Molecular Distance Measurements Reveal an \((\alpha\beta)_2\) Dimeric Structure of \(\text{Na}^+/\text{K}^+\)-ATPase

HIGH AFFINITY ATP BINDING SITE AND \(\text{K}^+\)-ACTIVATED PHOSPHATASE RESIDE ON DIFFERENT \(\alpha\)-SUBUNITs

(Received for publication, April 21, 1998, and in revised form, July 27, 1998)

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AtP hydrolysis by \(\text{Na}^+/\text{K}^+\)-ATPase proceeds via the interaction of simultaneously existing and cooperating high (E\(_1\)ATP) and low (E\(_2\)ATP) substrate binding sites. It is unclear whether both ATP sites reside on the same or different catalytic \(\alpha\)-subunits. To answer this question, we looked for a fluorescent label for the E\(_2\)ATP site that would be suitable for distance measurements by Förster energy transfer after affinity labeling of the E\(_1\)ATP site by fluorescein 5'-isothiocyanate (FITC). Erythrosin 5'-isothiocyanate (ErITC) inactivated, in an E\(_2\)ATP site-blocked enzyme (by FITC), the residual activity of the E\(_2\)ATP site, namely K\(^+\)-activated \(p\)-nitrophenylphosphatase in a concentration-dependent way that was ATP-protectable. The molar ratios of FITC/\(\alpha\)-subunit of 0.6 and of ErITC/\(\alpha\)-subunit of 0.48 indicate 2 ATP sites per (\(\alpha\)\(\beta\))\(_2\) diprotomer. Measurements of Förster energy transfer between the FITC-labeled E\(_1\)ATP and the ErITC-labeled or Co(NH\(_3\))\(_4\)ATP-inactivated E\(_2\)ATP sites gave a distance of 6.45 ± 0.64 nm. This distance excludes 2 ATP sites per \(\alpha\)-subunit since the diameter of \(\alpha\) is 4–5 nm. Förster energy transfer between cardiac glycoside binding sites labeled with anthroyloaubain and fluoresceinyl ethylendiamino ouabain gave a distance of 4.9 ± 0.5 nm. Hence all data are consistent with the hypothesis that Na\(^+\)/K\(^+\)-ATPase in cellular membranes is an (\(\alpha\)\(\beta\))\(_2\) diprotomer and works as a functional dimer (Thoenges, D., and Schoner, W. (1997) J. Biol. Chem. 272, 16315–16321).

\(\text{Na}^+/\text{K}^+\)-ATPase is an integral membrane protein that transports sodium and potassium ions against an electrochemical gradient. The transport of Na\(^+\) and K\(^+\) ions is presumably connected to an oscillation of the enzyme between two major conformational states, namely the E\(_1\)Na\(^+\) and the E\(_2\)K\(^+\) conformations. The E\(_1\) and E\(_2\) states have different affinities for ATP. The pumping mechanism may be described by conformational changes of a single ATP site of the catalytic \(\alpha\)-subunit between a high affinity E\(_1\)ATP\(^*\) site (from where Na\(^+\) export starts by phosphorylation) and a low affinity E\(_2\)ATP site (which is involved in K\(^+\) import) (1–3). Yet a model assuming consecutive changes of a single ATP site during the catalytic process, the so-called Albers-Post model, is inconsistent with the recent kinetic demonstration of simultaneously existing and cooperating ATP binding sites (4, 5) and the finding that specific labeling of the E\(_1\)ATP or the E\(_2\)ATP sites does not block labeling and partial activities of the other empty site (6–13). The recent demonstration of a “superphosphorylation,” i.e. that at least 2 mol of phosphate can be incorporated into the catalytic \(\alpha\)-subunit per mol of ouabain binding sites (14), is consistent with the coexistence of phosphorylated intermediates (E\(_1\)P, E\(_2\)P, and E\(_2\)P (15)) at different places in an oligomeric enzyme. Also, the observations of phosphorylation from P\(_i\) during Na\(^+\)/K\(^+\)-ATPase activity (16) and in an FITC-treated enzyme (10) are consistent with the possibility that Na\(^+\)/K\(^+\)-ATPase is phosphorylated from both ATP sites (12, 17).

Since two ATP binding sites of Na\(^+\)/K\(^+\)-ATPase cooperate during ATP hydrolysis (4, 5), it is of great interest to obtain more detailed information on the mutual interaction of both ATP sites in the absence (8, 10, 18, 19) and presence of the transported cations (20) and on their distance. There are a great number of experiments favoring the idea that both ATP sites reside on different \(\alpha\)-subunits (21, 22). But studies with detergent-solubilized Na\(^+\)/K\(^+\)-ATPase seem to contradict this assumption (13, 23, 24). Ward and Cavieres (13, 24) demonstrated that the detergent-solubilized putative (\(\alpha\)\(\beta\)) promoter of Na\(^+\)/K\(^+\)-ATPase shows negative cooperativity of ATP hydrolysis. This finding is in conflict with the assumption of cooperating catalytic \(\alpha\)-subunits during ATP hydrolysis but supports the possibility of two interacting ATP sites residing on the same catalytic \(\alpha\)-subunit. The amino acid sequence forming the high affinity E\(_1\)ATP site has been defined by affinity labeling with protein-reactive ATP derivatives and by specific labeling with the pseudo-ATP analog FITC (25–31). Information on the
location of the low affinity E1ATP binding site may be obtained by similar means, i.e. affinity labeling followed by amino acid sequencing and by energy transfer experiments after specific modification of the E1ATP site by a fluorophor. Affinity labeling of the low affinity E1ATP site needs the availability of specific ATP derivatives as probes (4). Since many derivatives of fluorescein (pyrene, eosin, or erythrosin) bind to the E1ATP site as well (32–35), the possibility arose that fluorescent pseudo-ATP derivatives may exist that label the E1ATP site as well. Provided specific labeling of the E1ATP binding site by a fluorescent pseudo-ATP derivative can be achieved, Förster energy transfer measurements may give information on the distance of this E1ATP site to the E1ATP site, since the latter can be labeled specifically by FITC at Lyso501 (26, 27, 36). Hence, information may be obtained from the availability of such a system on the question as to whether both ATP binding sites are close or distant from each other. This paper shows that a pseudo-ATP binding for the E1ATP site exists. Erythrosin 5′-isothiocyanate (ErITC) inactivates the residual K+–activated phosphatase (an activity of the E2ATP site) in an enzyme whose E1ATP had already been blocked by labeling with FITC. Energy transfer from the E1ATP-labeled fluorescein to the E2ATP-labeled erythrosin (as well as the Co2+-ions sitting in the E1ATP site) was so low that it is unlikely that both ATP sites reside on the same catalytic α-subunit.

