Time Resolution of Fluorescence Changes Observed in Titrations of Fluorescein 5′-Isothiocyanate-Modified Na,K-ATPase with Monovalent Cations†

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ABSTRACT: Equilibrium fluorometric titrations of fluorescein 5′-isothiocyanate-modified Na,K-ATPase with cations have usually been interpreted by assuming that an enhancement reports the conformational change from E_2 to E_1. We report time resolution of the fluorescence change into three phases when fluorophore-modified enzyme is mixed with the chloride salt of either sodium or choline in a stopped-flow instrument. The first phase is an increase in fluorescence within the dead time of the instrument that is also observed when fluorescein 5′-isothiocyanate (FITC) reacted with lysine is substituted for fluorescein-labeled enzyme. The other two phases occur on millisecond and second time scales. Three phases are also observed when fluorophore-modified enzyme preincubated in KCl is mixed with NaCl, but in this case the slowest phase is absent when choline chloride replaces NaCl. The two faster effects in phases one and two can be eliminated either by controlling the ionic strength or by anti-fluorescein antibody. Labeling the enzyme with fluorescein 5′-isothiocyanate in the presence of its substrate, adenosine 5′-triphosphate, practically eliminates the slowest effect. These results demonstrate that fluorescein reports three events that occur on three different time scales. The fastest phase reports the ionic strength jump of unbound fluorophore. The intermediate phase reports the ionic strength jump of fluorescein at "antibody-accessible" sites [Abbott, A. J., Amler, E., & Ball, W. J., Jr. (1991) *Biochemistry 30,* 1692–1701]. Only the slowest phase reports the enzyme conformational change implicated in transport. The results of this study reinforce the conclusion of Abbott *et al.* that caution must be exercised in interpreting equilibrium titrations of FITC-modified enzymes and show that artifacts can be avoided by kinetic titrations.

It is generally believed that protein conformational changes explain the obligatory coupling of ATP[1] hydrolysis to countertransport of Na^+ and K^+ ions (Post *et al.*, 1969) and might also explain the movement of ions through the sodium pump (Kyte, 1975). Consequently, a number of experimental approaches have been devised to demonstrate the existence of different protein conformations and to study the mechanisms of their interconversion. Perhaps the most direct and compelling evidence for a transition between protein conformations is that Na^+ and K^+ cause different proteolytic cleavage patterns (Jorgensen, 1975). However, the more rapid response and greater sensitivity of fluorophores to changes in protein environment are required for the more demanding purpose of studying dynamic events in the reaction cycle.

Although the intrinsic fluorescence of tryptophan residues in the protein is affected by Na^+ and K^+, the changes are too small for detailed mechanistic studies (Karlish & Yates, 1978). Therefore, Karlish (1979) introduced an extrinsic fluorophore that reports the same effects of Na^+ and K^+, but with bigger signals, by chemically modifying the enzyme with FITC. An advantage of FITC is that fluorescein is covalently bound to Lysine 501 (Farley *et al.*, 1984), so that analysis of the observed fluorescence emission is not complicated by ligand-induced changes in the amount of probe bound or in the stoichiometry of probe binding (Faller, 1990). A disadvantage of FITC is that the covalently modified enzyme does not react with ATP, so that only conformational changes in the dephosphoenzyme half of the reaction cycle have been studied by FITC modification of Na,K-ATPase.

K^+ quenches the fluorescence of FITC-modified Na,K-ATPase, and Na^+ reverses the K^+ quench (Karlish, 1980). Although stopped-flow fluorometry was used to measure the rates of the K^+ quench and Na^+ reversal, until recently measurements of the amplitudes of the K^+ quench and Na^+ reversal have been restricted to equilibrium fluorometric titrations. Four conformations of dephosphoenzyme have been inferred from the effects of monovalent and divalent cations on the intensity of fluorescein fluorescence at equilibrium. In order of decreasing fluorescence quantum yield, they are designated E_1Na (Hegyvary & Jorgensen, 1981), E_1 or MgE_1, MgE_2K and E_2K (Karlish, 1980; Hegyvary & Jorgensen, 1981). Implicit in these assignments is the assumption that fluorescein only reports conformational changes in the enzyme.

