) or after treatment with dithiothreitol (T-F-E + DTT). At the arrows, MgCl₂ and NaCl were added to the final concentration shown.](image)

![Fig. 10. Quenching of fluorescence by p-nitrophenylphosphate plus Mg²⁺. The samples (2.5 ml) contained: 50 mM Tris-C1 (pH 8.0), 0.1 mM Tris-EDTA, and either 4.1 µg/ml of fluorescein-(Na,K)-ATPase protein (F-E) or 3.8 µg/ml of thimerosal/fluorescein-(Na,K)-ATPase protein (T-F-E). At the arrow an equimolar mixture of MgCl₂ and Tris-p-nitrophenylphosphate was added to a final concentration of 1.6 mM.](image)

![Fig. 11. Relative fluorescence and interconversion of different forms of fluorescein-(Na,K)-ATPase.](image)
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quenched fluorescence of the thimerosal/fluorescein-enzyme more rapidly (k = 0.21 + 0.01 s\(^{-1}\), n = 8) than Mg\(^{2+}\) plus P\(_i\) (0.14 s\(^{-1}\); see above). This difference in rates was statistically significant (p < 0.01). Because of the instability of p-nitrophenylphosphate, the reaction mixture contained a small amount of P\(_i\); that, however, was not enough to quench fluorescence measurably (final concentration of P\(_i\) in the experiment shown in Fig. 10, 0.005 mM). Quenchings of fluorescence by P\(_i\) and p-nitrophenylphosphate were similar in two ways: both required Mg\(^{2+}\) and both were accelerated by K\(^{+}\). This analogy suggests that p-nitrophenylphosphate might have phosphorylated the enzyme as P, did (25, 26), but we did not attempt to measure this phosphorylation.

Fig. 11 summarizes schematically all forms of fluorescein-(Na,K)-ATPase and the paths of their interconversion that we could distinguish by the intensity and by the rate of change of their fluorescence with two exceptions. E\(_1\)-Mg and Mg-E\(_1\)-Na are probably not separate conformations relative to E\(_1\) and E\(_1\)-Na, respectively, and they are included in Fig. 11 only to show that addition of Mg\(^{2+}\) to E1 or E\(_1\)-Na did not change the level of their fluorescence.

DISCUSSION

Conformations of Fluorescein-(Na,K)-ATPase

To study conformational changes of (Na,K)-ATPase we used fluorescein as reporter group (9, 10) and purified plasma membranes as source of the enzyme. These membranes contained only the a and \(\beta\)-subunits of (Na,K)-ATPase (15) and thus artifacts, due to labeling of other membrane proteins, were absent. Since fluorescein binds only to the \(\alpha\)-subunit, we assumed that any change in fluorescence reflected conformational changes of primarily this subunit. Such conformational changes were studied earlier by digestion with proteolytic enzymes (see Refs. 2-5 and the introduction to the text) or by the fluorescence of intrinsic tryptophan (6-8). Fluorescein as a reporter group offered several advantages: the fluorescent signal was much larger and more stable than that of tryptophan (6-8) and, because of the fast response, the rate of conformational change could be measured, which measurement is not possible with proteolysis. The disadvantage of fluorescein is that it blocks ATP-binding with ATP (6, 13) and prevents the study of conformations induced by ATP or other nucleotides. Conformations of the fluorescein-enzyme are summarized on Fig. 11 and their properties are discussed as follows.

The E\(_1\), Conformation—E\(_1\), the form of the enzyme in Tris buffer, was highly fluorescent. A small, but significant increase in this fluorescence by Na\(^+\) (Fig. 1) indicated that the conformation of the Na-bound form was similar to E\(_1\). Earlier E\(_1\) and E\(_1\)-Na were described as a single conformation, the Na form, because of the identical pattern of inactivation by trypsin (2, 3, 5) and the similarity of tryptophan fluorescence (6, 7). ATP-binding experiments, however, showed a Na\(^+\)-dependent increase in the amount of ATP-binding sites (Table I in Ref. 27). Our fluorescence measurements provided evidence that this change in ATP binding is accompanied by a change in the structure of the \(\alpha\)-subunit.

The E\(_2\)-K Conformation—This is the second major conformation detected by tryptic digestion (2, 3, 5) and by tryptophan fluorescence (6-8). K\(^{+}\), as a single ligand, induced this conformation of fluorescein-(Na,K)-ATPase at a fast rate (Fig. 1) and at a single site of high affinity (K\(_{a_5}\) = 0.07 mM).

