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Characterization of Conformational Changes in (Na,K) ATPase Labeled with Fluorescein at the Active Site

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Abstract

Conformational changes have been studied in (Na,K) ATPase labeled at or near the ATP binding region with fluorescein following incubation with fluorescein isothiocyanate (FITC). One or two fluorescein groups are bound per ATPase molecule. (Na,K) ATPase activity, phosphorylation from ATP, and nucleotide binding are abolished in labeled enzyme, but phosphorylation from inorganic phosphate or K-phosphatase activity are only partially inactivated. The fluorescein groups are incorporated only into the 96 KD catalytic chain of the (Na,K) ATPase, and presence of ATP during the incubation with FITC protects against the incorporation and inhibition of enzymic activity. Upon trypsin treatment of labeled membranes the fluorescein appears first in a 58 KD fragment and eventually is released into the medium. The fluorescein-labeled (Na,K) ATPase shows a large quenching of fluorescence (15–20%) on conversion of the E_1 or $E_1 \cdot Na$ conformation in cation-free or Na^+-rich media to the $E_2 \cdot (K)$ form in K^+ (or congeners Tl^+, Rb^+, Cs^+, NH_4^+) rich media. Cation titrations suggest that K^+ and Na^+ ions compete at a single binding site and stabilize $E_1 \cdot Na$ or $E_2 \cdot (K)$ respectively; $K_K \approx 0.23$ mM, $K_{Na} \approx 1.2$ mM. The rate of the conformational transition $E_2 \cdot (K) \rightarrow E_1 \cdot Na$ is slow, $k = 0.3$ sec^{-1}, but contrary to previous experience [7, 8] ATP does not stimulate this rate. The rate of the transitions $E_1 + K^+ \rightarrow E_2 \cdot (K)$ rises sharply with K^+ concentration and shows saturation behavior, from which a $k_{max} \approx 286$ sec^{-1} and $K_k \approx 74$ mM are deduced. The data support and extend the previous suggestion that K^+ ions bound initially at a low-affinity (probably cytoplasm oriented) site in state E_1 are trapped in the occluded form $E_2 \cdot (K)$ by the conformational change poised far ($K_c \approx 1000$) in the direction of $E_2 \cdot (K)$. It is proposed in addition that at least two binding sites for K^+ exist at the cytoplasmic surface of isolated (Na,K) ATPase in state E_1 but a large difference in affinities precludes detection in fluorescence titrations of more than one site. A variety of ligands in addition to K^+ produce fluorescence-quenched or E_2 forms of the labeled (Na,K) ATPase. These include Mg^{2+} plus inorganic phosphate, without or with K^+ ions (E_2P or $E_2P \cdot K$) or with ouabain (E_2-ouabain or $E_2P \cdot$ ouabain). Na^+ ions antagonize these effects. The collected data support the notion that there may be many subspecies of the E_1 and E_2 forms (either phosphorylated or nonphosphorylated) with different numbers of Na^+ and/or K^+ ions bound or occluded, each subspecies having a

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characteristic ability to catalyze reactions and/or transport cations. The relationship between the conformational changes in fluorescein-labeled enzyme and the subunit structure of the (Na,K) ATPase is discussed with particular reference to "half of the site" models for ATP hydrolysis.

Introduction

In recent years protein conformation changes in Na,K) ATPase have been studied in detail by a number of experimental approaches [1–10]. We have investigated conformational transitions in (Na,K) ATPase purified from kidney membranes utilizing as probes the fluorescent formycin nucleotides and intrinsic protein fluorescence [6–8]. The probes allow easy detection of the two major forms of nonphosphorylated pump E_1 or $E_1 \cdot Na$ which exist in monovalent cation-free or Na-rich media and $E_2 \cdot (K)$ which predominates in K^+-rich media. Also E_1P and E_2P conformations of the phosphorylated protein can be detected in conditions of ATP or FTP hydrolysis [8, 11]. Kinetic measurements [6, 8] of the conformational transitions in conditions of nucleotide hydrolysis or studied in isolation from other steps of the substrate turnover cycle were consistent with the classical Post–Albers scheme [1, 2, 12]. In addition, the fluorescence studies lead to an extension of the conventional formulations by implying that the Na^+ efflux requires both phosphorylation and a conformational change in the sequence $E_1 \rightarrow E_1P \rightarrow E_2P$, followed by K^+ influx which involves both a dephosphorylation step and reverse conformational change in the intermediate sequence $E_2\,P \rightarrow E_2 \cdot (K) \rightarrow E_1$ [7]. The rate of the transition $E_2 \cdot (K) \rightarrow E_1 \cdot Na$ was measured using the fluorescent probes [7, 8] and was found to be very slow, consistent with the proposal that K^+ ions are occluded from free exchange with Na^+ ions in the medium [2]. Binding of nucleotide with a low affinity greatly accelerated the rate of this step [7, 8]. At physiological ATP concentrations the occluded species $E_2 \cdot (K)$ amounts to about half the total enzyme molecules and may be the site for kinetic regulation of the Na pump cycle *in vivo* [9]. Very recently we have also utilized the intrinsic protein fluorescence signal to detect defective conformational response in a selectively trypsinized (Na,K) ATPase [11].

The accuracy and versatility of the fluorescence measurements with formycin nucleotides or intrinsic fluorescence was limited by the relatively small signal changes. We have therefore invested some effort in obtaining (Na,K) ATPase labeled with a suitable fluorescent reporter group, namely fluorescein, as the results in this paper describe. It is important to bear in mind that if a label is to monitor events which occur, say in the active site of an enzyme, it must interact with that region of the protein and inevitably influence those events. Therefore in general one will aim to choose a label

which binds to the protein in a known region and affects activity in a defined way. Reagents such as N-ethylmaleimide, NEM, or 2,2[1]-dithiobis(2-nitrobenzoic acid), DTNB, are known to react with many sulfhydryl groups on the enzyme [13]. Therefore the choice of fluoresceinisothiocyanate (FITC) was dictated initially by the wish to avoid sulfhydryl reagents. It turns out that the FITC acts as a highly selective label for the ATP binding region and although nucleotide binding and substrate hydrolysis is abolished, the cation-induced conformational responses of the nonphosphorylated protein are intact. The $E_1 \cdot Na$ or $E_2 \cdot (K)$ forms are easily distinguished by rather large changes in the fluorescein fluorescence emission, and the improved accuracy of both kinetic and equilibrium measurements has provided important new information on the binding of the Na^+ or K^+ ions and stabilization of the $E_1 \cdot Na$ and $E_2 \cdot (K)$ states. Also the fluorescein-labeled (Na,K) ATPase is a convenient tool for investigating the interactions of pump-specific ligands such as ouabain, phosphate, or vanadate [14].

A preliminary account of this work has been reported in the proceedings of the Second International Conference on Properties and Functions of (Na,K) ATPase [9].

Experimental

(Na,K) ATPase was prepared from red outer medulla of pig kidney by the rapid procedure of Jorgensen [15]. The membranes were washed twice in a medium containing 25 mM Imidazole, pH 7.5, and 1 mM EDTA, suspended in this medium to which sucrose mM 250 had been added and stored frozen at −20°C. Specific (Na,K) ATPase activities were 17–23 units per milligram protein and there was no ouabain-insensitive activity in these preparations. The micromolar concentration of enzyme was estimated approximately by assuming a molecular weight of 270,000, and from the measured specific activity assuming that pure enzyme has a specific activity of 37 units/mg protein [16].