Several more recent papers suggest that the interpretation of fluorescence changes observed when FITC-modified Na,K-ATPase is titrated with monovalent or divalent cations may be more complicated. First, Xu (1989) showed that FITC also reacts covalently with Lysine 480 and Lysine 766. Second, Abbott *et al.* (1991) identified two classes of FITC-labeled sites that differ in their reactivity with anti-fluorescein antibody. Third, Grell *et al.* (1991) have reported that a number of monovalent cations other than Na^+ and K^+ can cause the fluorescence quench and subsequent reversal normally associated with the E_1 and E_2 conformations of Na,K-ATPase. Fourth, in a study of the role of Mg^{2+} ions in the conformational change reported by FITC modification of

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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; FITC, fluorescein 5′-isothiocyanate; FITC-X, fluorescein 5′-isothiocyanate-modified lysine or Na,K-ATPase; P_i, inorganic phosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; ChoCl, choline chloride.

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Na,K-ATPase, we have found discrepancies between the fluorescence changes that were observed in equilibrium fluorometric titrations (Karlish, 1980; Hegyvary & Jorgensen, 1981) and the amplitudes measured in "kinetic" titrations by stopped-flow fluorometry (Smirnova & Faller, 1993a). Therefore, we have investigated whether all of the fluorescence change observed in titrations of FITC-modified Na,K-ATPase with monovalent cations is caused by a protein conformational change.

In this paper, we show that the response of FITC-modified Na,K-ATPase to Na^+ can be resolved by stopped-flow fluorometry into three phases on order-of-magnitude different time scales. The fastest fluorescence change can be observed in the absence of protein, so it is attributed to unbound fluorophore. The fluorescence change that occurs with intermediate rate is protein-dependent but is eliminated by anti-fluorescein antibody, so that it results from fluorescein labeling of antibody-accessible sites. Only the longest relaxation time reports the enzyme conformational change from E_2 to E_1. An abstract communicating some of the results of this study has been published (Lin & Faller, 1993).

EXPERIMENTAL PROCEDURES

Materials

Na,K-ATPase. Membrane fragments containing Na,K-ATPase were purified from the outer medulla of pig kidneys as described by Jorgensen (1974). The specific activity of the preparations used in the measurements reported ranged from 12 to 16 μmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$ (Lowry *et al.*, 1951) at 37 °C. At least 97% of the total enzymatic activity could be inhibited by 0.1 mM ouabain. Although only representative results are shown in the figures, all of the experiments described in this paper were shown to be reproducible by replicate measurements with different enzyme preparations and with batches of the same preparation modified in separate reactions.

Chemical Modification. The purified Na,K-ATPase (2 mg mL^{-1}) was reacted with 20 μM FITC at pH 9.2 for 30 min using the experimental protocol of Farley *et al.* (1984). The reaction was terminated by lowering the pH to 7.4 with 50 mM Tris-HCl buffer containing 1 mM EDTA. The residual ATPase activity was less than 5%. FITC labeling in the presence of ATP was done similarly with 3 mM ATP and 10–100 μM FITC. FITC-Lysine was prepared by incubating 5 μM FITC with 100-fold excess lysine in 20 mM Tris-HCl at pH 9.2 in the dark at room temperature for 10 min.

Reagents. Fresh pig kidneys were a gift from Farmer John Clougherty Packing Co. (Los Angeles, CA). FITC (isomer I) and anti-fluorescein rabbit antibody (Watt & Voss, 1978) in 0.1 M potassium phosphate (10 μL = 1 unit) were purchased from Molecular Probes Inc. (Eugene, OR). The chloride salts of choline, potassium, and sodium were purchased from Sigma (St. Louis, MO). All other reagents were the highest grade commercially available.

Methods

Stopped-Flow Measurements. Stopped-flow measurements were made in the instrument described previously (Faller *et al.*, 1991) with a T-mixer installed. A novel feature of the On Line Instrument Systems software is the ability to resolve events occurring on different time scales by acquiring data from a single mix with two different collection times. For example, in most of the figures that will be shown the first 500 data points were collected in 0.2 s, and the second 500 data points were collected in 150 s. Unless otherwise indicated, 100 μL of solution containing 180 μg mL^{-1} FITC-modified protein in 0.13 mM EDTA and buffered by 6.6 mM Tris-HCl at pH 7.4 and 15 °C was mixed with an equal volume of the same buffer containing 200 mM NaCl or 200 mM ChoCl. Therefore, the final concentration of titrant in the stopped-flow mixing experiments that are described is half the quoted concentration. For example, mixing enzyme with 200 mM NaCl results in a final added salt concentration of 100 mM. The baseline was obtained by mixing FITC-modified enzyme, or lysine, with buffer containing no added salt. Empirical first-order rate constants (k_1 and k_2) and amplitudes (ΔF_1 and ΔF_2) for the observed fluorescence enhancements were estimated by fitting the equation for two parallel first-order reactions

$$F = \Delta F_1(1-e^{-k_1 t}) + \Delta F_2(1-e^{-k_2 t}) + F_i$$

to the data either by successive integration or with a nonlinear least-squares algorithm. F is the fluorescence intensity at time t, and F_i is the fluorescence intensity of the first data point. In the figures, fluorescence intensity is expressed either in arbitrary units (au) or as relative fluorescence (F/reference) with the baseline fluorescence level in the absence of K^+ as reference.

