Estimation of the Distance Change between Cysteine-457 and the Nucleotide Binding Site When Sodium Pump Changes Conformation from E_1 to E_2 by Fluorescence Energy Transfer Measurements†

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ABSTRACT: The first indication of the size of a conformational change implicated in ion transport by sodium pump has been obtained by measuring the change in efficiency of fluorescence energy transfer between two specific locations on the α-subunit. The donor (5′-(iodoacetamido)fluorescein) attaches covalently to cysteine-457, and the acceptor (2′(or 3′)-*O*-(trinitrophenyl)adenosine 5′-triphosphate) binds reversibly to the active site. The acceptor binds nearly 2 orders of magnitude tighter to the Na^+ than to the K^+ conformation of the enzyme and quenches donor fluorescence more efficiently in the Na^+ than in the K^+ conformation. The estimated distance between donor and acceptor, assuming random orientation of their emission and absorption dipoles, increases 2.9 ± 0.6 Å when the enzyme changes from the Na^+ to the K^+ conformation. Stopped-flow measurements of the change in fluorescence energy transfer efficiency with time when the doubly-labeled pump is mixed with Na^+ or K^+ demonstrate that the donor/acceptor pair reports the change between the E_1 and E_2 conformations of unphosphorylated enzyme. The observed first-order rate constant for the change in energy transfer efficiency depends sigmoidally on [K^+] and inversely on [Na^+], and both rate and amplitude data for the change in energy transfer efficiency can be fit with the same values of the rate and ion-dissociation constants as published data for the conformational change between E_1 and E_2 obtained by singly labeling the enzyme with fluorophores that report changes in protein microenvironment. The prerequisite for successfully measuring the distance change and equating the protein rearrangement with a step in the catalysis-transport cycle is that the donor by itself does not report the conformational change.

The working hypothesis in the sodium pump field for more than two decades has been that ATP[1] hydrolysis is coupled to ion transport by a series of protein conformational changes. Historically the conformational changes were thought to be directly involved in ion movement, and structural rearrangements large enough to alternately expose the ion binding sites on opposite sides of the membrane were envisioned. Today there is a growing consensus from analysis of the primary structures of P-type pumps and comparisons with other integral membrane proteins of known tertiary structure that membrane traversing α-helices are arranged into a pore through which ions pass. At the same time, there is increasingly strong physicochemical evidence that the characteristics which distinguish facilitated from passive transport are explained by conformational changes. One way of reconciling the structural and functional data might be a relatively small change in protein shape that determined the limited rate, specificity, and stoichiometry of active Na^+ and K^+ countertransport by gating a channel. The purpose of this study is to begin placing constraints on the possible roles of a conformational change in ion movement by determining the size of a protein rearrangement that has been implicated in ion transport by sodium pump experimentally.

Distances between specific locations in a protein of unknown secondary, tertiary, and quaternary structure can be measured by fluorescence resonance energy transfer (FRET), and several laboratories have mapped sites on the sodium pump by this technique. Sodium pump is constructed from a catalytic α-subunit and a β-subunit of unknown function. The distance from the ouabain binding site on the catalytic subunit to lucifer yellow attached to sugar residues of the glycoprotein β-subunit has been measured by FRET (Lee & Fortes, 1986). Evidence the enzyme might function as an $(\alpha\beta)_2$ dimer was obtained by measuring the distance between erythrosin and fluorescein attached to lysine-501 in different α-subunits (Amler *et al*., 1992). Within the α-subunit, FRET has been used to measure the distance from a fluorescent analogue of ouabain bound at the cardiac glycoside site to fluorescein attached to sulfhydryl groups (Jesaitis & Fortes, 1980), to lysine-501 (Carilli *et al*., 1982), and to cysteine-457 (Fortes & Aquilar, 1988). The distances from fluorescein-labeled cysteine-457 to two other locations,

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[1] Abbreviations: Na,K-ATPase, Mg^{2+}-dependent and Na^+- and K^+-stimulated ATPase (EC 3.6.1.37); ATP, adenosine 5′-triphosphate; TNP-ATP, 2′(or 3′)-*O*-(trinitrophenyl)adenosine 5′-triphosphate; P_i, inorganic phosphate; IAA, iodoacetate; IAF, 5′-(iodoacetamido)fluorescein; FITC, fluorescein 5′-isothiocyanate; ErITC, erythrosin 5′-isothiocyanate; DTT, DL-dithiothreitol; BIPM, *N*-[*p*-(2-benzimidazolyl)phenyl]maleimide; ANM, *N*-(1-anilinonaphth-4-yl)maleimide; EDTA, ethylenediaminetetraacetic acid; Tris (Tris$^+$), tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; ChoCl, choline chloride; FRET, fluorescence resonance energy transfer.

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erythrosin on lysine-501 (Amler *et al*., 1992) and a fluorescent substrate analogue bound at the active site (Fortes & Aguilar, 1988), have also been measured.

More recently, Taniguchi and co-workers have shown that dynamic changes in the protein can be followed by combining FRET and stopped-flow measurements (Taniguchi *et al*., 1988, 1994; Taniguchi & Mårdh, 1993). The distances between two different fluorescent derivatives of maleimide, BIPM attached to cysteine-964 and ANM attached to cysteine-802 (Shinoguchi *et al*., 1991), and the distances from BIPM to fluorescein attached to lysine-501 with FITC (Tosa *et al*., 1994) have been measured in different conformations of the enzyme. The conclusion drawn from both studies was that any change in distance between donor and acceptor in alternative conformations of the enzyme is less than 1 Å. However, the interpretation of both stopped-flow/FRET and FRET measurements of distance changes with the cited donor/acceptor pairs is complicated because changes in protein microenvironment, as well as the distance between donor and acceptor, affect the fluorescence signal.

The conformational change in unphosphorylated Na,K-ATPase has been implicated in ion transport by stopped-flow studies that utilize a single fluorescent label to follow the reaction by responding to the change in protein environment. Post *et al*. (1972) predicted that there is a rate-limiting change in the conformation of unphosphorylated Na,K-ATPase because the rate of rephosphorylation depends upon the K^+ congener used to accelerate dephosphorylation. The existence of a slow conformational change was confirmed by directly observing a slow reaction reported by both intrinsic tryptophan fluorescence (Karlish & Yates, 1978) and fluorescein attached to lysine-501 (Karlish, 1980). A recently-proposed, equivalent-site mechanism for regulation of the conformational change in dephosphoenzyme by Na^+ and K^+ binding permits estimation of the true (intrinsic) K^+ and Na^+ dissociation constants (Smirnova *et al*., 1995a). The conclusion drawn from following the conformational change with both fluorescein and eosin (Smirnova & Faller, 1995) is that Na^+ binds more than an order of magnitude tighter than K^+ from the cytosolic side of the plasma membrane, consistent with Na^+ export and K^+ import because of different ion specificities in different enzyme conformations. The consensus stoichiometry of ion transport is exchange of 2 K^+ for 3 Na^+. Two K^+ affinities can be resolved experimentally, and both sites must be filled before the conformational change occurs (Faller & Smirnova, 1993a), consistent with the stoichiometry of K^+ transport. At least two sites are required to explain reversal of the conformational change by Na^+ (Faller *et al*., 1994). Therefore, the properties of the conformational change reported by fluorescein and eosin can plausibly explain (1) rate limitation of the pump cycle, (2) the specificity of the enzyme for Na^+ and K^+, and (3) the stoichiometry of ion countertransport.