Concentrated stock solutions of fluorescein isothiocyanate were prepared by dissolving the solid in dry dimethyformamide, and were diluted with the solvent as necessary. Labeling of (Na,K) ATPase was performed routinely at 20°C by incubating enzyme (1–3 mg/ml) suspended in a medium containing 100 mM Tris-HCl, pH 9.2, and 2 mM EDTA (Tris) for 15–30 min with 0.5–10 μM FITC. Labeling was stopped usually by diluting the suspension severalfold with an ice cold solution containing 50 mM Tris-HCl, -H 7.5, or 25 mM Imidazole, pH 7.5, and 1 mM EDTA (Tris). Excess FITC and fluorescent hydrolysis products were removed either by dialysis at 0°C for 24–48 h against 1–3 liters of the diluting solutions containing also 1 mg/ml

bovine serum albumin, or by centrifuging the diluted membranes in a Beckman L 2–65 centrifuge (rotor SW 65) for 1.5 h and 50,000 rpm. In the latter case the pellet was resuspended at a known protein concentration in the diluting solution. (Na,K) ATPase activity was measured as described in [17]. K^+-dependent phosphatase was estimated as in [17] in a reaction medium containing 50 mM KCl, 50 mM Tris-HCl, pH 7.5, at 20°C, 6 mM $MgCl_2$, and 20 mm p-nitrophenyl phosphate (Tris salt), PNPP. The PNPP disodium salt was converted to the Tris salt by passage down a small column of Amberlite resin 1R-120 (H), and the pH of the effluent was brought to 7.5 with Tris solution. Formycin triphosphate (FTP) binding was assayed fluorimetrically as described in [6] in a medium containing enzyme (300 μg), 50 mM Tris-HCl, pH 7.7, 50 mM NaCl, 10 mM CDTA (Tris salt), and 1–10 μM FTP. Phosphorylation from ATP was performed at 0°C essentially as in [6], in 0.2 ml of the following medium: 50 μg enzyme, 80 mM NaCl, 20 mM Tris-HCl, pH 7.4, at 0°C, 2 mM $MgCl_2$, and 10 μM ATP + (γ-^{32}P)-ATP. Phosphorylation from inorganic phosphate was measured in the following medium (total volume 30 μl): 50 μg protein, 2.5 mM phosphate (Tris salt) + $^{32}P_i$, 50 mM Tris acetate, pH 7.5, 2 mM $MgCl_2$ and 1 mM ouabain. After incubation for 5 min at room temperature 5 ml of ice cold 5% perchloric acid solution containing 2 mM P_i was added. The denatured protein was then filtered on Whatmam GF/C paper discs, washed with 40 ml of 5% TCA, and put into a scintillation vial to which 10 ml Brays solution was added. Lowry protein assays were performed as in [17].

SDS-polyacrylamide gel electrophoresis was carried out by the method of Weber and Osborne [18] using 7.5% acrylamide and 0.2% bisacrylamide. Gels were stained with Coomassie Blue 0.25% and destained in a solution containing 7% acetic acid and 5% methanol. The fluorescent bands in unstained gels were visualized by illumination in a dark room with a long-wavelength UV lamp. Such gels were photographed using Kodak Plus X film and a camera fitted with an orange filter. Quantitative estimates of fluorescein bound to polypeptides in the gels were obtained in the following way: The fluorescent bands were cut out (2–3 mm of gel) and homogenized in 0.5 ml of a 1% solution of Triton X-100. After 2–3 days at room temperature 2 ml of water was added and the polyacrylamide was removed by centrifugation. Aliquots of the supernatant containing the extracted protein were scanned in the fluorimeter for their fluorescence emission as in Fig. 6. The area under the emission spectra in different samples was compared by cutting around the spectral trace and weighing the recorder paper.

Fluorescence measurements except for the stopped flow work were performed on a Perkin-Elmer HP44 spectrofluorimeter. The fluorescence cell was fitted with a mixing device which allows addition of known-volumes of solution, during continuous stirring of the enzyme suspension. Fixed volumes

were added manually with Hamilton syringes. Equilibrium titrations were carried out by continuous injection of solutions from a Hamilton syringe connected to a Harvard Apparatus Infusion System. For routine measurements excitation and emission wavelengths were 495 and 525 nm respectively; the slitwidths on both monochromators were 10 nm and the time response of the instrument was set to 0.3 sec. For measurement of ligand-induced fluorescence changes the signal was amplified 10-fold.

Stopped-flow measurements were carried out in the instrument referred to previously [6–8, 19]. Fluorescein fluorescence was excited at 480 nm and the emitted light was detected after passage through a Wratten No. 16 filter which block effectively transmission of wavelengths at 510 nm or below. Signals were recorded on a Data Lab Transients Recorder, or photographs were made of the oscilloscope traces. Rate constants were obtained graphically.

Materials

ATP disodium salt was obtained from Boehringer. FITC (isomer I), *p*-nitrophenyl phosphate (disodium) phosphatase substrate, ouabain, trypsin (type 1), and trypsin inhibitor (type 1–S) were from Sigma. FTP was prepared as described in [6]. $^{32}P_i$ was purchased from the Israel Atomic Energy Centre, Beer Sheba, and (γ-^{32}P)-ATP was purchased from the Radiochemical Centre, U.K.

Results

Enzyme Inactivation

Incubation of the kidney (Na,K) ATPase with a 1.6-fold molar excess of FITC at pH 9.2 resulted in inactivation of ATPase activity, along the time course shown in Fig. 1. The presence of ATP during the incubation provided essentially complete protection against the reagent. This result suggests that inactivation is caused by a highly selective reaction between the FITC and one or possibly two protein side groups in or near the ATP binding region. The concentration of ATP required for protection was no more than 10 μM, implying binding to a high-affinity site, but detailed titration was not carried out. Loss of ATPase activity in Fig. 1 was both incomplete and nonexponential. This is probably due to lability of the FITC at alkaline pH's which causes the effective reagent concentration to fall below that of the enzyme during the course of the reaction. A more rapid and essentially complete inactivation could be achieved with a somewhat higher molar ratio of FITC to enzyme, for example 4:1 as in the experiment of Fig. 2. In the range of FITC to enzyme

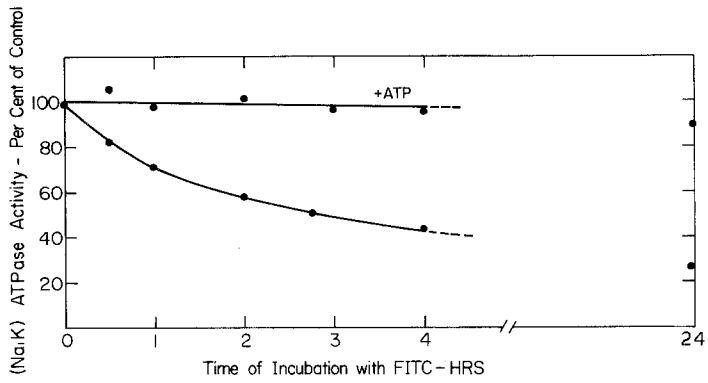

Fig. 1. Inactivation of (Na,K) ATPase by FITC and protection by ATP. (Na,K) ATPase, 1.09 mg/ml and specific activity 20 units/mg (≈2.4 μM enzyme), was incubated at 20°C with 4μM FITC in the standard incubation medium, with or without 3 mM ATP (Tris) (total volume 70 μl). At the times indicated, small aliquots were removed into 1 ml of the assay medium for (Na,K) ATPase and the activity was measured. Inactivation by FITC was stopped instantaneously both by the large dilution and the low pH (7.5) in the assay medium.

4–10:1, ATP protected to an extent of 75–90%. At much higher FITC concentrations a rapid but less selective inhibition was observed, and was therefore of less interest.

The rate of loss of the ATPase increased sharply when the pH of the medium for incubation with FITC was raised from 7 to 8 to 9 (now shown). This is consistent with involvement of a lysine side chain(s) in the isothiocyanate reaction, but definite proof was not obtained. At pH 10 a slow and incomplete inhibition was noted and was attributed to instability of FITC.

Table I summarizes a number of striking properties of the fully fluorescein-labeled enzyme. Nucleotide binding to the high-affinity site, monitored with the use of the fluorescent formycin nucleotides, was totally absent. In consequence, phosphorylation by ATP in the presence of Na^+ ions was prevented and hence also the enzyme turnover. These findings complement nicely the ATP protection data (Fig. 2) in pointing to involvement of the high-affinity nucleotide binding site as the position of the attachment of the fluorescein group(s). By contrast, reactions associated with later steps in the turnover cycle such as the K^+-phosphatase and phosphorylation by inorganic phosphate were only partially inactivated. Reduction in maximal rate of the phosphatase was accompanied by an increase in *K*m for the PNPP substrate from 2.7 with control enzyme to 5.5 mM in the fully labeled ATPase. The relative insensitivity of the phosphatase to FITC was studied further by comparing the time course of inactivation by FITC of phosphatase with ATPase. As seen in Fig. 2, although the maximal activity is reduced eventually to a level 30 to 40% of control, much of the ATPase activity

Table I. Enzymic Properties of Control and Fluorescein-Labeled (Na,K) ATPase[a]

Enzyme	(Na,K) ATPase, μmoles P_i · mg protein^{-1} · min^{-1}	FTP binding fluorescence change, arbitrary units	Phosphorylation by ATP, nmoles · mg protein^{-1}	Phosphorylation by P_i, nmoles · mg protein^{-1}	K-Phosphatase, nmoles · mg protein^{-1} · min^{-1}
Control enzyme	18.9	20	2.1	1.94	1.69
Fluorescein-labeled enzyme	0.94	0	0.14	0.86	0.71

[a]Enzyme was incubated with FITC for 4 h in the conditions of Fig. 2 and then dialyzed extensively as in the Experimental section. Control and fluorescein-labeled enzymes were then assayed for each reaction in identical conditions.