**EXPERIMENTAL PROCEDURES**

All chemicals were of the highest purity available and were obtained from Bio-Rad, Boehringer Mannheim, Merck, and Molecular Probes (Eugene, OR). Lab-Trol protein standard is a product of Merz & Dade (Munich, Germany). [γ-32P]ATP was from Amersham Pharmacia Bio-Tech. Calculation and presentation of data were performed with the program Graph-Pad Prism 2.01 (Graph Pad Software Inc., San Diego, CA).

**Preparation of the MgATP Complex Analogues**—The synthesis of Cr(H2O)4ATP, Cr(H2O)4AdoP(CH2)4P, and Co(NH3)4ATP was performed by the aniline method of Cleland et al. (37) with the variations described earlier (22).

**Enzyme and Assays**—Na+/K+/ATPase from pig kidney with a specific enzymatic activity of 25–27 units/mg of protein was isolated by a modification of Jørgensen’s procedure (38) and measured by coupled spectrophotometric assay (39). One enzyme unit is defined as the amount of enzyme hydrolyzing 1 μmol of ATP/min at 37 °C. Protein was determined by the method of Lowry et al. (40) using Lab-Trol as the protein standard. Lab-Trol is a mixture of proteins and enzymes used for the calibration of assays in clinical chemical analysis. All buffers used were made up to their respective pH value at room temperature.

K+-activated p-nitrophenolphosphatase activity was measured on a multiter plate by incubating Na+/K+/ATPase at 37 °C in a total volume of 150 μl containing 61 mM Tris/HCl (pH 7.25), 6.4 mM MgCl2, 12 mM KCl, and 5 mM p-nitrophenolphosphate. The reaction was started after 15 min by the addition of 200 μl of 3 n NaOH. The p-nitrophenolate formed was measured at 405 nm by an enzyme-linked immunosensor assay reader (5).

**Inactivation of Na+/K+/ATPase with FITC**—Na+/K+/ATPase at a final concentration of 1 unit/ml (65 μg/ml) was incubated overnight at 37 °C in a solution containing 20 mM Tris/HCl (pH 7.25) and 50 mM Co(NH3)4PO4 (a control without Co(NH3)4PO4 was run in parallel). The inactivated enzyme was sedimented at 100,000 × g, washed in 20 mM Tris/HCl (pH 7.25), and incubated in 20 mM Tris/HCl, 15 mM NaCl, and 2 μM ErITC. After different incubation times, the enzyme was centrifuged (the control was treated in the same way but without FITC). The inactivated enzyme (residual Na+/K+/ATPase activity of 1%) was spun down in Eppendorf tubes at 100,000 × g. The pellet was washed in 20 mM Tris/HCl (pH 7.25) and resuspended in a solution of 20 mM Tris/HCl (pH 7.25), 15 mM NaCl, and various concentrations (0–10 μM) of ErITC. After incubation for 15 min at 37 °C, the K+-activated p-nitrophenolphosphatase activity was estimated.

**Kinetic Analysis of the Inactivation of K+-activated p-Nitrophenolphosphatase by ErITC in a FITC-inactivated Na+/K+/ATPase—**FITC is a covalent label for the high affinity E1ATP binding site of Na+/K+/ATPase that leaves the activity of the E2ATP site almost unaffected (8, 11, 25, 36). Therefore, the effects of ErITC on K+-activated p-nitrophenolphosphatase, residual activity of the low affinity E2ATP site, were tested in the following way.

Na+/K+/ATPase at a final concentration of 1 unit/ml (65 μg/ml) was incubated overnight at 37 °C in a solution containing 20 mM Tris/HCl (pH 7.25) and 10 μM FITC. A control enzyme was treated in the same way but without FITC. This control was set at 100%. The inactivated enzyme (residual Na+/K+/ATPase activity of 1%) was spun down in Eppendorf tubes at 100,000 × g. The pellet was washed in 20 mM Tris/HCl (pH 7.25) and resuspended in a solution of 20 mM Tris/HCl (pH 7.25), 15 mM NaCl, and various concentrations (0–10 μM) of ErITC. After incubation for 15 min at 37 °C, the K+-activated p-nitrophenolphosphatase activity was estimated.

**Analysis of the Effect of Co(NH3)4ATP on the Incorporation of ErITC in FITC-treated Na+/K+/ATPase**—Co(NH3)4ATP is a specific inhibitor of the E1ATP site of Na+/K+/ATPase (9, 22). It was used, therefore, to detect interferences of ErITC with this site.

Na+/K+/ATPase at a final concentration of 2.5 units/ml (163 μg/ml) was incubated overnight at 37 °C in a solution containing 20 mM Tris/HCl (pH 7.25), 15 mM NaCl, and 10 μM FITC. The enzyme was spun down at 100,000 × g, and washed with 20 mM Tris/HCl (pH 7.25), and resuspended in 20 mM Tris/HCl (pH 7.25), 15 mM NaCl, and 1 mM Co(NH3)4ATP (the control was treated in the same way but without FITC). After 1-h incubation at 37 °C, the enzyme was spun down and washed again. After incubation at 37 °C for 1 additional h in a solution containing 20 mM Tris/HCl, 15 mM NaCl, and different concentrations of ErITC (0–1 μM), the enzyme was spun down at 100,000 × g and washed three times with 20 mM Tris/HCl (pH 7.25). The fluorescence of ErITC was detected in a Hitachi F-3000 spectrophotometer at λex 530 nm and λem 555 nm. A control enzyme labeled with FITC was treated in the same way but without FITC.