In this paper, we show by stopped-flow/FRET measurements that FRET between the donor/acceptor pair IAF/TNP-ATP reports the same conformational change as FITC modification of Na,K-ATPase or eosin binding. We estimate the distance from fluorescein-labeled cysteine-457 to TNP-ATP bound at the active site in the Na^+ and K^+ conformations. We show that a reasonably precise estimate of the change in distance when the conformation changes can be obtained because the donor does not report the protein rearrangement in hog enzyme by itself. An abstract communicating some of the results of this study has been published (Lin & Faller, 1995).

EXPERIMENTAL PROCEDURES

Materials

Enzyme Preparation. Na,K-ATPase was purified from the outer medullae of pig kidneys by the method of Jørgensen (1975). Protein concentration in mg mL^{-1} was determined with the assay of Lowry *et al*. (1951) using bovine serum albumin as the standard. Specific activity was measured by a coupled assay (Nørby, 1988) and varied from 12 to 16 μmol of P_i released min^{-1} (mg of protein)$^{-1}$ before chemical modification.

Chemical Modification. The protocol developed by Tyson *et al*. (1989) for modifying a specific residue in dog kidney enzyme identified as cysteine-457 was followed with minor changes designed to avoid nonspecific labeling (Results).

First, pig kidney enzyme was reacted with IAA (Castro & Farley, 1979). The reaction was initiated by adding IAA (25 mM) from a 0.5 M stock solution neutralized to pH 7.4 with NaOH to 4 mg mL^{-1} protein in 0.1 mM EDTA and 25 mM sodium phosphate at pH 7.0. The reaction with IAA was stopped after 30 min at 37 °C in the dark by diluting with an 8-fold excess of cold buffer containing 4 mM DTT, 20 mM KCl, and 50 mM imidazole-HCl at pH 7.5. The unreacted IAA that is scavenged by DTT was removed by alternately centrifuging and resuspending the IAA-treated membrane fragments in the same buffer without DTT two times.

Second, the IAA-enzyme was reacted with IAF (Kapakos & Steinberg, 1982). The washed pellet of membrane fragments containing IAA-protein was resuspended (2.5 mg mL^{-1}) in 20 mM KCl and 50 mM imidazole-HCl buffer at pH 7.5 and reacted with 70 μM IAF in the dark at 25 °C for 120 min. The reaction with IAF was stopped by adding 70 μM DTT, and unreacted IAF was removed by centrifuging and resuspending twice in 50 mM Tris-HCl buffer containing 0.1 mM Tris-EDTA at pH 7.5. The final pellet of IAF-modified Na,K-ATPase was suspended in pH 7.4, 50 mM Tris-HCl buffer containing 0.1 mM Tris-EDTA and 15% sucrose. Stock solutions of IAF/IAA-enzyme were stored at 0 °C and used within 5 days.

Donor and Acceptor Stoichiometries. The molar concentration of bound IAF was estimated with the published extinction coefficient (ϵ) or absorptivity of 7.3×10^4 M^{-1} cm^{-1} at 492 nm (Tyson *et al*., 1989) from the difference in absorbance between suspensions of the same concentration of protein in 1% SDS and 1 M NaOH doubly labeled with IAF and IAA (IAF/IAA-enzyme) and singly labeled with IAA (IAA-enzyme) to correct for scattering. The donor stoichiometry per $\alpha\beta$ heterodimer or α-subunit was calculated from the concentration of bound IAF and the protein concentration estimated in mg mL^{-1} by the Lowry method with the molecular weight (1.47×10^5) calculated from the amino acid sequences of the subunits (Shull *et al*., 1985, 1986) and ranged from 0.51 to 0.66 mol of IAF/mol of "Lowry" protein.

The stoichiometry of TNP-ATP binding to sodium pump has been shown to equal the stoichiometry of ouabain binding (Moczydlowski & Fortes, 1981). Therefore, the fraction of IAF-labeled protein that can bind the acceptor TNP-ATP (χ_a)

was estimated by measuring [^{3}H]ouabain binding to IAF-protein (Resh, 1988). The value of χ_a depends on the batch of labeled protein and ranged from 0.31 to 0.36.

Reagents. Fresh pig kidneys were a gift from Farmer John Clougherty Packing Co. (Los Angeles, CA). IAF and TNP-ADP were purchased from Molecular Probes Inc. (Eugene, OR). IAA, DTT, the Tris salt of ATP, and the cloride salts of choline, potassium, and sodium were obtained from Sigma (St. Louis, MO). All other reagents were the highest grade commercially available.

Methods

Fluorescence Measurements. Fluorescence was measured at right angles to the incident light with a Perkin-Elmer LS50B fluorometer equipped with a stirrer in the sample compartment. The donor was excited at the absorption maximum (492 nm) of IAF/IAA-modified Na,K-ATPase, and fluorescence was detected at the emission maximum (520 nm) of the labeled enzyme. The excitation slit width was 3 mm, and the emission slit width was varied from 6 to 10 mm. The protein concentration was 20−25 μg mL^{-1} in either 20 mM NaCl or 20 mM KCl and 50 mM Tris-HCl buffer at pH 7.4.

In titrations with acceptor, measured fluorescence intensities were corrected experimentally for absorption of the emitted and/or incident light by the acceptor (inner filter effect) because the absorbance of TNP-ATP becomes too high for accurate correction for inner filter effect theoretically (Lakowicz, 1983). ATP, which does not absorb in the range 492−520 nm, was used to displace TNP-ATP in a reference cuvette, so that the effect of acceptor binding on the fluorescence of enzyme donor could be expressed as the ratio (F_{rel}) of the fluorescence intensities of sample and reference cuvettes containing the same concentration of TNP-ATP. At each TNP-ATP concentration in the titration, the same volume of IAF/IAA-modified enzyme from a stock solution containing either Na^+ or K^+ was placed in both cuvettes. Then the same aliquot of TNP-ATP was added to each cuvette followed by equal volumes of water (sample cuvette) or Tris-ATP (reference cuvette) from stock solutions that gave final ATP concentrations of 2 mM when [TNP-ATP] < 1 μM or 8 mM when [TNP-ATP] ≥ 1 μM. The donor dissociation constant (K_d) was estimated by fitting the quadratic equation relating F_{rel} to acceptor binding at a single class of sites (Faller, 1990) to the data.

$$F_{rel} = 1 - \frac{(1-\gamma)}{2E_0} \times [(E_0 + L_0 + K_d) - [(E_0 + L_0 + K_d)^2 - 4E_0L_0]^{1/2}] \quad (1)$$

In eq 1, L_0 is the total ligand concentration, and E_o is the concentration of enzyme sites that bind TNP-ATP determined by measuring ouabain binding. The maximum quench or quench factor (γ) is defined as the ratio of the fluorescence intensities of IAF/IAA-enzyme with and without TNP-ATP bound.