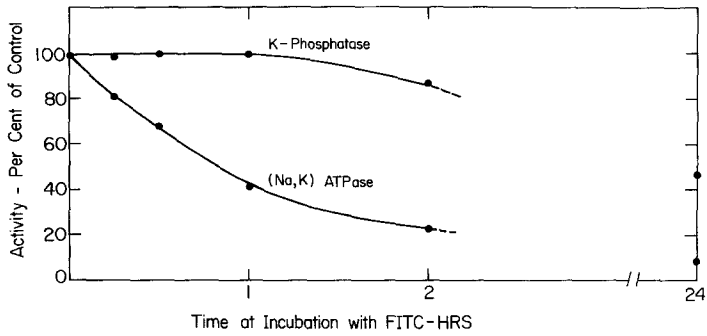

Fig. 2. Differential inactivation of (Na,K) ATPase and K^+-dependent phosphatase by FITC. Enzyme, 1.1 mg/ml (≈2.5 μM), was incubated at 20°C with 10 μM FITC for the times indicated and aliquots were then assayed for (Na,K) ATPase and K-dependent phosphatase.

disappears before there is any detectable effect on the phosphatase. This time lag suggests strongly that FITC must react with more protein side chains in order to inhibit phosphatase than is the case for the ATPase activity.

Fluorescence Labeling of the Catalytic Polypeptide

Further evidence for a highly selective incorporation of fluorescein into the active site of the (Na,K) ATPase is provided by the SDS-polyacrylamide gels shown in Fig. 3 and 4. Fluorescein labeled only the catalytic subunit; and glycoprotein was untouched and neither was there detectable binding to phospholipids (Figs. 3, 4a). Incorporation of fluorescein was largely prevented by the presence of ATP (Fig. 3). Measurements of the total fluorescence of the large polypeptide chains, eluted from the gels with Triton X-100, confirmed that ATP reduced covalent binding of fluorescein to the same extent that it hindered inhibition of enzyme activity (estimated separately on intact membranes). Protection was about 80% in Fig. 3 or essentially complete in the labeling conditions of Fig. 1.

Figure 4b, left and right traces, shows the localization of fluorescein in the polypeptide components of labeled enzyme subjected to a partial tryptic digestion prior to solubilization of the membranes in SDS. This degree of proteolysis cuts the catalytic polypeptide into two fragments of molecular weights about 58 and 47 kilodaltons, but the fluorescein is only in the larger fragment. Controlled trypsinization of unlabeled (Na,K) ATPase in K^+- or Na^+-rich media gives rise to different and distinctive polypeptides, providing convincing evidence for different Na^+ or K^+ stabilized protein conformations [4]. For the fluorescein-labeled (Na,K) ATPase this is not the case. One observes only the 58 and 47 kilodalton fragments in both K^+- and Na^+-rich media (not shown). Since it is clear from the fluorescence data (right) that the labeled protein is not fixed in a K^+ -like conformation, it may be that a

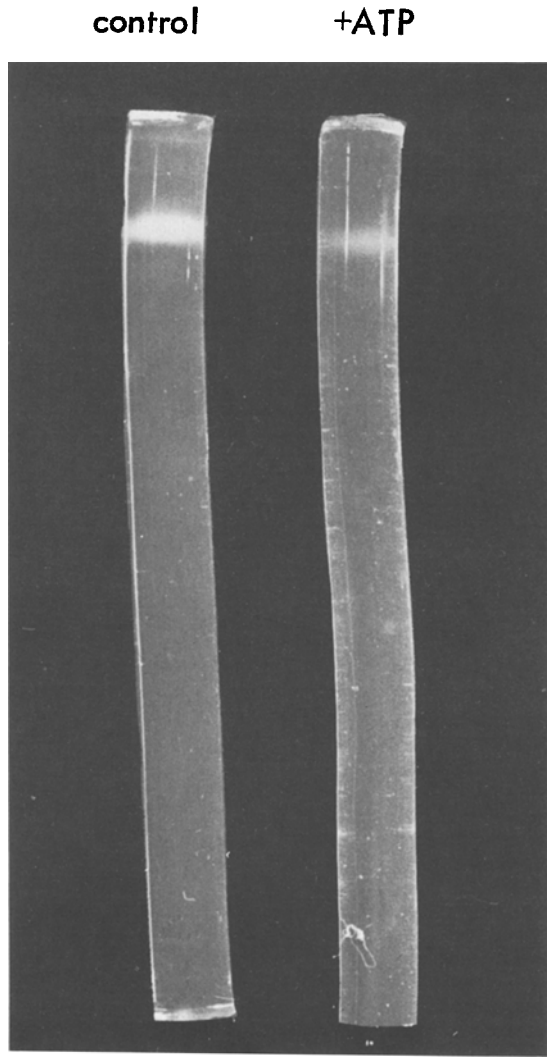

Fig. 3. Incorporation of fluorescein into the catalytic polypeptide and protection by ATP. Enzyme, 0.5 mg/ml (≈ 1 μM), was incubated at room temperature with 10 μM FITC for 30 min, without or with 3 mM ATP. Small aliquots were assayed for (Na,K) ATPase which was inhibited by 66 or 9% respectively. The membrane suspensions (200 μl) were diluted to 10 ml with a solution containing 25 mM Imidazole, pH 7.5, 1 mM EDTA at 0°C, and centrifuged as described in the Experimental section. The fluorescent pellets were dissolved in 2% SDS and portions containing 70 μg protein were boiled for 2 min with 1% mercaptoethanol and then applied to the gels. Gels were run for 12 h at a current of 2 mA per gel.

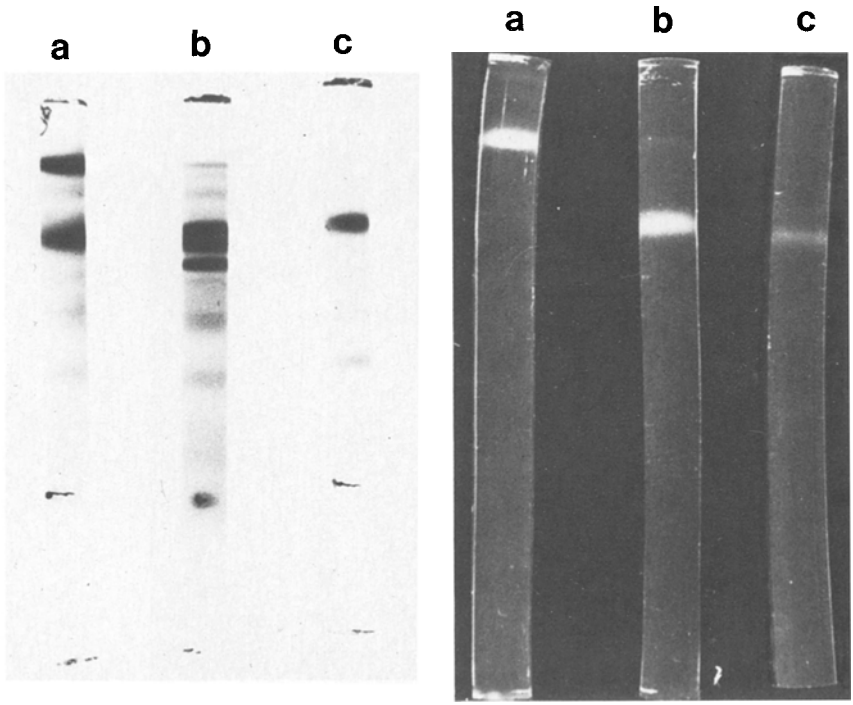

Fig. 4. Polyacrylamide gels of control and trypsinized fluorescein-labeled (Na,K) ATPase. (a) Control, (b) partial tryptic digest, and (c) extensive digestion. Labeled enzyme was prepared using a protein-to-FITC concentration ratio of about 1:10, and then dialyzed as described in the Experimental section. Protein (170 μg) was suspended in a medium of volume 1 ml containing 150 mM KCl, 1 mM $CaCl_2$, and 25 mM Imidazole, pH 7.5, at 20°C. Controlled proteolysis was achieved by addition of 6 μg trypsin, incubation at 37°C for 10 min, followed by addition of 20 μg trypsin inhibitor (in 1 ml). Extensive proteolysis was brought about by incubating labeled protein in the same KCl medium for 1 h at 37°C with 20 μg of trypsin. Control and proteolyzed membrane suspensions were then diluted, centrifuged, and the SDS-solubilized pellets applied to duplicate gels (80 μg protein) as described in Fig. 3. The photograph on the left is of Coomassie Blue stained gels; on the right unstained gels photographed as in the Experimental section (enlargement 1.17 times greater than in left photograph; both reduced 20% for reproduction).

lysine residue normally exposed to preferential tryptic attack in a Na^+ medium has been blocked by the fluorescein itself. Further proteolysis did not produce smaller fluorescent fragments in the membrane. After an extensive tryptic digestion of the fluorescein–(Na,K) ATPase (Fig 4c, left and right traces), nearly all the fluorescence was released from the proteolyzed membranes as soluble polypeptide fragments. This is expected, for it would be surprising if the ATP binding domain of the catalytic polypeptide was not susceptible to tryptic attack from the aqueous medium. Protein components remaining in the membrane include the glycoprotein, which is very resistant to trypsin, and lower-molecular-weight membrane-embedded polypeptides

[20] which are not visible in the gel on the left due to poor staining by the Coomassie Blue.