**Effect of ErITC on Na+/K+/ATPase—**

ErITC has been shown to incorporate into the E1ATP site of Na+/K+/ATPase for lifetime measurements of fluorescence (27). Since there is a K+–dependent phosphorylation of Na+/K+/ATPase for Lifetime Measurements of Fluorescence—Na+/K+/ATPase (6 units) was centrifuged in a total volume of 1 ml overnight at 37 °C in a solution containing 20 mM Tris/HCl (pH 7.25), 15 mM NaCl, and 10 μM FITC (a control contained no FITC). This inactivated enzyme was centrifuged in Eppendorf tubes at 100,000 × g. The pellet was resuspended in a total volume of 1 ml of 25% trichloroacetic acid prior to the addition of 200 μM ErITC. After different incubation times, the enzyme was centrifuged (the control was treated in the same way but without FITC). The inactivated enzyme was sedimented at 100,000 × g, washed in 20 mM Tris/HCl (pH 7.25), and incubated in 20 mM Tris/HCl, 15 mM NaCl, and 2 μM ErITC. After different incubation times, the enzyme was centrifuged (the control was treated in the same way but without FITC). After incubation at 37 °C for 1 h, the K+–dependent phosphorylation of Na+/K+/ATPase (27) was followed at 340 nm by a Hitachi spectrophotometer.

**Labeling of Na+/K+/ATPase for Lifetime Measurements of Fluorescence—Na+/K+/ATPase (6 units) was centrifuged in a total volume of 1 ml overnight at 37 °C in a solution containing 20 mM Tris/HCl (pH 7.25), 15 mM NaCl, and 2 μM ErITC (27). After the last centrifugation step, the protein was resuspended in 0.3 ml of 20 mM Tris/HCl buffer (pH 7.25) (final concentration 1.8 mg/ml). All lifetime measurements of FITC-labeled Na+/K+/ATPase were performed in the presence of 1 mg/ml antifluorescein antibodies to correct for contributions by free and nonspecifically attached FITC molecules (34).

To study the effects on the individual α-subunit, the labeled enzyme was solubilized by incubation with 5% SDS at 37 °C for 15 min to break...
any protein-protein interactions of the α-subunits. The energy transfer was measured in the presence of SDS in the same way.

Specific binding of the ouabain derivatives anthroylouabain (AO) and FEDO to Na\(^+/K\(^+\)-ATPase proceeded under conditions of backdoor phosphorylation (8, 42). Na\(^+/K\(^+\)-ATPase (6 units) was incubated in 200 mM imidazole/HCl buffer (pH 7.25), 3 mM MgCl\(_2\), 10 mM imidazole/phosphate (pH 7.25), and 150 mM AO for 1 h at 37 °C. To prepare the sample labeled with donor (AO) and acceptor (FEDO), the enzyme was incubated under the same conditions but in the presence of 150 mM AO and 300 mM FEDO. This concentration was used because the affinity of Na\(^+/K\)^+ATPase for AO was twice as high as that for FEDO (42).

**Determination of the Amount of FITC, ErITC, AO, FEDO, and Co(NH\(_3\))\(_4\)ATP Bound to Na\(^+/K\)^+ATPase—**A Specol 211 spectrophotometer was used for absorbance measurements. Steady-state fluorescence data were collected in quartz cuvettes on a Perkin-Elmer LS-5 fluorometer equipped with monochromators (34). Excitation and emission wavelengths were 500 and 520 nm, respectively, for FITC and FEDO, 362 and 471 nm for AO, and 530 and 555 nm for ErITC, respectively. Two Glan-Thompson polarizers were used for determination of the steady-state anisotropy values. All measurements were performed at 25 °C. The molar ratio of bound fluorophores per α-subunit was determined from steady-state fluorescence measurements based on the known quantum yield of standards of known concentrations. Quantum yield of bound FITC was compared with free FITC in ethanol, and bound ErITC was compared with free ErITC in water. Although the former increases about 10%, the quantum yield of the latter increases four times (34). For the determination of the molar FITC/ErITC ratio per mol of α-subunit, we labeled Na\(^+/K\)-ATPase at a spectrophotometric concentration of 25–27 units/mg of protein first with 10 μM FITC at pH 7.25 and subsequently with 2 μM ErITC (as described above). Steady-state fluorescence of the protein-bound fluorescein was corrected for unspecified fluorescence (outside the E\(_1\)ATP binding site) by the addition of 5 μg/mL antifluorescein antibodies (34). The molar concentration of Na\(^+/K\)-ATPase and its α-subunit was calculated from the protein concentration using the molecular weight of 113 kDa for the α- and 55 kDa for the β-subunit (3).

**Fluorescence Lifetime Measurements—**The samples were measured in a total volume of 600 μl at a final protein concentration of 1.8 mg/mL. The apparatus for lifetime measurements was based on a laser excitation source and on time-correlated single photon counting as the detection system. The excitation source consisted of a cavity-dumped dye laser (model 375, Spectra Physics, Mountain View, CA) synchronously pumped by the argon ion laser (model 171, Spectra Physics) and a frequency doubler. The excitation pulses (full width at half maximum about 10 ps) were generated at 356 nm with pyridine 1 as the laser dye. The required emission wavelength was selected by a monochromator with a proper cut-off filter in front of the input slit. The fluorescence decays were measured with the emission polarizer oriented at the “magic angle” of 54.7 degrees to the direction of the excitation polarization vector. The response function of the apparatus was determined by the REF procedure of Vecer (43) with FITC and potassium iodide-ER as a reference compound. All experiments were repeated with an unlabeled sample to correct for the fluorescence background and light scattering. The decay data were analyzed by a nonlinear least squares deconvolution procedure. The fit quality was evaluated from the randomness of the residual plot and the autocorrelation function together with the χ\(^2\) value.