Stopped-Flow Measurements. The stopped-flow instrument consists of a Hi-Tech PQ/SF-53 sample handling unit integrated into an On-Line Instrument Systems spectrofluorometer (Faller *et al*., 1991; Smirnova & Faller, 1993b; Lin & Faller, 1993). All of the measurements were made at 15 °C in 50 mM Tris-HCl buffer at pH 7.4 with the ionic strength (μ) adjusted to 250 mM with ChoCl. From 8 to 11 kinetic curves of 1000 points were stored and analyzed in each experiment at a single ion concentration. First-order rate constants and amplitudes were estimated by fitting the equation for a single exponential to the data by successive integration with the algorithm supplied by On-Line Instrument Systems. The concentration dependencies of the first-order rate constants and amplitudes were analyzed by fitting eqs 2, 6, and 7 in Smirnova *et al*. (1995a,b), which assume ion binding to two identical and independent sites, to the data with the SigmaPlot 5.01 nonlinear, least-squares curvefit program.

Anisotropy Measurements. Time-resolved decays of fluorescence anisotropy (r) were measured with a photon-counting, nanosecond instrument in Professor Herbert Cheung's laboratory at the University of Alabama in Birmingham. The decay of IAF/IAA-enzyme was monoexponential permitting estimation of the limiting (time zero) anisotropies of IAF in the Na^+ and K^+ conformations of the enzyme. However, the anisotropy decay of TNPATP could not be studied because of the short lifetime of the probe. Attempts to obtain the limiting anisotropy of the acceptor from steady-state measurements as a function of solution viscosity were also unsuccessful because of the low fluorescence quantum yield of bound TNP-ATP.

Theory of Energy Transfer. The theory of FRET is well understood (Stryer, 1978; Lakowicz, 1983). The efficiency (E) of FRET between donor (d) and acceptor (a) was calculated from the measured fluorescence intensities of donor in the presence (F_{da}) and absence (F_d) of acceptor with eq 2, which takes into account the fact that energy transfer occurs only in the fraction of donor molecules with acceptor bound (χ_a).

$$E = \frac{1}{\chi_a}\left(\frac{F_d - F_{da}}{F_d}\right) = \frac{(1-\gamma)}{\chi_a} \quad (2)$$

Transfer efficiency is related to the separation between donor and acceptor (R) by the Förster critical distance (R_0), which can be thought of practically as the separation between donor and acceptor at which energy is transferred with half-maximum efficiency.

$$R = R_0\left(\frac{1}{E} - 1\right)^{1/6} \quad (3)$$

The following equation is the theoretical expression for R_0 in cm.

$$R_0 = (9.79 \times 10^{-5})(\eta^{-4} Q_d J \kappa^2)^{1/6} \quad (4)$$

In eq 4, η is the refractive index of the bulk solution, Q_d is the quantum yield of the donor, J is the overlap integral between the emission spectrum of the donor and the absorption spectrum of the acceptor, and κ^2 is the orientation factor of the donor emission and acceptor absorption dipoles.

In practice, only the distance between the donor and acceptor assuming random orientation of the emission and absorption dipoles ($R_{2/3}$) can be calculated because in biological systems κ^2 cannot be uniquely determined experimentally (Cheung, 1991). The other quantities needed to calculate R_0 were either measured or taken directly from the literature. The fluorescence quantum yield of the donor in the absence of acceptor was estimated with quinine in 0.1

N H_2SO_4 ($Q = 0.51$) as the standard (Chen, 1974). The following equation was used to calculate the overlap integral between the corrected emission spectrum of the donor [$F_d(\lambda)$] and the absorption spectrum of the acceptor [$\epsilon_a(\lambda)$].

$$J = \frac{\int_0^\infty F_d(\lambda)\, \epsilon_a(\lambda)\, \lambda^4\, d\lambda}{\int_0^\infty F_d(\lambda)\, d\lambda} \quad (5)$$

The index of refraction of a dilute aqueous solution is 1.33 (Moog *et al*., 1984), and $\kappa^2 = 2/3$ when the orientations of the donor and acceptor dipoles randomize before energy transfer (Förster, 1948). The values of R_0 calculated with eq 4 and E calculated from the experimental estimates of χ_a, F_d, and γ with eq 2 were substituted into eq 3 to calculate $R_{2/3}$.

RESULTS

Effect of Donor on ATPase Activity. Tyson *et al*. (1989) have reported that pretreatment of Na,K-ATPase with IAA before reacting with IAF limits fluorescein attachment to a single amino acid residue and has no effect on ATPase activity. Our results confirm that there is no loss of ATPase activity when 2.5 mg mL^{-1} protein pretreated with IAA is incubated with 70 μM IAF for up to 3 h at 25 °C. However, longer reaction times decrease ATPase activity. The decrease in specific activity appears to result from nonspecific labeling because the specific fluorescence of the reaction mixture in arbitrary units (au) per milligram of protein increases during the first hour of incubation with IAF, remains essentially constant for the next 2 h, and increases again if the reaction runs longer. Therefore, to minimize nonspecific incorporation of fluorescein into the enzyme, the reaction with IAF was stopped after 2 h before the second increase in specific fluorescence and concurrent decrease in specific activity begin. To avoid confusion with fluorescein inserted into the protein at other sites with different reagents, we will refer to the donor as IAF.

Energy Transfer from IAF to TNP-ATP. Fortes and Aquilar (1988) have previously used IAF and TNP-ATP as a donor/acceptor pair for FRET studies of dog kidney Na,K-ATPase. Figure 1 confirms that TNP-ATP also quenches the fluorescence of IAF/IAA-modified porcine Na,K-ATPase. The solid line is the corrected (for wavelength-dependent effects) emission spectrum of the labeled enzyme. The absorption spectrum of TNP-ATP (dotted line) overlaps the emission spectrum of IAF, so covalently attached IAF can transfer energy to bound TNP-ATP. The dashed line is the emission spectrum of the labeled enzyme after addition of 20 μM TNP-ATP corrected for dilution and inner filter effect. TNP-ATP also fluoresces, and its emission spectrum overlaps the emission spectrum of IAF/IAA-enzyme. However, the quantum yield of TNP-ATP (0.22×10^{-3}) is so low (Moczydlowski & Fortes, 1981) compared to the quantum yield of IAF/IAA-enzyme (0.38) that bound TNP-ATP does not contribute detectably to the quenched emission spectrum. Therefore, the quench of donor fluorescence depends only on the efficiency of FRET between IAF and TNP-ATP.