Cation-Induced Conformational Changes

Emission and excitation spectra of the labeled (Na,K) ATPase characteristic for fluorescein bound to proteins are presented in Fig. 5, together with the cation effects. Addition of low concentrations of K^+ to membranes suspended in a Na^+- and K^+-free medium produced an immediate quenching of the fluorescence intensity by about 17% without shifting the wavelength optimum. Na^+ ions reversed or prevented the K^+ effect. The bound fluorescein was clearly reporting on the transitions between the E_1 or $E_1 \cdot Na$

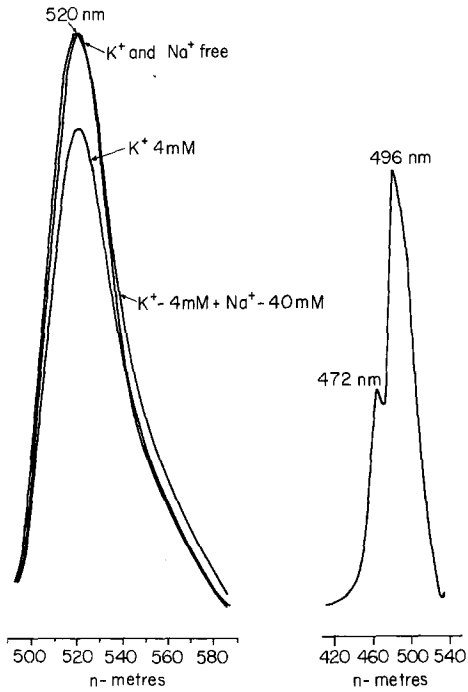

Fig. 5. Excitation and emission spectra of fluorescein-labeled (Na,K) ATPase and the effects of K^+ and Na^+ ions. Enzyme was labeled at a protein-to-FITC concentration ratio of 1:1.5 in a 30-min incubation at 24°C, and the suspension was then dialyzed for 48 h as described in the Experimental section. Labeled protein, 60 μg, was suspended in 2.5 ml of a solution containing 50 mM Tris-HCl, pH 7.7, at 20°C and 1 mM EDTA (Tris). Emission spectra (left) were run with a fixed excitation wavelength of 485 nm, and for the excitation spectrum (right) the fixed emission wavelength was 540 nm. Slit widths on both monochromators were 5 nm. These particular spectra were not corrected for wavelength sensitivity of the emission photo tube. 2.5 μl of 4 M KCl or 25 μl of 4 M NaCl were added as indicated and the spectra were rescanned.

conformation which have a "high" flourescence yield and the $E_2 \cdot (K)$ conformation which has a "low" fluorescence.

In order to establish optimal conditions for studying the cation effects we have measured the size of the K^+-dependent signal change in samples of the enzyme containing varying amounts of fluorescein bound per mole of protein (Fig. 6). Samples a–e were prepared by incubating increasing concentrations of FITC with a fixed concentration of protein. The linear relation between the amplitude of the K^+-induced change and the total fluorescence of fixed quantities of protein, seen for samples a–d, indicates a uniform behavior of the population of reporter molecules due presumably to labeling at a single side chain. As the FITC concentration was raised to that of sample e, or even higher (not shown), the K^+-dependent response was an increasingly small fraction of the total fluorescence, following incorporation of the fluorescein into additional side chains at which it is not responsive to changes in the protein conformation. Fluorescence measurements were of course routinely performed with protein labeled most selectively. ATPase activity would

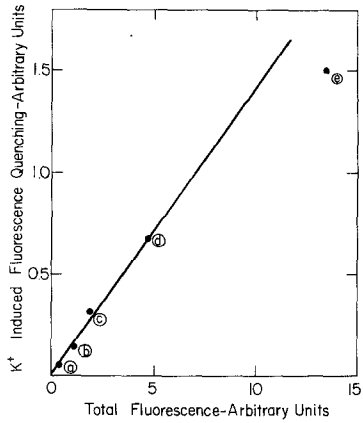

Fig. 6. Fluorescence quenching in (Na,K) ATPase labeled with different concentrations of FITC. (Na,K) ATPase, 0.4 mg/ml (≈0.8 μM), was incubated for 15 min at 23°C with FITC at final micromolar concentrations of (a) 0.4, (b) 1, (c) 2, (d) 4, and (e) 10. After dilution and dialysis as in the methods, aliquots of the suspension were centrifuged and equal amounts of protein (39 μg) from SDS-solubilized pellets were applied to gels and electrophoresed. The amounts of fluorescein in the catalytic unit for preparations a–e was estimated as described in the methods section. For measurement of the K-induced fluorescence quenching, dialyzed enzyme (60 μg of protein) was suspended in 2.5 ml of the Tris-HCl medium in Fig. 5. For each sample a–e the total fluorescence was recorded at a single emission and excitation wavelength of 525 and 495 nm respectively. Then the decrease in intensity following addition of 10 μl of 2 M KCl was recorded. Small variations in the amounts of protein added in samples a–e were estimated [8] by recording the membrane's intrinsic protein fluorescence (excitation 288 nm; emission 335 nm) and corrected for by dividing the K^+-induced change in fluorescein fluorescence by the total protein fluorescence in each case. Unfortunately, reliable ATPase assays were not obtained in this experiment.

normally be inhibited to 50–60% in condition d of Fig 6 or less in conditions a–c.

Monovalent Cation Titration

The conveniently large size of the fluorescence signals allows quite accurate determinations of the ion requirements for the conformational transitions. Figure 7A presents a typical titration performed by injecting a KCl solution at a known slow rate into the constantly and rapidly stirred enzyme suspension. Since the rate of injection of K^+ ions is much slower than the rate of equilibration between the two conformations, the change traces out the level of $E_2 \cdot (K)$ form as a function of the known K^+ concentration, discounting the continuous drop in signal at the end of the titration caused by the diluting effect of the injected fluid volume. The curve looks hyperbolic and the inverse plot of Fig. 7B is linear, suggesting, of course, interaction of a single K^+ ion per enzyme molecule. Strong support for that inference comes from titrations of the K^+ requirements in media containing different concentrations of NaCl. The apparent affinity for K^+ obtained from plots similar to that in Fig. 7B is linearly related to the Na^+ ion concentration (Fig. 8), implying that K^+ and Na^+ compete for a single binding site and stabilize the alternate conformations respectively. The data in Fig. 8 give $K_K = 0.23$ mM and $K_{Na} = 1.27$ mM. In media containing either 150 mM Tris-HCl (pH 7.7) or 140 mM NaCl plus 10 mM Tris-HCl (pH 7.7), continuous KCl titration curves were also hyperbolic and gave rise to apparent affinities for K^+ ions of 0.72 and 44.7 mM respectively. The $K_{0.5}$ value in the absence of Na^+ ions is somewhat higher than in the conditions of Fig. 7 and 8, perhaps due to the

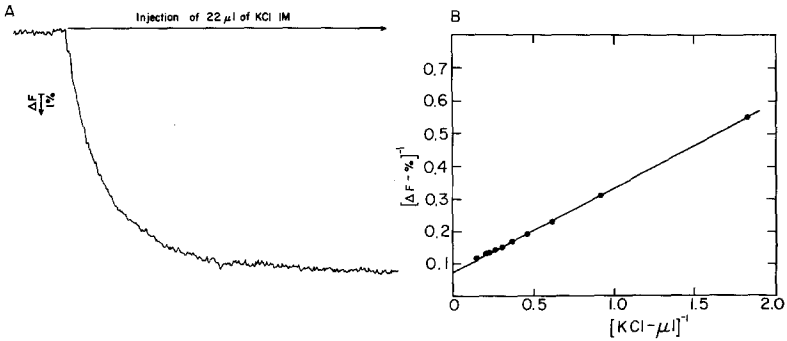

Fig. 7. Continuous equilibrium titration of K^+-induced fluorescence quenching. Membranes were labeled and suspended in the Na- and K-free medium as in Fig. 5. The constant rate of injection of KCl (1 M) was adjusted slow enough so that the concentration of KCl for a half maximal response was independent of the rate. The total time for completion of the titration was about 10 min (speed 5 or 6 on the infusion apparatus). A small correction for the effect of dilution on the extent of the fluorescence response was made from the final section of the trace.