**Calculation of the Förster Resonance Energy Transfer and of Molecular Distances—**Distances (R) between donor and acceptor pairs were derived from the apparent efficiencies of Förster energy transfer (E), which was calculated from the quenching of the steady-state fluorescence intensity of the donor or from the decrease of donor lifetime (τ\(_{ex}\), presence of donor only; τ\(_{DA}\), presence of donor and acceptor). The apparent efficiency of energy transfer is related to the absolute rate of energy transfer (k\(_{ET}\)) as shown below.

\[
E = (k_{ET}τ_0)(1 + k_{ET}τ_0) = 1 - (τ_{DA}/τ_0)
\]

(Eq. 1)

This rate has been defined by Förster (44) as

\[
k_{ET} = (Φ_{PL}λJ_0τ_{D}P_{BA} τ_{A}) \times 8.7 \times 10^{22} \text{ s}^{-1},
\]

(Eq. 2)

where Φ\(_{PL}\) is the quantum yield of the donor in the absence of acceptor, n is the refractive index of the solution, R is the distance between donor and acceptor (in cm), J is the spectral overlap integral (cm\(^2\)/ml) defined as

\[
J = \int \frac{F_{PL}(λ)λdλ}{\int F_{PL}(λ)λdλ} = \int \frac{F_{PL}(λ)λdλ}{\int F_{PL}(λ)λdλ}
\]

(Eq. 3)

where \(ε_0(λ)\) is the extinction coefficient of the acceptor, and \(F_{PL}(λ)λ\) is the fluorescence of the donor (excited at \(λ_0\)) that is emitted at wavelength \(λ\). Finally, \(κ^2\) is the orientation factor of the donor, which is defined as

\[
κ^2 = (\sin sinβ cosβ - 2cosα cosβ)^2,
\]

(Eq. 4)

and α and β are, respectively, the angles that the emission dipole of the donor and the absorption dipole acceptor’s form with vector R. Vector R connects these two dipoles. P is then the angle between the planes that contain α and β. The resulting Förster critical distance (R\(_{0}\)), which is the distance in the case of \(E = 0.5\), is

\[
R_{0}^6 = 8.7 \times 10^{22} \Phi_{PL}φ_{PL} \text{nm}^6.
\]

(Eq. 5)

Labeling of the enzyme used in this work (pH at 7.25) resulted in the donor and acceptor anisotropy values low enough to take the orientational factor \(κ^2\) for the mutual distance calculations as 23, assuming random mutual orientation of fluorophores (45). For determination of the overlap integral \(J_0\), the values of \(κ\) and \(φ\) were taken from Amler et al. (34). An excess of donor concentration over acceptor concentration was corrected for using rates of energy transfer (34). Briefly, the corrected rate of energy transfer \(k_{ET}\) was calculated as

\[
k_{ET} = k/n_{DA}/n_{PL}
\]

(Eq. 6)

where \(k\) is the observed rate of energy transfer, and \(n_{DA}\) and \(n_{PL}\) are the molar ratios of bound donor and acceptor, respectively.

**RESULTS**

**Characterization of Erythrosin Isothiocyanate as a Label of ATP Sites—**ErITC has been formerly used as a label for the high affinity E\(_{1}\)ATP site (34). To certify that ErITC is a useful label for both ATP sites of Na\(^+/K\)-ATPase, the action of ErITC on the overall Na\(^+/K\)-activated ATP hydrolysis as well as on partial activities of the enzyme was studied. When the enzyme was incubated with increasing micromolar concentrations of ErITC at 37 °C, Na\(^+/K\)-ATPase activity was lost as a time- and concentration-dependent process (Fig. 1A). The data were fitted with a two-site model (4), which revealed \(K\) values of 0.66 and 0.78 μM (Fig. 1B). Millimolar concentrations of ATP (0–25 mM) protected Na\(^+/K\)-ATPase against the inactivation by 0.2 μM ErITC (Fig. 2). Na\(^-\)dependent phosphorylation of the catalytic α-subunit from the E\(_{1}\)ATP site in Na\(^+/K\)-ATPase is a specific property of this site. To verify that ErITC interacts in fact with the E\(_{1}\)ATP site, we blocked the E\(_{1}\)ATP site with Co(NH\(_3\))\(_4\)PO\(_4\) (4, 12) and studied the effect of 2 μM ErITC on the Na\(^-\)-dependent formation of a phospho intermediate. Table I shows that the velocity of inactivation of the Na\(^-\)-dependent autoposphorylation of a Co(NH\(_3\))\(_4\)PO\(_4\)-treated and and -untreated control enzyme is the same. Hence, one of the two sites interacting with ErITC (Fig. 1B) is the E\(_{1}\)ATP site.

Furthermore, to learn whether ErITC may also affect the partial activity of the E\(_{2}\)ATP site, we blocked the activity of the E\(_{2}\)ATP site by incubation with FITC first (36) and then studied the action of ErITC on the remaining activity of the K\(^-\)-activated p-nitrophenylphosphatase (Figs. 3 and 4), which is an enzymatic property of the E\(_{2}\) conformation and the E\(_{2}\)ATP site (4, 8). It was inactivated by ErITC as well. The kinetics of the inactivation of K\(^-\)-activated p-nitrophenylphosphatase by ErITC gave a straight line in a reciprocal plot of inactivation velocity constant versus the ErITC concentration (41) (Fig. 3), indicating thereby the interaction of ErITC with a single site
Na⁺/K⁺-ATPase Is an (αβ)₂ Dimer