Effects of Na^+ and K^+ on Energy Transfer. Fortes and Aquilar (1988) did not report the effects of Na^+ and K^+ on the efficiency of energy transfer between IAF and TNP-ATP, presumably because IAF alone reports the conformational change in dog enzyme (Kapakos & Steinberg, 1986), obscuring any effect of the protein rearrangement on the change in energy transfer efficiency. Figure 2 shows titrations of IAF/IAA-modified hog Na,K-ATPase with TNP-ATP in each of the transported ions. Sample and reference cuvettes were compared at each TNP-ATP concentration as described in Methods because of the inner filter effect of the titrant. The data were fitted with eq 1 for TNP-ATP binding to a single site, and the parameter estimates are given in the figure legend. Replacing Na^+ (circles) by K^+ (triangles) reduces the affinity of IAF/IAA-enzyme for TNP-ATP by nearly 2 orders of magnitude, consistent with approximately 2 orders of magnitude tighter binding of ATP to the Na^+ (E_1) than to the K^+ (E_2) conformation of Na,K-ATPase (Nørby, 1983).

The 3.2% difference between the end points of the titrations (γ) in Na^+ and K^+ could result either from a change in the distance between IAF and TNP-ATP in the E_1 and E_2

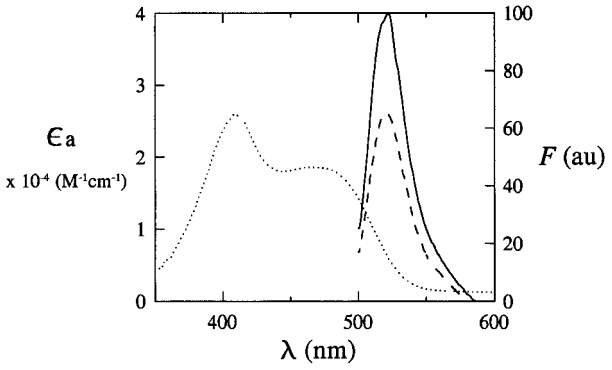

FIGURE 1: Absorption and fluorescence emission spectra showing energy transfer from IAF (donor) to TNP-ATP (acceptor). The extinction coefficient of the acceptor (ϵ_a) is plotted on the left, and fluorescence intensity (F) in arbitrary units is plotted on the right. The abscissa is wavelength (λ). The corrected fluorescence emission spectrum of IAF/IAA-modified Na,K-ATPase (solid line) was measured in 50 mM Tris-HCl buffer containing 20 mM NaCl at pH 7.4. The absorption spectrum of IAF/IAA-enzyme with the nucleotide site saturated by addition of 20 μM TNP-ATP (dotted line) overlaps the emission spectrum of IAF-enzyme and quenches the emitted fluorescence intensity 35% at the emission maximum after correction for dilution and the inner filter effect of TNP-ATP (dashed line).

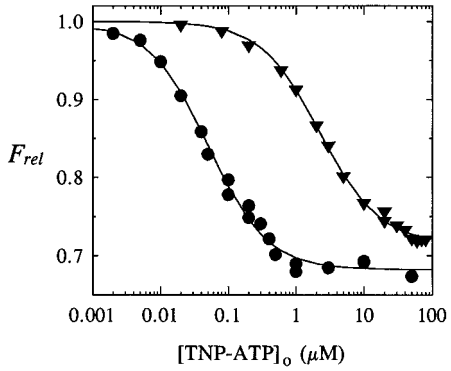

FIGURE 2: Titration of IAF/IAA-modified Na,K-ATPase by TNP-ATP in NaCl or KCl. Relative fluorescence, obtained by comparing sample and reference cuvettes as explained in Methods, is plotted against total TNP-ATP concentration on a logarithmic scale. Titrant was added to 20 μg mL^{-1} IAF/IAA-modified protein ($\chi_a = 0.36$) in 50 mM Tris-HCl and either 20 mM NaCl (●) or 20 mM KCl (▼) at pH 7.4. Instrument settings are quoted in Methods. The solid lines are the best fits of eq 1 to the data. The estimated parameters are $K_d = 30 \pm 0.3$ nM and $\gamma = 6.82 \pm 0.4\%$ in Na^+ and $K_d = 2.2 \pm 0.1$ μM and $\gamma = 71.4 \pm 0.2\%$ in K^+.

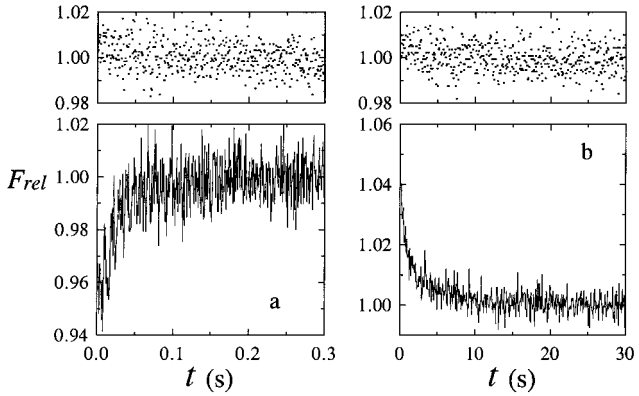

FIGURE 3: Time resolution of change in FRET efficiency. Relative fluorescence is plotted against time (t) in seconds. (a) IAF/IAA-modified Na,K-ATPase was mixed with KCl. Both syringes contained 2 mM NaCl, 198 mM (enzyme syringe) or 158 mM (reagent syringe) ChoCl, and zero (upper trace) or 32 μM (lower trace) TNP-ATP in 50 mM Tris-HCl at pH 7.4 and 15 °C. The concentrations of protein and KCl after mixing were 96 μg mL^{-1} and 20 mM, respectively. (b) IAF/IAA-modified Na,K-ATPase was mixed with NaCl. The contents of the enzyme and reagent syringes were the same except that 2 mM KCl was substituted for 2 mM NaCl in both syringes and the reagent syringe contained 40 mM NaCl. The first-order rate constants estimated from the lower traces are 40.5 $\pm$ 2.7 s^{-1} in (a) and 0.57 $\pm$ 0.02 s^{-1} in (b).

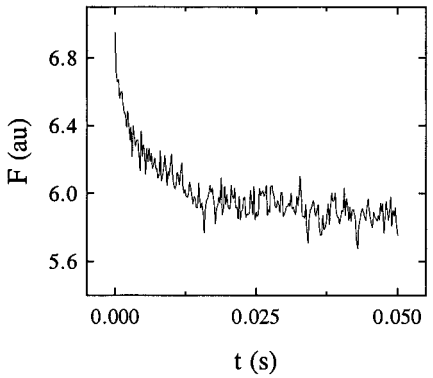

FIGURE 4: Rate of TNP-ATP binding to IAF/IAA-modified Na,K-ATPase. Fluorescence intensity is plotted against time in seconds. The enzyme syringe contained 192 μg mL^{-1} IAF/IAA-protein, and the reagent syringe contained 64 μM TNP-ATP. Both solutions contained 80 mM NaCl and 120 mM ChoCl and were buffered by 50 mM Tris-HCl at pH 7.4 and 15 °C.