The E\(_2\)-K-Mg Conformation—This was a new conformation that could be differentiated from E\(_1\) and E\(_2\)-K by three properties: first, the level of fluorescence of E\(_2\)-K-Mg was between that of E\(_1\) and E\(_2\)-K; second, E\(_2\)-K-Mg was converted to E\(_1\)-Na more slowly than E\(_2\)-K (Fig. 4), and third, titration of fluorescein-(Na,K)-ATPase showed that, in the absence of Mg\(^{2+}\), K\(^{+}\) bound to a single site but, in the presence of Mg\(^{2+}\), K\(^{+}\) bound to multiple sites (see Fig. 2 and "Results"). The composition of E\(_2\)-K-Mg is therefore E\(_2\)-K\(_\alpha\)-Mg, where the most likely value for n is 2, suggested by the stoichiometry of Na\(^+\)-K\(^+\) transport (1). These fluorescence measurements were in agreement with the results of structural studies made by tryptic digestion which showed that Mg\(^{2+}\) does not induce the K form (7, 8).

The E\(_2\)-P-Mg Conformation—This conformation had about the same low level of fluorescence as E\(_1\)-K (Fig. 1) and structural similarity between the two conformations was shown earlier by tryptic digestion (3, 5, 7) and tryptophan fluorescence (7, 8). Direct measurement of the incorporation of [\(^{32}\)P]PP, confirmed an earlier observation of Karlish (10, 24) that the fluorescein enzyme, though it does not have ATPase activity, can be phosphorylated by P\(_i\). The steady state level of the phosphorylated fluorescein-enzyme was however much lower than that of the native enzyme. It is likely therefore that fluorescein at least partially inhibited phosphorylation. The conformational transition was probably proportional to phosphorylation because both processes were half-maximal at about the same concentration of Mg\(^{2+}\) and P\(_i\) (see Fig. 5, "Results," and Ref. 12).

The E\(_2\)-P-Mg-ouabain Conformation—This was characterized by its low level of fluorescence and by the sensitivity of its formation to the blocking of some sulfhydryl groups. These results, together with the previous observation that ouabain binding exposes a peptide bond within the \(\alpha\)-subunit to chymotrypsin digestion (5), show that ouabain-binding induces an unique conformation of the \(\alpha\)-subunit of (Na,K)-ATPase. The rate of the conformational transition, E\(_1\) to (E\(_2\)-P)-Mg-ouabain, was similar to the rate of ouabain-binding per se (12).

The E\(_2\)-P-Mg-ouabain transition serves as an example of a conformational change induced by a large molecule. This transition cannot be directly measured by fluorescence because the change is too slow, and the fluorescence of P\(_i\) cannot be distinguished from that of Mg\(^{2+}\) and P\(_i\). However, the change can be studied by using ouabain as a ligand which mimics the action of Mg\(^{2+}\) and P\(_i\) on the enzyme.

The Chemical Basis of Conformational Changes

As sensed with proteolytic enzymes (2-5) and fluorescence probes (6-10) the structural transitions between dephosphoforms (E\(_1\)-Na and E\(_2\)-K) and phosphoforms (E\(_2\)-P and E\(_2\)-P) appear to be identical. Specific inhibition of functional groups of the enzyme, however, showed that notable differences in the structure of these forms can be detected. Thimerosal, the inhibitor we used, reacted with sulfhydryl groups only, because its effects could be reversed with dithiothreitol (Figs. 9 and 10). In the fluorescein-enzyme, thimerosal selectively blocked the E\(_1\) to E\(_1\)-K and the (E\(_2\)-P)-Mg to (E\(_2\)-P)-Mg-ouabain transitions.

These two conformational changes are therefore distinguished either by a need for reactive sulfhydryl groups or by a need for free space around those groups that thimerosal
may block. By this requirement we could subdivide conformations that appeared homogeneous by other methods, e.g. E₂-K and (E₂-P)-Mg conformations were classified as "E₁" or "K" form because they were indistinguishable by tryptic cleavage (2-4) or by tryptophan fluorescence (6-7), though they could be separated with thimerosal as selective inhibitor of the formation of E₂-K. The fluorescein-(Na,K)-ATPase therefore had two K forms and in these experiments E₂-K was not a precursor of (E₂-P)-Mg.