**Effect of ErITC on Na⁺-dependent phosphorylation from γ-32P/ATP**

Na⁺/K⁺-ATPase at a final concentration of 3 units/ml (195 μg/ml) was incubated overnight at 37 °C in a solution containing 20 mM Tris/HCl (pH 7.25) and 50 mM Co(NH₃)₄PO₄. A control without Co(NH₃)₄PO₄ was run in parallel. The control enzyme (100 pmoi/unit) and the Co(NH₃)₄PO₄-inactivated enzyme (33 pmoi/unit) were incubated with 2 μM ErITC in 20 mM Tris/HCl (pH 7.25) and 15 mM NaCl at 37 °C for the times indicated, and Na⁺-dependent phosphorylation from γ-32P/ATP was measured. Mean values and S.D. of five experiments are shown. For experimental details see “Experimental Procedures” and Refs. 4 and 54.

| Time of inactivation by ErITC | Na⁺-dependent phosphorylation by γ-32P/ATP of Co(NH₃)₄PO₄-treated enzyme | Na⁺-dependent phosphorylation by γ-32P/ATP of a native control enzyme |
|-----------------------------|---------------------------------------------------------------------------|---------------------------------------------------------------------|
| min                         | %                           | %                                 |
| 0                           | 100 ± 5                     | 100 ± 6                            |
| 5                           | 60 ± 3                      | 60 ± 4                             |
| 15                          | 46 ± 4                      | 45 ± 3                             |
| 30                          | 36 ± 5                      | 35 ± 5                             |
| 45                          | 34 ± 3                      | 34 ± 1                             |
| 60                          | 24 ± 2                      | 17 ± 3                             |

**TABLE I**

**Steady-state Fluorescence of FITC- and ErITC-labeled Na⁺/K⁺-ATPase—**Unlike previous reports (25, 34, 36), enzyme labeling with isothiocyanates was performed for a longer time period and at pH 7.25 (see “Experimental Procedures”). Similar to the FITC labeling conditions at pH 9.0 (25, 94, 36), inactivation of Na⁺/K⁺-ATPase by FITC at pH 7.25 was protectable by ATP, and the resultant protein was sensitive to Na⁺ and K⁺ (18) (but another residue may be modified within the E2ATP sites). For experimental details see “Experimental Procedures.”

**FIG. 1. Inactivation of the Na⁺/K⁺-ATPase activity by ErITC.** A, Na⁺/K⁺-ATPase was inactivated with ErITC as follows. 1 unit of Na⁺/K⁺-ATPase was incubated in a total volume of 1 ml with 200 nM ErITC in 20 mM Tris/HCl (pH 7.25) and 15 mM NaCl at 37 °C. The residual Na⁺/K⁺-ATPase activity was assayed by transferring aliquots of 50 μl to the optical assay (39). B, the apparent velocity constants of inactivation were calculated as monoeponential decay and plotted against ErITC concentration. The Kᵢ values were calculated by a fit of a two-site binding hyperbola $Y = \left(\frac{v_{max1} \times X}{K_{i1} + X}\right) + \left(\frac{v_{max2} \times X}{K_{i2} + X}\right)$. For experimental details see “Experimental Procedures.”

**FIG. 2. Protective effect of ATP against the inactivation of the Na⁺/K⁺-ATPase activity by ErITC.** 1 unit of Na⁺/K⁺-ATPase was incubated in a total volume of 1 ml with 200 mM ErITC in 20 mM Tris/HCl (pH 7.25) and 15 mM NaCl at 37 °C ( control without ErITC). The following concentrations of ATP were included in the inactivation test to probe for a protective effect of ATP: ● 0 mM; ○ 2.5 mM; □ 25 mM ATP. Na⁺/K⁺-ATPase activity was assayed by transferring aliquots of 50 μl to the coupled optical assay (39). For experimental details see “Experimental Procedures.”

(Kᵢ = 0.74 μM, Fig. 3, inset). The modification of this site was prevented by 10 mM ATP (Fig. 4). Hence, ErITC labeled under these specific conditions a site with low affinity for ATP.

It has been shown formerly that the MgATP complex analog Co(NH₃)₄ATP is a specific label of the E₁ATP binding site (4, 9). To certify that ErITC labels in fact the E₁ATP site in a FITC-pretreated Na⁺/K⁺-ATPase, we additionally blocked the free E₁ATP site in a FITC-pretreated Na⁺/K⁺-ATPase (whose E₂ATP site was blocked by FITC) by treatment with 1 mM Co(NH₃)₄ATP (Fig. 5). No incorporation of erythrosin from ErITC into such a double-modified enzyme (whose E₁ATP and E₂ATP sites had been blocked) was seen. Only a control enzyme, whose E₂ATP site was accessible (no pretreatment with Co(NH₃)₄ATP), was modified by ErITC (Fig. 5). In conclusion, ErITC can modify both ATP sites (Fig. 1B), but after specific blockade of the E₂ATP site, it is a modifier of the E₁ATP site (Figs. 3–5).

**FIG. 3. Determination of the affinity of ErITC for the low affinity binding site.** Na⁺/K⁺-ATPase at a concentration of 1 unit/ml was pretreated overnight by 10 μM FITC. This enzyme was incubated for 10 min at 37 °C in 20 mM Tris/HCl buffer (pH 7.25) with increasing concentrations (0–10 μM) of ErITC. Residual phosphatase activity was measured according to Linnertz et al. (8). By plotting the apparent velocity constants (double reciprocal) against the inhibitor constant, the inactivation constant was determined to be Kᵢ = 0.74 μM. Mean values of three experiments are shown. For details see “Experimental Procedures.”

(Continued)
enzyme. Fluorescence response of FITC-treated enzyme to Na\(^+\) and K\(^+\) ions, respectively, was quite similar and insensitive to pH of labeling. Labeling of Na\(^+/K^+\) -ATPase by ErITC at pH 9 did not show specific inhibition of the K\(^+\) -activated phosphatase in FITC-pretreated enzyme. An E\(_1\) → E\(_2\) transition was seen when the effects of Na\(^+\) and K\(^+\) ions were studied in such an enzyme preparation, observing the FITC fluorescence (with or without ErITC bound to the E\(_2\) ATP binding site (data not shown)).