conformations of the enzyme or from a change in the Förster critical distance if Q_d and/or J is different in E_1 and E_2. Therefore, the quantum yield of IAF/IAA-enzyme was measured ($n = 3$) in Na^+ ($Q_d = 0.38 \pm 0.05$) and in K^+ ($Q_d = 0.37 \pm 0.05$). In agreement with an earlier report (Steinberg & Karlish, 1989), the fluorescence of fluorescein covalently attached to cysteine-457 of the pig enzyme is not affected by the protein conformation. The overlap integral between the emission spectrum of the donor IAF and the absorption spectrum of the acceptor TNP-ATP evaluated with eq 5 ($n = 2$) is also the same in Na^+ ($J = (2.71 \pm 0.18) \times 10^{-14}$ M^{-1} cm^3) as in K^+ ($J = (2.76 \pm 0.20) \times 10^{-14}$ M^{-1} cm^3), so R_0 calculated assuming constant η and $\kappa^2 = 2/3$ is unaffected by the transported ions. Therefore, the difference between the end points of the titrations of IAF/IAA-enzyme with TNP-ATP in Na^+ and K^+ is the result of a decrease in the efficiency of energy transfer between donor and acceptor in the E_1 and E_2 conformations and is consistent with an increase in the distance between IAF and TNP-ATP when the enzyme changes conformation from E_1 to E_2.

Conformational Change Reported by FRET. Stopped-flow and FRET measurements were combined to determine the rate and functional dependence on ion concentrations of the conformational change reported by energy transfer between IAF and TNP-ATP. Figure 3 shows typical time curves obtained by mixing K^+ (a) or Na^+ (b) with IAF/IAA-enzyme in the presence (solid) or absence (dotted) of TNP-ATP. When no TNP-ATP is present (upper traces), there is no fluorescence change on the time scale of the conformational change in Na,K-ATPase (Lin & Faller, 1993), confirming that IAF by itself does not report a conformational change in the hog enzyme (Steinberg & Karlish, 1989). In the presence of TNP-ATP, approximately equal (4%) but opposite fluorescence changes are observed. The roughly 2 orders of magnitude difference between the time scales in Figure 3, panels a and b is consistent with the difference in rates of the conformational change reported by fluorescein when FITC-labeled sodium pump is mixed with K^+ or Na^+ (Smirnova *et al.*, 1995a). However, since the acceptor is reversibly bound and the affinity of IAF-modified Na,K-ATPase for TNP-ATP is dramatically different in Na^+ and K^+ (Figure 2), TNP-ATP reequilibration could also contribute to the observed fluorescence signal when [Na^+] or [K^+] is jumped in a mixing experiment (Smirnova & Faller, 1995).

A direct contribution of TNP-ATP fluorescence to the observed fluorescence signal because binding to the protein enhances TNP-ATP fluorescence (Moczydlowski & Fortes, 1981) can be ruled out for two reasons. First, fluorescence of the acceptor would be difficult to detect in the presence of donor because the quantum yield of IAF is more than 3 orders of magnitude larger than the quantum yield of bound TNP-ATP. Second, the expected fluorescence changes are in the wrong direction to explain the experimental observations in Figure 3. TNP-ATP binds tighter to the Na^+ than to the K^+ conformation of the enzyme (Figure 2), so the intensity of TNP-ATP fluorescence should decrease because of TNP-ATP dissociation when the equilibrated enzyme is mixed with K^+ (Figure 3a) and increase because of TNP-ATP association when the equilibrated enzyme is mixed with Na^+ (Figure 3b). In both experiments the observed fluorescence changes are in the opposite direction.

The fluorescence changes expected from the indirect effect of TNP-ATP binding or dissociation on IAF fluorescence because of FRET are in the right direction, but kinetic evidence that the fluorescence changes in Figure 3 do not result from reequilibration of acceptor with enzyme is shown in Figure 4. When TNP-ATP is mixed with the Na^+ conformation of IAF/IAA-enzyme to give the same final concentration of acceptor as in Figure 3 (32 μM), the expected fluorescence quench because of energy transfer to the acceptor is observed, but on a 1−3 orders of magnitude shorter time scale than either of the fluorescence changes in Figure 3. Therefore, the rate of TNP-ATP binding (Figure 4) is approximately 10−100 times faster, respectively, than the reactions in Figure 3a,b. This difference in rates combined with the smaller amplitude expected for reequilibration in a Na^+- or K^+-jump experiment because 32 μM TNP-ATP essentially saturates the enzyme in both the Na^+ and K^+ conformations (Figure 2) means that the conformational changes reported by FRET in Figure 3a,b can be studied without interference from acceptor binding.

Stopped-flow experiments designed to learn whether the conformational change reported by FRET is the conforma-

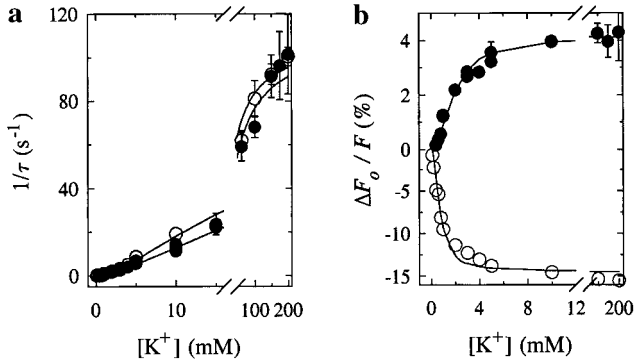

FIGURE 5: Comparison of K^+ titration curves reported by FRET (●) and FITC modification (○). In (a) $1/\tau$ is plotted against final (after mixing) $[K^+]$. The corresponding percentage changes in fluorescence are plotted in (b). The uncertainties in the estimates of the dependent variables are indicated by vertical bars. Equal volumes of an enzyme solution containing 192 μg mL^{-1} protein labeled with IAF or FITC in 2 mM NaCl and 198 mM ChoCl were mixed with reagent solutions containing twice the concentration of KCl recorded on the abscissa, 2 mM NaCl, and enough ChoCl for 200 mM added salt. Both solutions were buffered by 50 mM Tris-HCl at pH 7.4 and 15 °C, and both contained 32 μM TNP-ATP in the experiments with IAF/IAA-modified enzyme. The solid lines are the best fits of eq 2 (a) and eq 6 (b) in Smirnova *et al.* (1995a) to the data. The parameter estimates are reported in Table 1.