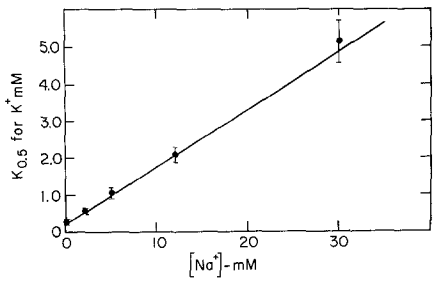

Fig. 8. Dependence of the apparent K^+ affinity on Na^+ concentration. Conditions were as in Fig. 7 except that NaCl replaced an equivalent concentration of Tris-HCl. The concentration and volume of the injected KCl solution was adjusted to meet the K^+ requirement.

weak Na^+-like action of $Tris^+$ ions at the higher concentration. One can also titrate the Na^+ ion requirement for reversing the fluorescence quenching at fixed K^+ concentrations. Such curves too are consistent with simple competition between Na^+ and K^+ ions (not shown), and lead to an estimated K_{Na} = 0.9 mM.

K^+ congeners produced K^+-like effects with the following order of affinities: Tl > Rb > K > NH_4 > Cs. Li^+ ions did not affect the fluorescence at concentrations up to 50 mM.

Recently we have reported that the K^+-induced change in the intrinsic protein fluorescence of unlabeled (Na,K) ATPase is antagonized by low concentrations of ATP or ADP, which stabilize the E_1 state [11]. This phenomenon cannot be observed with fluorescein-labeled enzyme since nucleotide binding has been abolished. Also the apparent K^+ affinity for sustaining the conformational change (Kapp = 0.2 mM) is quite unnaffected by the presence of ATP even at a concentration of 5 mM (not shown).

Rates of the Conformational Transitions

Stopped-flow fluorimeter traces of the conformational changes monitored with the use of fluorescein-labeled (Na,K) ATPase are shown in Fig. 9. The signals are of better quality than those obtained with the other probes, and allow resolution of certain problems previously unanswered.

The slow transition $E_2 \cdot (K)$–$E_1 \cdot Na$ (Fig. 9A, left) has a rate of 0.3 sec^{-1} which is essentially the same as found in experiments with formycin nucleotides or protein fluorescence, when performed at low or zero nucleotide concentrations [7, 8]. But a striking difference is the fact that millimolar concentrations of ATP did not stimulate the rate of the transition in contrast to the previous findings (Fig. 9B, left).

The right-hand traces of Fig. 9 are signals of the transition $E_1 + K \rightarrow E_2 \cdot (K)$ measured at increasing concentrations of K^+ ion (A, B, and C). The

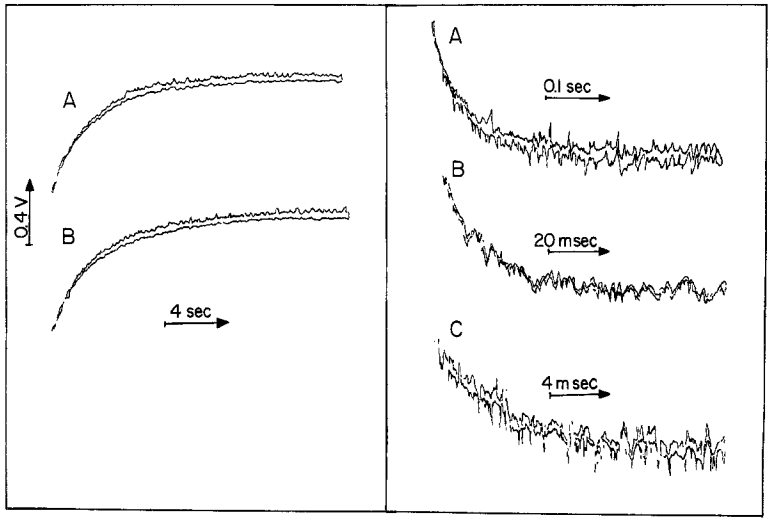

Fig. 9. Stopped-flow traces of conformational transitions in fluorescein-labeled (Na,K) ATPase. Left: transition $E_2 \cdot K \rightarrow E_1 \cdot Na$. Syringe I contained, in 5 ml, 150 μg (Na,K) ATPase labeled as in Fig. 5, 150 mM Tris-HCl, pH 7.7 at 20°C, 1 mM EDTA (Tris), and 3 mM KCl. Syringe II contained (A) 150 mM NaCl and (B) 150 mM NaCl and 6 mM $ATP(Na)_2$. The time constant on the oscilloscope was 100 msec. Right: transition $E_1 + K^+ \rightarrow E_2 \cdot (K)$. Syringe I contained, in 5 ml, 300 μg (Na,K) ATPase labeled as in Fig. 5, 150 mM Tris-HCl, pH 7.7, and 1 mM EDTA (Tris). Syringe II contained KCl and Tris-HCl, pH 7.7, at a total concentration of 150 mM, 1 mM EDTA $(Tris)_2$, and (A) KCl 4 mM, time constant 5 msec; (B) KCl 20 mM, time constant 1 msec; (C) KCl 150 mM, time constant 0.1 msec. The final concentration of all materials not present in both syringes was half that given above. All experiments were conducted at room temperature, 22°C.

very large increase in rate on raising the K^+ concentration is visualized in the plot of Fig. 10. These results essentially duplicate those of the FTP experiments for the KCl range 2–20 mM [7], but the new feature is the saturation phenomenon which begins to be apparent at about 40 mM KCl. The data can be fitted rather well by the following parameters: maximal $k = 286\ sec^{-1}$, $K_{0.5}$ for $K^+ = 74$ mM, and the Hill coefficient $n = 1.09$. The deviation of the n value from 1 is almost certainly insignificant.

Our experience with the various fluorescence probes has led to the conclusion that K^+ and Na^+ ions bind to the protein in a mutually exclusive fashion and stabilize alternate conformations [7]. This behavior seems consistent with consecutive cation transport models [21]. On the other hand, concerted cation transport models require simultaneous attachment of Na^+ and K^+ to the pump [22]. In an attempt to test the prediction of hybrid cation binding, we have studied the rate of the slow transition $E_2 \cdot (K)$–$E_1 \cdot Na$ with fixed K^+ concentrations but variable Na^+ in a high concentration range. The enzyme was suspended in a medium containing 1.45 M Tris-HCl, pH 7.6, and

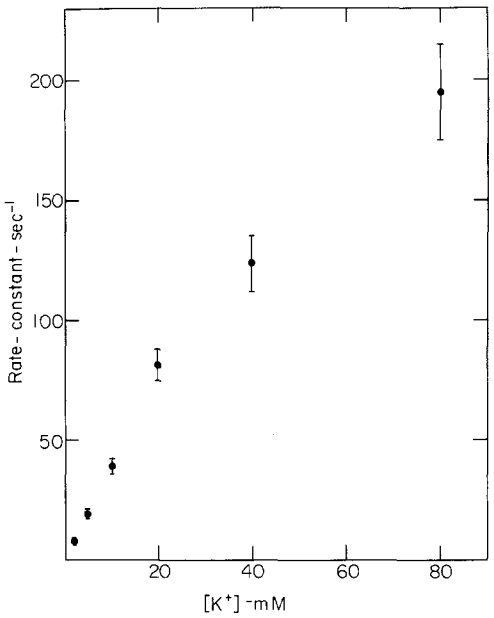

Fig. 10. Variation of rate constant for transition $E_1 + K^+ \rightarrow E_2 \cdot (K)$ with K^+ concentrations. Rate constants are from the experiment of Fig. 9. Each point is the average value ± 1 SEM from at least three replicate traces.

0.05 M KCl, and was mixed in the stopped flow fluorimeter with the following solutions: (A) 1.3 M Tris-HCl, 0.2 M NaCl; (B) 1 M Tris-HCl, 0.5 M NaCl; and (C) 1.5 M NaCl. Final concentrations after mixing were 25 mM KCl and 0.1, 0.25, and 0.75M NaCl respectively. Rate constants observed were (A) 0.73 sec^{-1}, (B) 0.81 sec^{-1}, and (C) 0.67 sec^{-1}. These rates are two to three times higher than in the experiment reported in Fig. 9. The difference is due probably to the influence of the high-ionic-strength media. But there is clearly no evidence for a differential effect of Na^+ ion in the range 0.1–0.75 M on the rate of the $E_2 \cdot (K)$–$E_1 \cdot Na$ transition.