Since the nature of the fluorophor is important for the ATP site specificity, we also tried to learn whether the change of the reactive group may affect the interaction with the E\(_2\) ATP site. When cysteine-reactive erythrosin 5'-iodacetamide was used, a modification of the E\(_2\) ATP site was also seen, as checked by the loss of the activity of K\(^+\) -activated p-nitrophenylphosphatase in a FITC-inactivated Na\(^+/K^+\) -ATPase. The apparent affinity of the drug, however, was 10 times lower, and about 20 mol of SH groups/mole of Na\(^+/K^+\) -ATPase were labeled. Hence, this substance is not useful for Förster energy transfer measurements.

Interestingly, previous studies with 5-iodacetamidofluorescein revealed that it is bound outside the ATP binding site (47, 48) at Cys\(_{457}\) (49).

Distance between the High Affinity and Low Affinity ATP Binding Sites—As is evident from the experiment shown in Fig. 4, labeling of the high affinity E\(_1\) ATP site by FITC and of the low affinity E\(_2\) ATP site by ErITC should allow determination of the distance between both ATP binding sites. The donor-acceptor pair FITC/ErITC is well known for its high overlap integral, its high quantum yield of FITC, and the high extinction coefficient of ErITC. Thus, this donor-acceptor pair is a suitable tool to study long distance interactions of enzyme subunit as well as short distances. Short distances would be indicated by a high Förster energy transfer, whereas low energy transfer would indicate long distances between the fluorophores. Therefore, Na\(^+/K^+\) -ATPase was labeled at a FITC concentration of 30 nmol/mg of protein. This procedure achieved a total inhibition of Na\(^+/K^+\) -ATPase activity (Fig. 4). Half of the FITC-labeled enzyme preparation was subsequently labeled with ErITC (6 nmol/mg of protein). The K\(^+\) -activated p-nitrophenylphosphatase was inhibited, thereby, by more than 99%. After removal of free fluorophore by 3-fold centrifugation after each labeling step, the steady-state anisotropy values were measured (r = 0.27 for FITC, and r = 0.23 for ErITC, respectively, at the doubly labeled sample), and the fluorescence lifetime of the covalently attached FITC was determined in a FITC/ErITC-doubly labeled and a control FITC-labeled enzyme. As is evident from Fig. 6, energy transfer from the fluorescein residue to the erythrosin residue attached to Na\(^+/K^+\) -ATPase was not very pronounced. The average lifetime of the FITC-excited state was \(\tau = 3.21\) ns (Table II). A two-component lifetime decay was found to fit best to the experimental data obtained in the absence of ErITC as the energy acceptor. However, the fluorescence intensity decay became more heterogeneous in the presence of ErITC. In this case, only a three-component fit was adequate. The experimental data did not support more complex theoretical models of the fluorescence decay. Under these conditions, the average lifetime of the attached FITC decreased very slightly to only \(\tau = 2.88\) ns (Table II), due to Förster resonance energy transfer. The tiny decrease by about 10.5% of the average lifetime of the excited state of fluorescein in the double-labeled FITC/ErITC enzyme (as compared with a control enzyme) indicates a very long distance between the FITC label attached to the E\(_2\) ATP binding site and the ErITC attached to the E\(_1\) ATP site. Some slightly elevated \(\chi^2\) values in Table II could result as a consequence of differences in background fluorescence between the

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**FIG. 4.** Effect of 2 μM ErITC on K\(^+\) -activated p-nitrophenylphosphatase in FITC-pretreated enzyme. FITC (10 μM) was added after 10 min to 6 units of Na\(^+/K^-\) -ATPase, and the change of Na\(^+/K^-\) -ATPase (×) and K\(^+\) -activated p-nitrophenylphosphatase at 5 mM p-nitrophenylphosphate (■) was observed. 2 μM ErITC was added at 70 min, 60 min after inactivation with FITC, and K\(^+\) -activated p-nitrophenylphosphatase was measured in the presence (□) and absence (○) of 10 mM ATP. The residual K\(^+\) -activated p-nitrophenylphosphatase activity of the enzyme without ErITC remained during the "Experimental Procedures" and Ref. 54.

**FIG. 5.** Co(NH\(_3\))\(_4\)ATP protects against ErITC-labeling of a FITC-labeled Na\(^+/K^-\) -ATPase. 2.5 units (163 μg/ml) of FITC-labeled Na\(^+/K^-\) -ATPase was treated with 1 mM Co(NH\(_3\))\(_4\)ATP (●) in 20 mM Tris/HCl (pH 7.25) and 15 mM NaCl at 37 °C for 1 h. A control without Co(NH\(_3\))\(_4\)ATP (□) was performed in parallel. To both samples increasing concentrations of ErITC were added, and the fluorescence of erythrosin attached to protein was determined after washing. Mean values and S.D. of three experiments are shown. For experimental details, see "Experimental Procedures" and Ref. 54.
untreated sample, which was taken as a background correction, and the treated enzyme (50). Such minor differences can be hardly controlled and avoided.

Calculation of the distance between the \( E_A \) and \( E_B \) binding sites needs, besides knowledge of the efficiency of the Förster energy transfer, the molar ratio of the attached fluorophores to acceptor ratio. This ratio was determined fluorescently based on the increases in quantum yield of FITC and ErITC, respectively (see “Experimental Procedures”). Known concentrations of FITC in ethanol, ErITC in water, and of the enzyme allowed us to calculate the molar ratio from steady-state fluorescence data. The calculated molar ratio of total FITC/\( \alpha \)-subunit was 0.8. There was, however, a 25% unspecific labeling by FITC. Hence, 1 mol of ErITC/mol of \( \alpha \)-subunit was obtained. The molar ratio of ErITC/\( \alpha \)-subunit in an ATP-prelabeled enzyme was 0.48. Hence, 1 mol of ErITC and FITC were bound/2 mol of \( \alpha \)-subunits under the above conditions. However, when ErITC labeling was performed in an enzyme preparation that had not been previously preincubated with FITC, a molar ratio of 1.3 mol ErITC/mol of \( \alpha \)-subunit was obtained. In lifetime measurements, similar corrections of the molar donor to acceptor ratio using antifluorescein antibodies were performed (34, 46). The corrected rate of energy transfer was thus, 0.108 ns\(^{-1}\), and the calculated distance between the donor and the acceptor was 6.9 ± 0.9 nm. These results were calculated from the energy transfer rate according to Amler et al. (34, 51). Inclusion of 5% SDS into the assay system decreased the energy transfer between FITC and ErITC to 3% of the original. Thus, subunit separation resulted in the disappearance of Förster energy transfer.