(*a*) *Slit Widths.* Both the reporter group, fluorescein, and the protein are sensitive to high-intensity radiation. Therefore, it is important to establish that photobleaching and/or photodenaturation are not affecting fluorometric titrations of FITC-labeled sodium pump. In our instrument a super-quiet 75-W high-pressure xenon short-arc lamp (Hamamatsu Corp.) is focused on the entrance slit of a holographic diffraction grating monochromator (Instruments SA, Inc.). The stable baselines shown in the figures demonstrate that with the 1-mm entrance and exit slits used in our experiments the emission intensity from FITC-modified Na,K-ATPase does not change perceptibly in the several minute time course of Na^+ reversal experiments.

(*b*) *Dead Time.* Some understanding of the rate limitation of the stopped-flow methodology is essential for interpretation of the measurements reported in this paper. The time required to mix two solutions in a stopped-flow machine and begin observing the time course of the resulting reaction is called the dead time (t_d) of the instrument. t_d limits the rate of reactions that can be measured and is important for correction of observed amplitudes when the reaction is so fast that only a fraction of the signal change can be time-resolved. The measured t_d of our instrument is 2.2 ± 0.7 ms for mixing two 100-μL aqueous solutions with 7 atm drive pressure near ambient temperature (Smirnova & Faller, 1993a). We have published raw data showing that an empirical first-order constant as large as 340 ± 18 s^{-1} (n = 11) for a conformational change in sodium pump reported by fluorescein can be inferred from observation of 35% of the reaction (Faller *et al.*, 1991). The percentage of a first-order reaction actually observed, and therefore the fluorescence intensity at the start of the reaction, can be calculated from the estimates of k, ΔF, and t_d with eq 2 in the cited reference.

Equilibrium Fluorescence Measurements. Conventional measurements of fluorescence intensity at equilibrium were made with a Perkin-Elmer LS 50 fluorometer. Unless stated otherwise, FITC-modified protein (40–60 μg) was suspended in 2.5 mL of 6.6 mM Tris-HCl buffer at pH 7.4 and ambient temperature with continuous stirring. Samples were excited at 495 nm, and the emission was recorded at 520 nm. Both slit widths were 2.5 mm. Salts were added from concentrated

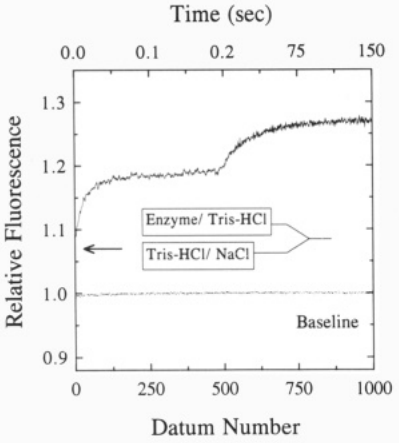

FIGURE 1: Time resolution of Na^+ reversal. The solid (upper) trace is a recording of the fluorescence changes that occur when 100 μL of 180 μg mL^{-1} FITC-modified Na,K-ATPase in 0.13 mM EDTA and 6.6 mM Tris-HCl adjusted to pH 7.4 at 15 °C is mixed with 100 μL of 200 mM NaCl in the same buffer. The design of the experiment is indicated by the schematic showing the drive syringes and the mixing chamber. The stippled (lower) trace was obtained at the same photomultiplier high voltage by mixing buffered enzyme with buffer. The first 500 data points were collected in 0.2 s, and the second 500 data points were collected in 150 s. The arrow indicates the estimated starting point of the faster exponential increase in fluorescence, which depends on $\Delta F_1 = 0.98$, k_1, and t_d as explained in the text.

stock solutions, e.g., 4 M NaCl, to minimize the volume correction.