Phosphorylation, Ouabain, and Divalent Metal Cation Binding

Since the fluorescein-labeled (Na,K) ATPase incorporates inorganic phosphate covalently (Table I), it seemed natural to inquire whether P_i or other ligands which effect phosphorylation influence the fluorescence changes. It turns out that either phosphate (Fig. 11A) or ouabain (Fig. 11C) at optimal concentrations or a combination of both ligands at suboptimal concentrations (Fig. 11B) quenches the fluorescence to about the same level as does K^+. The phosphate-dependent signal requires the presence of Mg^{2+} ions, it is prevented altogether by addition of Na^+ before the phosphate, and

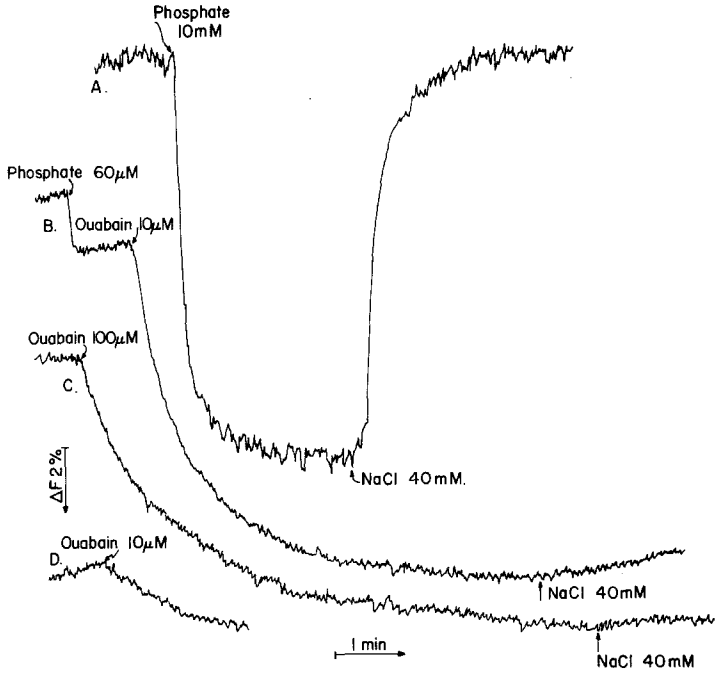

Fig. 11. Quenching of fluorescein fluorescence by inorganic phosphate or ouabain. Enzyme (60 μg) labeled as in Fig. 5 was suspended in a medium containing 40 mM Tris-HCl, pH 7.8, 2 mM EDTA (Tris), and 4 mM $MgCl_2$, and the fluorescein fluorescence was recorded as described in the Experimental section. Small volumes (not greater than 25 μl) of concentrated solutions of phosphate (Tris), ouabain, or NaCl were added to the fluorescence cell where indicated.

addition of Na^+ ions after phosphate reverses the quenching (Fig. 11A). The $K_{0.5}$ for phosphate is about 1 mM in these conditions (not shown). These phenomena almost certainly reflect the interchanges between high fluorescence E_1 or $E_1 \cdot Na$ states and low fluorescence phosphoenzyme E_2-P [3]. The rates of fluorescence quenching by phosphate or reversal by Na^+ ions were not studied, but such measurement might give valuable information on the rates of phosphoylation and dephosphorylation of the protein.

Ouabain at high concentrations (100 μM) was clearly capable of stabilizing a form E_2- ouabain provided Mg^{2+} ions were present (Fig. 11C). Na^+ ions prevented ouabain-induced quenching (not shown), but hardly reversed the change if added to the fluorescence-quenched enzyme. The slow rate of the ouabain-induced fluorescence change provides a convenient measure of the net ligand binding rate at a concentration for which direct binding assays are usually impossible. Ouabain and inorganic phosphate added together gave rise presumably to the species $E_2P \cdot$ ouabain, which too is stable upon addition of Na^+ ions.

The properties of an E_2 conformation of the enzyme produced in the presence of Mg^{2+}, K^+, and phosphate ions are shown in Fig. 12. KCl solution was first mixed into the enzyme suspension to give the standard fluorescence quenching. Subsequent addition of Mg^{2+} ions produced a partial reversal of the fluorescence change. At this stage the protein must be bound with both Mg^{2+} and K^+ ions. Thirdly addition of inorganic phosphate brought the signal back to maximal quenching. This effect was not due to chelation of Mg^2 ions since at the prevalent concentrations no more than 20% of Mg^{2+} would be bound by phosphate ions. Finally the fluorescence intensity could be rapidly restored to near the original level by Na^+ ions. This finding contrasts strikingly with a similar experiment using fluorescein ATPase in the presence of Mg^{2+}, K^+, and low concentrations of vanadate ions in which we have shown that reversal of the fluorescence quenching by the added Na^+ ions does not occur [14].

It seemed from Fig. 12 that Mg^{2+} ions influenced the conformational state of the protein, and so the divalent metal cation effects were studied in further detail. Low concentrations (1 mM) of Mg^{2+}, Mn^{2+}, and Ca^{2+} ions reduce the amplitude of the fluorescence change produced by a saturating K^+ concentration or partially reverse the change if added after the K^+ (seen for

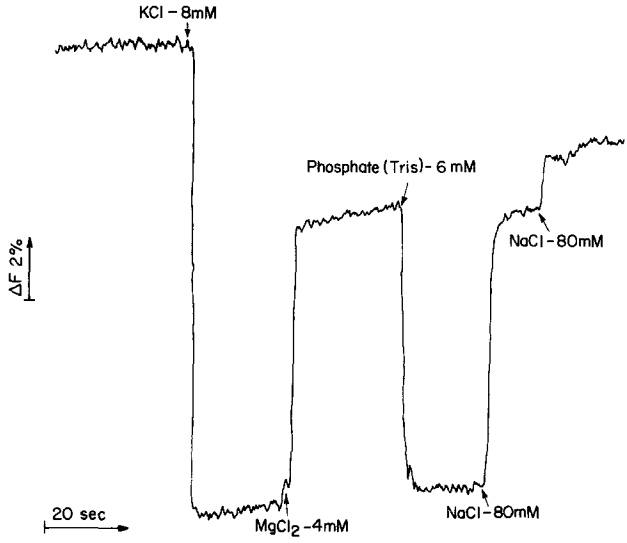

Fig. 12. Interaction of potassium, magnesium, and phosphate ions on fluorescein-labeled (Na,K) ATPase. Enzyme (70 μg) labeled as Fig. 5 was suspended in the fluorescence cell in 2.5 ml of medium containing 30 mM Tris-HCl, pH 7.7. 10-μl volumes of 2 M KCl, 1 M $MgCl_2$, and 1.5 M phosphate (Tris) were added as shown. Additions of 50 μl of 4 M NaCl (80 mM final) dilutes the volume by 2%, which accounts for the apparent lack of complete reversibility of the initial fluorescence drop. Control experiments with 80 mM Tris-HCl show that the effect of NaCl is not due to the change in ionic strength of the medium.

Mg^{2+} in Fig. 12). These metal cations do not affect the fluorescence in the absence of K^+, and so it appears that they are behaving like Na^+ ions. Titrations of the K^+ requirement for fluorescence quenching in the presence of Mg^{2+} ions show, however, that this is not the case. Although as seen in Table II the apparent $K_{0.5}$ for K^+ ions is raised modestly by the presence of Mg^{2+} ions, the major effect of the divalent cation is to reduce the size of the maximal signal change at saturating K^+ levels. By extrapolating to infinite Mg^{2+} concentration, one can predict a reduction to only 35% of the control signal.

Similarly titrations of the Mg^{2+} ion requirement for reversing the K^+-induced quenching reveal that in no circumstance was the fluorescence reversible to the minus K^+ level even by saturating concentrations of Mg^{2+}, although the apparent Mg^{2+} affinity was lowered by increasing the K^+ concentration (K_{Mg} in the range 0.1–0.3 mM.)

Discussion

Fluorescein isothiocyanate appears to act as a selective label for the high-affinity ATP binding site on (Na,K) ATPase, and it seems probable that a lysine side chain is involved. A lysine residue lies next to the aspartic acid phosphorylated by ATP [23], but we do not know if it is the one labeled by FITC. The fact that occupation of the ATP binding region by the fluorescein does not prevent phosphate ions from binding and phosphorylating the protein makes it likely that the fluorescein interferes mainly with attachment of the purine nucleus of ATP.