An alternative approach to determine the distance between the low and high affinity ATP binding sites on Na\(^+\)/K\(^+\)-ATPase was to measure the distance between FITC attached to the high affinity \( E_1\) ATP binding site and Co\((\text{NH}_3)_4\)ATP bound to the \( E_2\) ATP site (8, 9). Since the absorption spectrum of Co\((\text{NH}_3)_4\)ATP overlaps well with the fluorescence emission spectrum of FITC, this was possible. We calculated a critical distance for this donor-acceptor pair as \( R_0 = 4.0 \) nm. We have to take into account that Co\((\text{NH}_3)_4\)ATP does not bind covalently. Hence, the presence of some free Co\((\text{NH}_3)_4\)ATP or Co\(^{2+}\) ions has to be considered. Since Co\(^{2+}\) ions are known to be potent collisional fluorescence quenchers, the lifetime of the excited state of FITC has always been measured in the presence of 1 mM Co\(^{2+}\) ions. The lifetime of FITC-labeled Na\(^+\)/K\(^+\)-ATPase in the presence of 1 mM CoCl\(_2\) was found to be 2.3 ns, whereas after inactivation with 1 mM Co\((\text{NH}_3)_4\)ATP, it decreased to 2.0 ns (Table II). The data in both cases were collected in the presence of antifluorescein antibodies to avoid contribution of nonspecifically attached fluorophores (34, 46). This decrease was due to Förster resonance energy transfer between FITC and Co\((\text{NH}_3)_4\)ATP bound to the \( E_2\) ATP site. Clearly, the 12.2% resonance energy transfer efficiency obtained points to a remote molecular distance between the protein-bound FITC and Co\((\text{NH}_3)_4\)ATP. Taking into account the critical distance of the energy donor-acceptor pair, we calculated a distance between \( E_1\) and \( E_2\) ATP sites of 6.0 ± 0.9 nm from this experiment (Table II).

**Distance Measurements between Ouabain Binding Sites**—To verify that the calculation of the distance between the \( E_1\) and \( E_2\) ATP binding sites represents the actual distance between two \( \alpha \)-subunits, we tried to measure the distance between ouabain binding sites as well. It is generally accepted that each \( \alpha \)-subunit has a molar ratio of ATP binding sites to ouabain derivatives, FEDO (8, 42). Steady-state fluorescence measurements of Na\(^+\)/K\(^+\)-ATPase labeled with both ouabain derivatives revealed a binding ratio of AO to FEDO of 0.53 AO/0.55 FEDO per \( \alpha \)-subunit, which are values within the theoretical error of 10% per label and result in a ratio of 1:1.2. This ratio was sufficient for distance measurements between two similar ouabain binding sites. On average, only half of the enzyme molecules had one labeled \( \alpha \)-subunit with AO and the other one with FEDO. The rest of the functional units of the enzyme had both \( \alpha \)-subunits labeled by either FEDO or AO only.

AO in the ouabain binding site revealed two different lifetimes, which were an intrinsic property of the label. A long-lived component of 10.7 ns and a short-lived component of 3.4 ns. The average lifetime was calculated to be 7.7 ns (Table II). The average lifetime of the excited state of AO decreased in the presence of FEDO to the value of 6.05 ns (Table II). In addition, the decay became more complicated, and a three component decay had to be used to fit the measured data. A calculation of the average distance between attached AO and FEDO from the average lifetime resulted in a molecular distance of 4.9 ± 0.5 nm.

**DISCUSSION**

This study shows, consistent with the idea that the interaction of the \( E_1\) ATP site is not specific for a distinct fluorophore of the eosin type (53), that in addition to eosin (32, 33) and...
fluorescein (36), erythrosin isothiocyanate also binds to the E1ATP site (Table I). The modification of this site is clearly evident after the blockade of the E1ATP site by Co(NH4)2ATP (Table I). ErITC inactivated the Na+-dependent phosphorylation of the α-subunit from the E1ATP site with the same rate constant as the overall Na+/K+ ATPase activity (Table I). The inactivation is prohibited by an excess of ATP (Fig. 3). This overall activity is, however, affected by ErITC with two different inactivation constants of 0.66 μM and 0.78 μM (Fig. 1), indicating a possible interaction with a second ATP site. In fact, after specific blockade of the E1ATP site by FITC, the protein-reactive ErITC inactivated the K+-activated p-nitrophenylphosphatase, a partial activity of the E2ATP site, with a K0 of 0.74 μM (Fig. 3). Also, labeling of this site is prevented by an additional blockade of the E2ATP site by Co(NH4)2ATP (Fig. 5). Hence, after specific protection of the E1ATP site by FITC (8), ErITC covalently labels the E2ATP site.

FITC and ErITC are very similar in their chemical structures, except for the four bulky iodides in the erythrosin molecule. It is surprising, therefore, that FITC is a label of the E1ATP site and not of the E2ATP site, whereas ErITC binds to and modifies both ATP sites. The idea has been put forward more recently that the E2ATP site has a broader binding pocket than the E1ATP site, since after modification of the E2ATP site with 7-chloro-4-nitrophenyl-2-oxa-1,3-diazole chloride, the latter site was easily accessible to a number of fluorescence quenchers (54). This explanation, however, cannot be applied to the differential labeling of ATP sites by ErITC and FITC, since the more bulky ErITC should not bind to the E1ATP site, which is what actually happens (Table I). Due to the close similarity of both fluorophores, the possibility exists that the π electron system of the aromatic ring system contributes to the interaction as well. It is certainly less displaced in the case of FITC as compared with ErITC.