(K)-NPPase Activity of the Thimerosal/Fluorescein-Enzyme

Hydrolysis of p-nitrophenylphosphate was thought to be catalyzed by the K-bound form of the dephosphoenzyme (28) in the presence of Mg₂⁺. By the criteria of fluorescence changes defined in this paper the thimerosal/fluorescein-enzyme did not assume a K-bound conformation, E₂-K, either in the presence or in the absence of Mg₂⁺, yet it had very high (K)-NPPase activity (Fig. 7). The question is then, which configuration catalyzed the hydrolysis of p-nitrophenolphosphate? In the presence of Mg₂⁺ p-nitrophenylphosphate quenched the fluorescence of the thimerosal/fluorescein-enzyme about the same extent as Mg₂⁺ and Pₐ, although at a faster rate (see Fig. 10 and "Results") and K⁺ accelerated the rate of quenching by both combinations of ligands (see "Results"). These observations suggested that perhaps hydrolysis of p-nitrophenylphosphate by the thimerosal/fluorescein-enzyme required prior phosphorylation and the formation of a conformation similar to, although not necessarily identical with, (E₂-P)-Mg. (Quenching by p-nitrophenylphosphate plus Mg₂⁺ was faster than by Pₐ plus Mg₂⁺; compare Figs. 1 and 10.) Phosphorylation of native (Na,K)-ATPase by p-nitrophenolphosphate is controversial (25, 26) and our observation of fluorescence quenching by p-nitrophenolphosphate plus Mg₂⁺ does not prove conclusively phosphorylation of the thimerosal/fluorescein-enzyme. With the use of thimerosal, however, we could show that the conformation of the thimerosal/fluorescein-enzyme with nitrophosphophosphatase activity was not the E₂-K form.

Mg²⁺ and the Conformational Transitions

We made two major observations on the interaction of Mg²⁺ with different forms of fluorescein-(Na,K)-ATPase: first, at least one of the conformational changes, the E₂-K to E₁-Na transition, was slowed by Mg²⁺ at a site of low affinity (Fig. 4); second, in the absence of nucleotides the affinity of the phosphoenzyme for Mg²⁺ (Kₐ = 0.03-0.09 mM) was more than 10-fold higher than that of the dephosphoenzyme (Kₐ = 1.2 mM; see also Fig. 6 and "Results"). The enzyme, therefore, must possess sites with alternating affinity for Mg²⁺ at which Mg²⁺ may control different steps of the reaction cycle. Earlier findings also support this view. Mg²⁺ is required for phosphorylation by ATP or P, (1) and for the conversion of the ADP-sensitive phosphoenzyme, E₁-P, into the K⁺-sensitive phosphoenzyme, E₁-P (11). Tightly bound Mg²⁺ is necessary for fast dephosphorylation by K⁺ (12) and for the binding of ouabain (1). Binding sites with different affinities for Mg²⁺ were reported for these processes: sites with high affinity for ADP-ATP exchange (Kₐ = 5 mM; Ref. 13); sites with lower affinity for (K)-NPPase (Kₐ = 0.08 mM; Ref. 13), for ADPase (Kₐ = 0.5-1.5 mM; Ref. 14), and for the formation of the K⁺ form (Kₐ = 1.2 mM; Ref. 7). On the basis of these findings we propose the following cycle for the interaction of Mg²⁺ with the native enzyme. Phosphorylation of the enzyme by ATP and conversion of E₁-P to E₂-P require Mg²⁺ (1, 11, 12); upon phosphorylation, the affinity for Mg²⁺ increases and some Mg²⁺ remains tightly bound during dephosphorylation by K⁺ as was shown earlier (12). After dephosphorylation, the E₂-K-Mg form is likely to release Mg²⁺ because the affinity for Mg²⁺ decreases. ATP may enhance this process and this may also be one way by which ATP accelerates the E₂-K to E₁-Na transition. Finally, Mg²⁺ will rebind to the E₁-Na form before phosphorylation takes place. It follows from this hypothesis that (Na,K)-ATPase binds and releases Mg²⁺ during the catalytic cycle as it does Na⁺ and K⁺, although apparently without transporting Mg²⁺ across the cell membrane. Scheme 1 summarizes the sequence of conformational changes that this hypothesis envisions; a difference between the conformation of E₁-P and E₂-P was shown by tryptophan fluorescence (7, 8).

![Scheme 1](image-url)

**Scheme 1**

Post et al. (28) observed that, in the absence of ATP, the E₂-K to E₁-Na conversion is slow and attributed this slow exchange of ions to "occlusion" of K⁺ during translocation across the membranes. Our results suggest that the E₂-K-Mg conformation may be the occluded form and it may have more than one molecule of bound K⁺ (Figs. 2 and 4).

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