The inactivation data (Fig. 1), covalent incorporation before and after trypsin treatment seen in the gels (Fig. 4), and the fluorescence experiment of Fig. 5 are consistent with reaction of FITC in selective conditions with a

Table II. Effect of Mg^{2+} Ions on K^+-Dependent Fluorescence Quenching[a]

$MgCl_2$, mM	Maximal fluorescence change, %	$K_{0.5}$ for K^+, mM
0	100	0.25
0.50	83	0.78
1	67.5	0.9
4	47.5	0.83
10	42.0	1–2 mM

[a]Enzyme labeled as in Fig. 5 was suspended in 2.5 ml of a medium containing 40 mM Tris-HCl, pH 7.8, and 0–10 mM $MgCl_2$. Continuous K^+ titrations were performed as described in the Experimental section and Fig. 7. The relationship between the fluorescence change at maximal K^+ concentrations and the Mg^{2+} concentration was essentially hyperbolic, which allow extrapolation of the signal amplitude to be expected at infinite Mg concentrations by an inverse plot.

single residue. In addition the phosphatase inactivation data of Fig. 2 implies that FITC reacts first with one residue which reduces the ATPase by 60–70% without affecting phosphatase, and then with a second residue which results in total inhibition of ATPase and reduces the phosphatase activity to its final level. But it is difficult to state with certainty the exact stoichiometry of fluorescein binding, i.e., 1 or 2 moles per mole of ATPase, because the kinetics of inactivation cannot be analyzed easily due to instability of the FITC. Also the alternative and direct approach of measuring absorption of the fluorescein-labeled ATPase requires more protein than was easily available and does not give an exact estimation.

The uncertainty can probably be resolved with the aid of peptide maps and amino acid analysis of the fluorescent fragments released from the membrane by trypsin (Fig. 4c). Indeed the fluorescein-labeled ATPase may eventually prove to be valuable for structural studies including sequencing the region of the catalytic polypeptide involved in the ATP binding.

The fluorescein label turns out to be a very successful reporter, since although enzyme turnover is prevented, the conformational changes seem to be normal and indeed have been studied in greater detail than was possible with the formycin nucleotides or intrinsic protein fluorescence. The fluorescence yield of the label responds to the conformational state of the ATPase due presumably to a change in its environment.

Consider the following scheme for cation binding and the conformational change [7, 8]:

$$E_1 \cdot Na \overset{K_s}{\rightleftharpoons} Na^+ + E_1 + K^+ \overset{K_p}{\rightleftharpoons} E_1 \cdot K \overset{K_c}{\rightleftharpoons} E_2 \cdot (K)$$

Here K_p and K_s are cation binding constants and K_c is the conformational equilibrium constant.

Na^+ and K^+ ions compete for a single site in the E_1 form. Binding of K^+ allows the conformational transition to the $E_2 \cdot (K)$ form in which the K^+ ions are thought to be occluded because they exchange very slowly with the medium cations (see Fig. 9, left). The suggested order of events, namely K^+ binding followed by the conformational change, is strongly supported by the new finding (Fig. 10) of saturation by high K^+ concentrations of the rate of $E_1 + K \rightarrow E_2 \cdot (K)$. This experiment leads to a binding constant K_p of 74 mM. The conformational equilibrium constant can be estimated from the ratio of the maximal rate of $E_1 + K^+ \rightarrow E_2 \cdot (K)$ ($k = 286\ sec^{-1}$) and the rate of the change $E_2 \cdot (K) \rightarrow E_1 \cdot Na$ ($k = 0.3\ sec^{-1}$). Here K_c equals 953. Therefore, in equilibrium binding titrations a weak initial attachment of K^+ ions to E_1 coupled to the conformational change poised far in the direction of $E_2 \cdot (K)$ effectively traps the K^+, giving an apparent high affinity. In the absence of Na^+ ions the values were in the range 180–250 μM for fluorescein

ATPase, compared with 130 μM and 50 μM using formycin nucleotides [7] or intrinsic fluorescence [8] respectively.

The low-affinity K^+ and high-affinity Na^+ binding sites in state E_1, evident from both kinetic experiments and equilibrium titrations (see text), lead us to suggest [7] that the site involved is one normally facing the cell interior. However, the clear indication for a single site raises the question whether it is involved in the active extrusion of Na from the cell [21]. Transport data in red cells indicate the involvement, in the normal Na–K exchange mode of pumping, of three high- and equal-affinity Na^+ binding sites at the inner surface, competed for equally by K^+ ions with a relatively low affinity [21, 24]. The anomaly of a single K^+ or Na^+ site in the conformational response parallels an observation by Mardh and Post [25] that Na^+ activates phosphorylation in isolated membranes along a hyperbolic curve and this Na^+ site is now known conclusively to be at the cytoplasmic surface [26]. We have attempted to explain the apparent single-site K^+ binding in the fluorescence titrations by assuming that, although three cation binding sites of equal Na^+ or equal K^+ affinity exist at the inner surface, binding of only one K^+ ion per protein molecule sustains the transition from E_1 to the occluded $E_2 \cdot (K)$ form [9]. Very recently Beauge and Glynn were able to measure directly occlusion of Rb^+ ions in the protein, and they suggested that two Rb^+ ions are occluded per enzyme molecule [27]. Also Matsui et al. [28] have measured ouabain-sensitive ^{42}K binding to a dog kidney ATPase preparation and have found two K^+ ions to be bound in the range of concentrations (10–100 μM) which induce the conformational transition, and it seems that the two sites have affinities which differ by up to one order of magnitude. These data imply that the characteristics of Na^+ and K^+ binding at the cytoplasmic surface in experiments with isolated membranes may not be the same as in the experimental conditions of active Na^+ efflux from whole cells. Therefore, one must consider also the alternative explanations summarized by schemes (A) and (B):

$$E_1 + K^+ \overset{K_{p_1}}{\rightleftharpoons} E_1 \cdot K + K^+ \overset{K_{p_2}}{\rightleftharpoons} E_1 \cdot KK \overset{K_c}{\rightleftharpoons} E_2 \cdot (KK) \qquad \text{(A)}$$

$$\begin{array}{ccc} E_1 + K^+ \overset{K_{p_1}}{\rightleftharpoons} E_1 \cdot K + K^+ & \overset{K_{p_2}}{\rightleftharpoons} & E_1 \cdot KK \\ \Big\| K_c & & \Big\backslash K_c \\ E_2 \cdot (K) + K^+ & \overset{K_{p_2}}{\rightleftharpoons} & E_2 \cdot (KK) \end{array} \qquad \text{(B)}$$

Here we assume for the isolated ATPase two K^+ binding sites on E_1 with different affinities; in both cases $K_{p_1} \ll K_{p_2}$. In scheme (A) both sites must be occupied prior to the transition, and if their affinities are sufficiently different, K^+ concentrations much lower than necessary for quenching

fluorescence can saturate the first site. In this situation our titrations may be too insensitive to detect sigmoidicity at the very foot of the curves and in essence give information only on the lower-affinity binding site. The second, possibility, scheme (B), is that it is not necessary to bind more than one K^+ ion in order to induce the conformational transition although at high enough concentrations more than one K^+ ion may become occluded. Again if the effective binding affinity to the first site is sufficiently higher than to the second site, the protein may have essentially undergone the transition at K^+ concentrations lower than are required to start filling the second site. In this case the fluorescence titrations would monitor binding to the highest-affinity site.

Table III lists the conformational species which can be detected by the probes we have used. The conformational transitions in the fluorescein-labeled ATPase produced by inorganic phosphate or ouabain coincide closely with expectations from studies of phosphorylation of the enzyme by phosphate [3] or of ouabain binding [10]. These point to the alternation between the Na-stabilized $E_1 \cdot Na$ and the phosphorylated protein in the E_2 conformation E_2P (perhaps E_iP in the terminology of Post et al. [3]), $E_2P \cdot K$, or the ouabain-bound species E_2-ouabain and $E_2P \cdot$ ouabain. Experiments with vanadate using the fluorescein ATPase [14] show that in the absence of K^+ ions, the vanadate acts very similarly to inorganic phosphate in producing at high concentrations (80 μM) an E_2 form which is easily reverted to the E_1 state by Na^+ ions. In the presence of K^+ ions, however, vanadate at very low

Table III. Conformational Forms of the (Na,K) ATPase Distinguishable by Fluorescent Probes[a]

Ligand binding conditions	Conformation	Probe	Reference
Na- and K-free	E_1	FN, IPF, FA	7, 8, 9
Na	$E_1 \cdot Na$	FN, IPF, FA	7, 8, 9
ATP	$E_1 \cdot ATP$	FN, IPF	6, 11
Mg	$E_1 \cdot Mg$	FA	This paper
K	$E_2 \cdot (K)$	FN, IPF, FA	7, 8, 9
Mg + K	$E_2 \cdot (K) \cdot Mg$	FA	This paper
Mg + phosphate + K	$E_2P \cdot K \cdot Mg$	FA	This paper
Mg + phosphate	$E_2P \cdot Mg$ (E_iP)	FA	This paper
Mg, vanadate	E_2 vanadate $\cdot$ Mg	FA	14
Mg, vanadate + K	E_2 vanadate $\cdot$ K $\cdot$ Mg	FA, IPF	14, 11
Mg, ouabain	$E_2 \cdot$ ouabain $\cdot$ Mg	FA	This paper
Mg, ouabain + phosphate	$E_2P \cdot$ ouabain $\cdot$ Mg	FA	This paper
Turnover conditions			
ATP, Mg Na (10–100 mM)	mainly E_2P	IPF, FN	6, 8, 11
ATP, Mg high Na (300 mM)	mainly E_1P	IPF	11
ATP, Mg Na (10–100 mM), K (1–10 mM)	$E_2 \cdot (K)$	FN, IPF	6, 8, 11

[a]Abbreviations: FN, formycin nucleotides; IPF, intrinsic protein fluorescence; FA, fluorescein ATPase. Terminology of the intermediates is that in refs. 2, 3, 7, and 45.

concentrations (10^{-7}–10^{-6} M) gives rise to a very stable state MgE_2 vanadate · K, which is quite unlike the situation with phosphate (Fig. 12).