It has been known for a long while that labeling of the E1ATP site at Lys1901 by FITC records the interaction of Na+/K+-ATPase with Na+ and K+ ions (36). Since ErITC is labeling the E1ATP site as well (Table I), one might assume that addition of K+ ions to an ErITC-labeled Na+/K+-ATPase should lead to a decrease of fluorescence as well as does with a FITC-labeled enzyme. This does not occur, however, as very careful studies with such an enzyme have revealed (34). It has been shown recently that labeling of the E1ATP site by fluorescent ethylanthroyl-Co(NH4)2ATP freezes the conformational flexibility of the E1ATP site of Na+/K+-ATPase, as studied by the fluorescence change of the FITC-labeled enzyme upon the addition of K+ ions (18). Therefore, to exclude the possibility that labeling of the E1ATP and E2ATP sites by ErITC may lead to a loss of the conformational flexibility in the presence of the transport substrates Na+ and Na+ as well, additional studies on an enzyme were carried out whose E1ATP site was labeled by FITC followed subsequently by the covalent modification of the E2ATP site with ErITC. When the effect of K+ ions was studied in such an enzyme preparation, an E1–E2 transition was seen when observing the FITC fluorescence. Hence, the conformational flexibility of the E2ATP site is lost only when ATP and not a pseudo-ATP analog is bound to the E2ATP site. These studies, moreover, seem to indicate that FITC and ErITC may not modify exactly the same site, even though they are modifying the E1ATP site. Consistent with the idea of a different microenvironment of the ATP sites labeled by FITC or ErITC is the observation that the quenching constants of iodide differ for FITC- (Kq = 0.4 m−1) and ErITC-modified (Kq = 3.5 m−1) enzymes. A definitive answer to this question can only be given by the demonstration that ErITC labels Lys1901 or another amino acid. Experiments to answer this question have been started.

Although, a definitive answer as to which amino acid might be modified by ErITC is lacking, there is no doubt as to the conclusion that ErITC labels ATP sites (Figs. 3 and 4) and that, in an FITC-treated Na+/K+-ATPase, ErITC interacts with the E2ATP site (Figs. 4 and 5). Labeling of Na+/K+-ATPase by ErITC is suppressed by ATP (Figs. 3–5) (34) as is the labeling of the enzyme by FITC (36). There were 0.48 mol of ErITC/α-subunit incorporated into an enzyme whose E1ATP site had been modified already by FITC. Moreover, the molar ratio of specifically incorporated FITC/α-subunit was 0.6. Hence, 1 mol of ErITC and FITC were bound 2 mol of α-subunits when both ATP sites were modified by different fluorescent isothiocyanates. The stoichiometry is consistent with the previous assumption that the two interacting ATP sites (4, 5) reside on different α-subunits (22). Consistent with this assumption are also the data obtained for Förster energy transfer measurements (Fig. 6, Table II). Fluorescein and erythrosin show a high overlap integral. Hence a close location of the two interacting ATP sites on the same α-subunit, as postulated by Ward and Cavieres (13), should lead to a high fluorescence quench. This is apparently not the case (Fig. 6, Table II). The data obtained

### Table II

|                      | FITC | FITC/ErITC | FITC/Co(NH4)2ATP | AO | AO/FEDO |
|----------------------|------|------------|------------------|----|---------|
| f1                   | 0.32 | 0.42       | 0.45             | 0.51 | 0.59  |
| τ1 (ns)              | 1.76 | 1.77       | 1.79             | 1.06 | 10.74  |
| f2                   | 0.68 | 0.57       | 0.39             | 1.38 | 0.31   |
| τ2 (ns)              | 3.90 | 3.79       | 3.88             | 3.69 | 3.43   |
| f3                   | 0.03 | 0.06       | 0.07             | 0.51 | 0.49   |
| τ3 (ns)              | 0.48 | 0.53       | 0.53             | 2.02 | 7.73   |
| τ2 (ns)              | 2.21 | 2.58       | 2.3              | 1.2  | 1.30   |
| τ2 (ns)              | 1.2  | 1.5        | 1.3              | 1.2  | 1.48   |
| Efficiency E         | 10.3%|            |                  | 12.2%| 21.7%  |
| Distance             | 6.9 ± 0.9 nm |            |                  | 6.0 ± 0.9 nm | 4.9 ± 0.5 nm |
(Table II) show that the distance between FITC- and ErITC-labeled ATP sites is 6.9 ± 0.9 nm. Because of different conditions of labeling in previous experiments (pH 9 versus pH 7.25, this study), the distance between FITC and ErITC (Table II) was somewhat higher than formerly published (r = 5.6 nm (34)). But as stressed previously, such long distances can only be explained by assuming a (αβ)2 diprotomeric structure, since the diameter of the α-subunit is 4.5 nm (34, 55). Independent measurements on the distance between the E1ATP site labeled by FITC and the E2ATP site labeled by Co(NH₃)₄ATP gave a value of 6.0 ± 0.9 nm (Table II). Both distances agree favorably well within the S.E. and reveal a total distance of 6.45 ± 0.64 nm (according to the law of propagation of errors) between both ATP-sites. In support of the conclusion that the E1ATP and E2ATP binding sites reside on different α-subunits is also the observation that solubilization of an FITC/ErITC doubly labeled enzyme showed no energy transfer any more. Hence, all data do not support the postulate that the two interacting ATP sites reside on the same α-subunit (13). Consistent with an (αβ)2 diprotomeric structure is also the finding that the distance between the cardiac glycoside receptor sites labeled with TNP-ATP (2.9 ± 0.6 nm) and ErITC (3.2 nm), respectively, (34, 56); the distance between N-β-(2-benzimidazolophenyl)maleimide (BIPM) bound to Cys580 and FITC (3.6 nm) and AO (3.9 nm) (60).

**Acknowledgments**—We are grateful to W. Mortens for technical assistance and Dr. Roger D. Dennis for reading the manuscript.

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