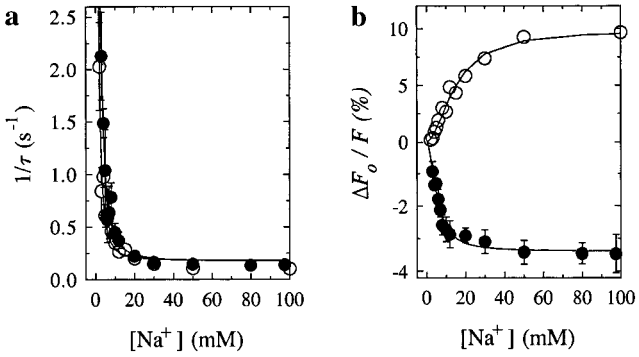

FIGURE 6: Dependence of the reactions reported by FRET (●) and FITC modification (○) on $[Na^+]$. In (a) $1/\tau$ is plotted against $[Na^+]$, and the corresponding percentage changes in fluorescence are graphed in (b). The experimental protocol in the legend to Figure 5 was followed in the FRET measurements, except that the roles of Na^+ and K^+ were reversed. In particular, the enzyme solution initially contained 5 mM KCl and the reagent syringe contained twice the NaCl concentration reported on the abscissa. Part of the FITC data (amplitudes) was published previously in Figure 3 of Smirnova *et al.* (1995a). The solid lines are the best fits of eq 2 (a) and eq 7 (b) in the cited reference to the data. The parameter estimates are recorded in Table 1.

tional change that has been implicated in ion transport are shown in Figures 5 and 6. The criteria used to decide whether FRET reports the same conformational change reported by fluorescein and eosin are, first, whether the shapes of the FRET titration curves are predicted by the mechanism proposed to explain the conformational change reported by FITC modification (Smirnova *et al.*, 1995a) and, second, whether the FRET parameter estimates are the same, within experimental error, as the values that have been shown to fit fluorescein and eosin data (Smirnova & Faller, 1995).

Figure 5 compares the functional dependence of the rates and amplitudes of the reactions reported by FRET (closed circles) and FITC modification (open circles) on $[K^+]$. The reciprocal relaxation times ($1/\tau$) are essentially superimposable and depend sigmoidally on $[K^+]$. The changes in corrected (for instrument dead time) fluorescence (ΔF_0), expressed as a percentage, reported by the change in FRET efficiency are smaller than the percentage changes in fluorescein fluorescence resulting from different protein environments in the E_1 and E_2 conformations and opposite in sign, but both amplitude titration curves appear visually to be hyperbolic. The theoretical curves are fits of eq 2 (a) and eq 6 (b) in Smirnova *et al.* (1995a,b) for K^+ binding to two identical and independent sites to the data. The strategy used to obtain best estimates of the parameters by combining rate and amplitude data for different choices of the independent variable is described in the cited reference. In Table 1, the estimated parameters are indicated by reporting the standard deviation in the estimate.

Figure 6 shows the functional dependence on $[Na^+]$ of $1/\tau$ and $\Delta F_0/F$ for the reaction reported by a change in FRET efficiency (closed circles). Data from an earlier publication obtained by attaching fluorescein to the enzyme with FITC is plotted (circles) on the same graphs for comparison. The reciprocal relaxation time (Figure 6a) depends inversely on $[Na^+]$ as predicted by eq 2 in Smirnova *et al.* (1995a). The fixed $[K^+]$ is too low to clearly see the sigmoidal dependence of the amplitude of the fluorescence change (Figure 6b) on $[Na^+]$ predicted by eq 7 (*ibid.*). The theoretical curves are fits of the referenced equations to the data, and the parameter estimates are recorded in Table 1.

DISCUSSION

The average distance between attached fluorescein and bound TNP-ATP is measured by FRET. The distance change calculated from different FRET efficiencies in the Na^+ and K^+ conformations can be interpreted as relative motion between two defined points on the enzyme only if IAF modifies cysteine-457 specifically and TNP-ATP binds specifically to the active site. Therefore, we begin the Discussion with evidence that the donor and acceptor are each attached to a single, unique site on the functional enzyme.

Evidence IAF and TNP-ATP Are Specifically Attached. According to Tyson *et al.* (1989), a "single, unique" residue is covalently modified when dog kidney Na,K-ATPase is preincubated with IAA before reacting with IAF. In support of their conclusion, the authors showed that the fluorescence response to Na^+ increased 50% and the stoichiometry of IAF incorporation decreased to 1.2 mol of IAF/mol of α-subunit following pretreatment with IAA. The actual stoichiometry may have been higher because the Lowry method overestimates protein in eel electroplax membranes by 40% (Moczydlowski & Fortes, 1981). However, all of the peptides sequenced, which accounted for most of the fluorescent material, contained fragments of the decapeptide surrounding the amino acid numbered 457 in the sheep α sequence. Identification of the residue as a cysteine was confirmed by blocking IAF incorporation with phenylarsine oxide, which reacts with vicinal sulfhydryls.

This study and the two previous FRET studies of IAF/IAA-enzyme confirm that 1 mol of fluorescein is incorporated per mole of α-subunit if correction is made for overestimation of protein by the Lowry assay. Specific labeling was assumed in the earlier FRET studies because of approximately stoichiometric incorporation of 1−1.3 (Fortes & Aguilar, 1988) and 0.78 (Amler *et al.*, 1992) mol

Table 1: Summary of Parameter Estimates

Figure	reagent/method	variable: independent	variable: dependent	k_f (s^{-1})	K_K (mM)	K_{Na} (mM)	k_r (s^{-1})	$\Delta F_{max}/F$ (%)
5	FRET	K^+	$1/\tau$	118 ± 4	4.3 ± 0.3	0.5	0.15	
			$\Delta F_o/F$	118	4.2 ± 0.2	0.18	0.15	4.1 ± 0.1
	FITC	K^+	$1/\tau$	120 ± 3	3.1 ± 0.2	0.5	0.15	
			$\Delta F_o/F$	120	3.9 ± 0.3	0.5	0.15	−15.0 ± 0.3
6	FRET	Na^+	$1/\tau$	118	4.3	0.44 ± 0.01	0.16 ± 0.04	
			$\Delta F_o/F$	118	4.3	0.18 ± 0.01	0.15	-3.4 ± 0.1
	FITC	Na^+	$1/\tau$	120	3.9	0.25 ± 0.01	0.18 ± 0.04	
			$\Delta F_o/F$	120	3.9	0.44 ± 0.03	0.15	9.8 ± 0.4

of IAF/mol of α-subunit, respectively, into the dog and lamb enzymes. The corrected stoichiometries of IAF incorporation into the hog preparations used in this study varied from 0.72 to 0.93 mol of IAF/mol of $\alpha\beta$. Another reason for concluding that a single site was labeled in our experiments is that the progress of reactions with IAF was monitored and stopped before concurrent increases in specific fluorescence and decreases in specific activity indicated nonspecific incorporation (Results).

According to Moczydlowski and Fortes (1981), one TNP-ATP binds per α-subunit to the catalytic site of Na,K-ATPase with different affinities in the E_1 and E_2 conformations. The authors inferred the stoichiometry from an upper limit on the combining weight of $(1.76 \pm 0.06) \times 10^5$ compared with the $\alpha\beta$ molecular weight from amino acid composition of 1.47×10^5 and essentially equivalent incorporation of TNP-ATP and ouabain (1.1 TNP-ATP per ouabain). Binding to the active site was demonstrated by showing that ATP binds competitively with TNP-ATP and that TNP-ATP competitively inhibits ATPase activity. The ATP dissociation constant deduced from competitive binding was in quantitative agreement with the Michaelis constant estimated from activity measurements, and the TNP-ATP inhibition constant evaluated from activity measurements agreed with the dissociation constant from direct binding measurements. The TNP-ATP dissociation constant reported in the FRET study (Fortes & Aguilar, 1988) increased from 43 ± 18 nM at 24 °C in imidazole, which has an unselective Na^+-like effect on the enzyme conformation (Lin & Faller, 1993), to 5.2 μM in 100 mM K^+.