A major point of Table III is that we can distinguish two major conformations E_1 or E_2 of either phosphorylated or nonphosphorylated protein, and these states are stabilized alternately by a variety of ligands. The covalent bond-forming and bond-breaking steps, $E_1 \rightarrow E_1P$ and E_2P–$E_2 \cdot (K)$, are not accompanied by changes in the E_1 and E_2 status of the protein, and seem to be associated with Na^+ and K^+ binding and dissociation at the surfaces respectively [7, 11]. The transport steps for Na^+ and K^+ would then involve the conformational transitions $E_1P \rightarrow E_2P$ and $E_2 \cdot (K) \rightarrow E_1 \cdot Na$ respectively, and E_1P may be an occluded Na^+-binding form just as the $E_2 \cdot (K)$ contains occluded K^+ ions [7].

In the human red cell it is usually found that three Na^+ ions are extruded for two K^+ ions taken up per ATP hydrolyzed [21] and so the conformational transitions $E_1P \rightarrow E_2P$ and $E_2 \cdot (K) \rightarrow E_1$ may occur at a significant rate only with three Na^+ ions occluded in E_1P and two K^+ ions occluded in $E_2 \cdot (K)$. However, the different cation requirements for transport in whole cells and partial reactions in isolated membranes discussed above suggest that several subspecies of the E_1 or E_2 conformations may exist, with different numbers of Na^+ and/or K^+ bound or occluded, each subspecies having its local conformation and catalytic abilities. The E_1 forms may bind one, two, or three Na^+ or K^+ ions at the inner facing sites. The consequences of binding one or more K^+ ions to E_1 were discussed above. For Na^+-dependent reactions, phosphorylation seems to require no more than one Na^+ ion to be bound to E_1 [25] and, as found recently, ADP–ATP exchange is most rapid with two Na^+ ions bound [29]. Na^+ efflux in the red cell would require presumably that all three sites be occupied (and become occluded) in the phosphoenzyme E_1P prior to the conformational transition (see also [26]). Reduction of the normal Na^+/K^+ ratio from 3:2 to 1:2 as reported recently for a reconstituted trypsinized (Na,K) ATPase preparation [30] could result in principle from a significant rate of the $E_1P \rightarrow E_2P$ transition with less than three Na^+ ions occluded. At the external surface two K^+ ions bound in conformation E_2P would normally become occluded in $E_2 \cdot (K)$ from where they are transported into the cell. But there is also evidence that the rate of dephosphorylation of E_2P in isolated membranes is activated by K^+ ions along a hyperbolic curve ([31, 32] and R. L. Post, personal communications), suggesting that binding of only one K^+ ion is necessary to accelerate hydrolysis of E_2P. Formation of the occluded species $E_2 \cdot (K)$ may require that two K^+ ions be bound.

The interaction of divalent metal cations and K^+ on the fluorescence signals are of interest because of the importance of Mg^{2+} for enzyme activity. A Mg^{2+}-dependent decrease in apparent K^+ affinity (Table II) could result from binding of Mg^{2+} to E_1 forms with a higher affinity than to E_2 forms,

leading to a Mg^{2+}-dependent shift of three to four times in the conformational equilibrium toward E_1 forms [7]. This reasoning, however, cannot also account for the fall in the signal amplitude at saturating K^+ and Mg^{2+} concentrations (to 35% of the maximal value), for this would require a Mg^{2+}-dependent shift of about 3000 times in the conformational equilibrium. Probably the simplest explanation is that bound Mg^{2+} ions greatly reduce the efficiency of fluorescence quenching in the $E_2 \cdot (K)$ form, either by a direct interaction with the fluorecein (carboxyl) or by inducing a local conformational change [and phosphate relieves this effect (Fig. 12)]. Localized effects of Mg^{2+} may influence reactivities and rates of processes without drastically altering the equilibria between the E_1 and E_2 conformations.

The subunit organization of the (Na,K) ATPase must be considered when discussing the role of the major conformational transitions. Most investigators now agree that the Na pump is arranged as a dimer. Each half consists of one catalytic peptide containing both ATP and ouabain binding sites [33–35] and one or two copies of the glycoprotein [16, 36] (and possibly [35] a proteolipid). Only one ATP or ouabain is bound with high affinity per ATPase dimer, or one P_i may be incorporated covalently. The possible importance of negative cooperativity in ligand binding has found its expression in flip-flop or alternating site mechanisms of the (Na,K) ATPase (see [37–39, 40] for a discussion of previous misconceptions). There is also good steady-state kinetic evidence for participation of ATP at both high-(phosphorylating) and low-affinity (regulatory) binding sites in the normal hydrolysis cycle [41–43]. But it is still not clear whether these appear sequentially on different enzyme conformations or co-exist on opposite halves of the dimer throughout the turnover cycle. The finding in Fig. 9B (left) that high concentrations of ATP do not accelerate the $E_2 \cdot (K) \rightarrow E_1 \cdot Na$ transition of the fluorescein-labeled (Na,K) ATPase suggests most simply that ATP will not bind to the $E_2 \cdot (K)$ form. Conversely for the unlabeled (Na,K) ATPase it is reasonable to assume that the high-affinity site for ATP in the E_1 form is converted into a low-affinity site in $E_2 \cdot (K)$. On the basis of this experiment, the steady-state kinetic data, and the ligand binding alone, it is not necessary to assume the existence of more than a single nucleotide binding site, which could straddle both halves of the dimer and have high or low affinity in E_1 or $E_2 \cdot (K)$ states respectively. But other evidence from tryptic digestion [5] and studies of enzyme inactivation by methylene blue [43], NBD-chloride [44], and vanadate [45] is consistent with the simultaneous presence of nucleotide binding sites on both halves of the dimer. E_1 would have high- and low-affinity sites (implying intrinsic assymetry in the dimer [44]), while $E_2 \cdot (K)$ could have low-affinity ATP binding on both chains (contrary to the suggestion of Cantley et al. [44]). The experiment of Fig. 9B (left) does not absolutely preclude this situation since the enzyme-bound fluorescein in the state $E_2 \cdot$

(K) can prevent ATP binding both to its own half and also to the opposite chain by steric hindrance, or prevent ATP bound to the opposing half from stimulating the conformational transition. If there are ATP binding sites on the two halves of unlabeled ATPase, the low-affinity sites should always be occupied at physiological ATP concentrations in all the conformations E_1, E_1P, E_2P, and $E_2 \cdot (K)$. Binding of ATP to a regulatory site prior to dissociation of the products ADP and P_i could be kinetically advantageous compared to the mechanism with only a single nucleotide binding site. But clearly more critical evidence for or against ATP binding to low-affinity sites in phosphorylated and nonphosphorylated conformations of the (Na,K) ATPase is required in order to decide on the status of the "half of the site" models.

Much less weight has been attached to the possible significance of dimeric organization for the cation binding and translocation mechanism. Thermodynamic reasoning suggests that substrate and ion binding sites lie at the subunit interface [46]. The fact that ligands which bind at different sites such as K^+, phosphate, or ouabain all induce the same E_1 to E_2 transition (Table III) makes it unlikely that the change is a local one, and since ouabain bound to the external face of the catalytic subunit affects the fluorescein bound at the inner face, it is likely that the reorganization of the catalytic chain is felt throughout the body of the transmembrane protein. Therefore it is attractive to imagine that the cation transport involves a movement of subunits and rearrangement of the protein side chains around the cation binding sites in one conformation leading effectively to transport of the sites to the opposing face of the protein.

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