Our results support the conclusion that TNP-ATP binds stoichiometrically to the active site of Na,K-ATPase. Titrations of porcine enzyme with TNP-ATP could be fit with eq 1 for binding to a single site (Figure 2). The dissociation constant increased from 30 nM in Na^+ to 2.2 μM in K^+ at 15 °C (Figure 2 legend) in good agreement with the literature values cited in the preceding paragraph. We took advantage of equivalent ouabain binding to count the number of protein molecules that bind TNP-ATP and of competitive binding to circumvent the problem of inner filter effects in titrations with TNP-ATP by displacing TNP-ATP with ATP in the reference cuvette (Methods). The measurements of ouabain binding indicated that only about one-third of the protein labeled with IAF binds TNP-ATP ($0.31 \leq \chi_a \leq 0.36$). Multiplying the measured activities of the preparations titrated with TNP-ATP after modification with both IAA and IAF (8.5−12.5 μmol of P_i min^{-1} mg^{-1}) by 3 to estimate the activity of completely active enzyme gives values (26−38 μmol of P_i min^{-1} mg^{-1}) consistent with the highest activities (32−37 μmol P_i of min^{-1} mg^{-1}) that have been reported for Na,K-ATPase purified by SDS extraction (Jørgensen, 1974). Fortes and Aguilar (1988) also found that "IAF reacted with all the α-subunits in the preparation, but only a fraction of those α-subunits (∼1/3rd) were able to bind TNP-ATP", and speculated that the remaining protein is inactive (possibly denatured).

Distance from IAF to TNP-ATP in E_1 and in E_2. The distances between IAF attached to cysteine-457 and TNP-ATP bound at the active site calculated with eqs 2−4 and the parameter estimates given in the text are 25.2 ± 5.1 Å in E_1 and 28.1 ± 3.5 Å in E_2. The $R_{2/3}$ in the E_1 conformation is in excellent agreement with the value (24 Å) estimated by Fortes and Aguilar (1988) from measurements in 50 mM imidazole. Since different protocols that would not be expected to uniformly label nonspecific sites contributing to the observed fluorescence were used to label the enzyme with IAF, quantitative agreement between the distances measured with IAF/IAA-enzyme (this study) and IAF-enzyme (Fortes & Aguilar, 1988) supports the conclusion that IAF and TNP-ATP attach to specific sites.

Only the end points of the titrations were determined for four other preparations of IAF/IAA-enzyme by adding an excess of TNP-ATP in 20 mM Na^+ because complete titrations like those in Figure 2 are tedious and require considerable protein. The maximum fluorescence quench ($1 - \gamma$) depended linearly (not shown) on the specific activity after labeling with IAA and IAF over the range (8.5−12.5 μmol of P_i min^{-1} mg^{-1}), confirming experimentally that E and therefore the derived value of $R_{2/3}$ are independent of the modified-enzyme preparation. The specific activity of fully active enzyme, estimated from E for the Na^+ conformation (88%) and the slope (intercept zero within error of estimation) of the straight line, is 32 μmol min^{-1} mg^{-1} in good agreement with the previously quoted estimates of the specific activity of "pure" SDS-extracted enzyme (Jørgensen, 1974). In other words, both the ouabain binding stoichiometry and specific activity of IAF/IAA-enzyme indicate that only about 1/3rd of the modified protein is competent to bind TNP-ATP in agreement with the conclusion of Fortes and Aquilar (1988).

Change in Distance when the Conformation Changes. The change in distance between donor and acceptor ($\Delta R_{2/3}$) for the pair IAF/TNP-ATP (2.9 Å) is less than the uncertainty in the estimate of $R_{2/3}$ for either the E_1 (±5.1 Å) or the E_2 (±3.5 Å) conformation. However, the uncertainty in $\Delta R_{2/3}$ is smaller than the uncertainty calculated by propagating the errors in the individual estimates of $R_{2/3}$ in Na^+ and K^+ because the measured fluorescence quench results only from FRET between donor and acceptor. We have confirmed that IAF by itself does not report the change between Na^+ and

K^+ conformations of pig kidney enzyme (Figure 3). There is no measurable change in Q_d or J (Results). The value of η is for bulk solution. The TNP-ATP binding capacity (maximum χ_a) was the same in the titrations with Na^+ and K^+ because the same IAF/IAA-enzyme preparation was used for both of the titrations shown in Figure 2. Therefore, the uncertainty in $\Delta R_{2/3}$ can be calculated with the following equation, in which the variable subscripts denote values for the Na^+ and K^+ conformations of the enzyme:

$$d(\Delta R_{2/3}) = \frac{\chi_a R_0^6}{6}\left[\frac{d\gamma_K}{R_K^5(1-\gamma_K)^2} + \frac{d\gamma_{Na}}{R_{Na}^5(1-\gamma_{Na})^2}\right] \quad (6)$$

The result is $\Delta R_{2/3} = 2.9 \pm 0.6$ Å. Since the distance from the luminal cardiac glycoside site to the cytosolic active site is 72−74 Å (Carilli *et al*., 1982; Amler *et al*., 1992), the measured distance change seems to favor a relatively small change in shape such as the gating of a channel. However, a larger protein rearrangement cannot be excluded. Cysteine-457 and the active site are both in the largest hydrophilic domain of the protein, so they could be located near the joint of subdomains that function like a hinge. For example, a number of ATP-binding proteins of known three-dimensional structure have clefts that close when the substrate binds. In hexokinase, atoms of the polypeptide backbone move as much as 8 Å (Anderson *et al*., 1979). Additional measurements of changes in the distances between different points on the protein are needed to visualize the conformational change in three dimensions.

The distances from BIPM, which may be located in a transmembrane segment (Taniguchi *et al*., 1994), to ANM (Shinoguchi *et al*., 1991) and to FITC (Tosa *et al*., 1994) have been measured in the Na^+ and K^+ conformations of the enzyme. Unfortunately, the calculated $\Delta R_{2/3}$ values are less than the uncertainty in the reported $R_{2/3}$ estimates for both the Na^+ and the K^+ conformations, and eq 6 cannot be used to calculate the uncertainty in $\Delta R_{2/3}$ because in both of these donor/acceptor pairs Q_d as well as $R_{2/3}$ is affected by the conformational change.

Conformation-dependent changes in the fluorescence properties of the acceptor and/or donor complicate the interpretation of FRET measurements in another way. There is the problem of resolving the change in FRET efficiency from the contributions to the observed fluorescence signal of acceptor and/or donor acting alone as reporters groups. The problem is illustrated by stopped-flow/FRET studies of the BIPM/FITC pair (Taniguchi & Mårdh, 1993). The quantum yields of both members of this donor/acceptor pair are changed by different protein microenvironments in the Na^+ and K^+ conformations of the enzyme. Therefore, the emissions from FITC ($\lambda_{max} = 520$) in doubly-labeled enzyme when both BIPM and FITC were excited by 305 nm light and when only FITC was excited by 470 nm light were measured as a function of time, and their ratio (305 nm excitation/470 nm excitation) was assumed to reflect the rate of change of FRET efficiency. The result when enzyme in K^+ was mixed with Na^+ in experiments comparable to those described in this study was different rates for the changes in FITC emission (470 nm excitation) and FRET efficiency (*ibid*., Figure 6b). The rate of change of BIPM emission was different from the rate of change of either FITC emission or FRET efficiency. Therefore, either BIPM, FITC, and FRET report different steps in the conformational change between E_1 and E_2, or the method of correcting the resultant signal for changes in the quantum yields of donor and acceptor is incorrect. This study demonstrates that both labeling Na,K-ATPase with FITC and FRET between IAF and TNP-ATP report the same reaction.

While this paper was in preparation, a paper appeared reporting a distance change of 2−3 Å between sites on the α- and β-subunits of Na,K-ATPase when K^+ binds (Amler *et al*., 1996). The fluorescence of the enzyme labeled only with the donor (lucifer yellow) was not affected by K^+, so the increase in donor emission caused by K^+ could be attributed to an increase in distance between donor and acceptor. However, calculation of distances from the fluorescence intensity data and estimation of the uncertainty in the distance change were complicated by lucifer yellow attachment to more than one site on the β-subunit and roughly equal degrees of specific and nonspecific labeling of the α-subunit by acceptor (ErITC). The distance change was attributed to the change between Na^+ and K^+ conformations even though no change in FRET efficiency was observed when Na^+ was added to the doubly-labeled enzyme. The conformational change was modeled as a lateral displacement of the hydrophilic domain of the β-subunit relative to the ouabain binding site on the α-subunit, presumably because no change in FRET efficiency was observed with Na^+ or K^+ in an earlier study when both donor (IAF) and acceptor (ErITC) were on the α-subunit (Amler *et al*., 1992). However, our experiments with the IAF/TNP-ATP donor/acceptor pair demonstrate that the conformational change between E_1 and E_2 does involve rearrangement of the α-subunit.

All of the change in IAF emission when porcine IAF/IAA-enzyme with TNP-ATP bound is titrated with Na^+ or K^+ can confidently be attributed to the conformational change in the protein because the fluorescence of IAF/IAA-enzyme is unaffected by the transported ions (top traces in Figure 3a,b) and TNP-ATP emission does not contribute to the observed fluorescence (Results). Although additional uncertainty in the estimates of $R_{2/3}$ cannot be avoided because κ^2 is unknown (Methods), the qualification that the uncertainty in the estimate of $\Delta R_{2/3}$ assumes random orientation of the absorption and emission dipoles could be removed by showing that the limiting anisotopies of the donor and acceptor are independent of the enzyme conformation. Therefore, we attempted to measure the limiting anisotropies of bound IAF and TNP-ATP. The limiting anisotropies of IAF in the E_1 (0.191) and E_2 (0.173) conformations of Na,K-ATPase are approximately the same and in good agreement with an anisotropy value (0.17) reported previously (Amler *et al*., 1992). The fundamental anisotropy of IAF at the excitation wavelength used in our experiments (492 nm) is 0.373. Unfortunately, we were unable to obtain the corresponding values for TNP-ATP. Therefore, the uncertainty we report in our estimate of $\Delta R_{2/3}$ depends upon the assumption that κ^2 is the same in E_1 and E_2. Conversely, if κ^2 does not change, the estimate of ΔR does not depend on the assumption that $\kappa^2 = 2/3$. The equivalence of ΔR and $\Delta R_{2/3}$ when κ^2 does not change can be illustrated by estimating the error introduced into R values calculated for the E_1 and E_2 conformations by assuming $\kappa^2 = 2/3$. The ranges of possible values estimated with Figure 9 in Dale *et al*. (1979) from the axial depolarization (square root of

limiting anisotropy/fundamental anisotropy) of IAF in the E_1 and E_2 conformations by letting the axial depolarization of TNP-ATP assume all possible values are $18.2 \leq R \leq 31.6$ Å (E_1) and $20.7 \leq R \leq 35.0$ Å (E_2). The small difference between the ΔR values calculated by taking the difference between the minimum R estimates (minimum κ^2) or the difference between the maximum R estimates (maximum κ^2) is the result of the slightly different axial depolarizations of IAF in the two conformations that were used to estimate the range of κ^2 values for E_1 and for E_2. The differences between both the minimum (2.5 Å) and maximum (3.4 Å) values fall within the quoted uncertainty in $\Delta R_{2/3}$.

The assumption that κ^2 does not change appreciably when the enzyme conformation changes from E_1 to E_2 is reasonable because the folding of the ATP-binding domain of sodium pump is thought to resemble adenylate kinase (Taylor & Green, 1989), which binds ATP in a cleft or pocket (Müller & Schulz, 1992). Published estimates of the anisotropy (0.29) and fundamental anisotropy (0.34) of TNP-ATP bound to eel enzyme (Moczydlowski & Fortes, 1981) are consistent with binding within a narrow cavity because the axial depolarization is approximately 0.92, which is close to the theoretical value (1.0) for complete immobilization of the fluorophore. The TNP-ATP anisotropy values were measured in 44 mM HEPES−Tris at pH 7.5, so they are probably for binding to a mixture of E_1 (∼75%) and E_2 (∼25%) conformations (Lin & Faller, 1993). Assuming that the axial depolarization of TNP-ATP bound to E_1 is 1.0 gives an estimate for the axial depolarization of TNP-ATP bound to E_2 of 0.68 from the value 0.92 for binding to the mixture. The trinitrophenyl moiety is thought to extend into a broader cavity (Taylor & Green, 1989), and its motion is probably not restricted by many more interactions with the protein in the E_1 conformation because the higher affinity for TNP-ATP in the E_1 than in the E_2 conformation can be accounted for semiquantitatively by the different affinities of E_1 and E_2 for ATP (Results).

FRET Reports the Conformational Change between E_1 and E_2. The data in Figures 5 and 6 and the parameter estimates in Table 1 demonstrate that FRET between IAF and TNP-ATP reports the same conformational change as covalent modification of the enzyme with FITC or reversible eosin binding. The functional dependence of $1/\tau$ for the change in FRET efficiency on $[K^+]$ and $[Na^+]$ is the same as the dependence reported by FITC or eosin, and there is no statistical difference between the parameter estimates that fit both $1/\tau$ and $\Delta F_o/F$ data obtained by following the reaction with FRET, FITC, or eosin. The justification for equating the reaction reported by fluorescein or reversible eosin binding and therefore by FRET with the change between protein conformations designated E_1 and E_2 that have been implicated in transport was reviewed briefly in the introduction and has been discussed in detail in the cited publications.

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