RESULTS

Time Resolution of Fluorescence Changes. Figure 1 is a typical stopped-flow experiment in which relative fluorescence is plotted versus datum number along the lower horizontal axis. Two different collection times were used, so the time scales corresponding to the data points in each half of the experiment are shown on the upper horizontal axis. The stippled lower trace is the baseline after dilution obtained without changing any instrument settings by mixing FITC-modified Na,K-ATPase with buffer to give a final low ionic strength (μ) of 6 mM. The solid upper trace shows the fluorescence changes that occur when the labeled enzyme is mixed with NaCl to give a final Na^+ concentration of 100 mM and a final $\mu = 106$ mM. Two time-dependent responses are evident that could be satisfactorily fitted by the biexponential equation in the Experimental Procedures with empirical first-order rate constants $k_1 = 50.0 \pm 5.3$ s^{-1} for the faster effect and $k_2 = 0.034 \pm 0.004$ s^{-1} for the slower effect. A third effect can be inferred from the trace by calculating the relative fluorescence at the beginning of the faster reaction from the estimates of k_1, ΔF_1, and t_d. The faster exponential increase in fluorescence starts well above the dilution baseline (arrow). Therefore, an approximately 8% shift in the baseline must have occurred within the stopped-flow dead time. Assuming the reaction that causes the baseline shift is first-order, the empirical rate constant is estimated to be greater than 1500 s^{-1}. Therefore, at least three fluorescence enhancements with half-times in the (1) microsecond, (2) millisecond, and (3) second ranges can be observed in the stopped-flow when a low ionic strength suspension of membranes containing FITC-modified Na,K-ATPase is mixed with NaCl.

Characterization of Empirical Rate Constants. Experiments designed to identify each of the reactions reported by fluorescein modification of sodium pump are summarized in Figure 2.

(*1*) *Microsecond Half-Time.* The half-time of the fastest fluorescence change is less than 450 μs, suggesting that this change reports an essentially diffusion-controlled reaction. Several observations indicate that some fluorophore is not covalently bound to protein, and therefore the immeasurably fast fluorescence enhancement might result from the 100 mM ionic strength jump of free fluorophore that occurs within the instrument dead time. The evidence that some fluorophore remains free is as follows: First, dilution of our preparation of FITC-modified enzyme is followed by a slow drift in emission intensity, whose magnitude depends on the magnitude of the dilution and is therefore consistent with redistribution of unreacted fluorophore between the lipid and aqueous phases. Experimentally, we minimize this artifact by preincubating the membrane fragments in buffer in the dark as close to the final protein concentration as possible until the signal is stable. Second, fluorescence was detectable in the supernatant even after our FITC-labeled membranes were washed as many as six times by repeated resuspension and centrifugation. Centrifugation through a Sephadex G50 column results in significant loss of protein without completely eliminating the drift following dilution of the sample. Third, Abbott *et al.* (1991) concluded from gel electrophoresis experiments that even though modification of the antibody-accessible class of FITC sites is covalent, the reaction is slowly reversible. Finally, Xu (1989) reported that only 57% of the fluorescein bound under his labeling conditions was recovered in tryptic peptides.

To confirm that the microsecond half-time could result from an effect of the ionic strength jump on fluorophore that is not attached to protein, we reacted FITC with an excess of lysine and mixed the product with NaCl. Figure 2a shows that an approximately 8% increase in fluorescence from the dilution baseline occurs within the instrument dead time. There is no change in fluorescence on the millisecond time scale or on the second time scale. No displacement of the baseline was observed when 200 mM ChoCl was added to the FITC-lysine syringe, so that the ionic strength was high before mixing and was unchanged by mixing.

(*2*) *Millisecond Half-Time.* The half-time of the intermediate velocity reaction in Figure 1 is 14 ms. Since this effect is not observed with FITC-lysine, fluorescein must be reporting a reaction of protein-bound probe. This fluorescence enhancement also results from the ionic strength jump, because no enhancement was observed in the millisecond time range when 200 mM ChoCl was added to the enzyme syringe, so that the ionic strength was unchanged by mixing. Figure 2b is an experiment designed to test whether the millisecond half-time observed with fluorescein is reporting an effect of the ionic strength jump on the antibody-accessible class of FITC modification sites described by Abbott *et al.* (1991).

According to Abbott *et al.*, two classes of FITC-labeled sites can be differentiated by anti-fluorescein antibody. Fluorescence from fluorescein at "antibody-accessible" sites is quenched by anti-fluorescein antibody. Fluorescence from the other population of labeled sites was unaffected by anti-fluorescein antibody, so the second class of FITC sites was designated "antibody-inaccessible". Figure 2b shows that both the fluorescence increase with microsecond half-time and the fluorescence increase with millisecond half-time are eliminated by anti-fluorescein antibody. Since anti-fluorescein antibody also quenches the fluorescence of free FITC in solution (Watt & Voss, 1978), the absence of a baseline shift in Figure 2b supports the conclusion drawn from Figure 2a that the microsecond enhancement in Figure 1 reports the ionic strength jump of free fluorophore. From the absence of a reaction

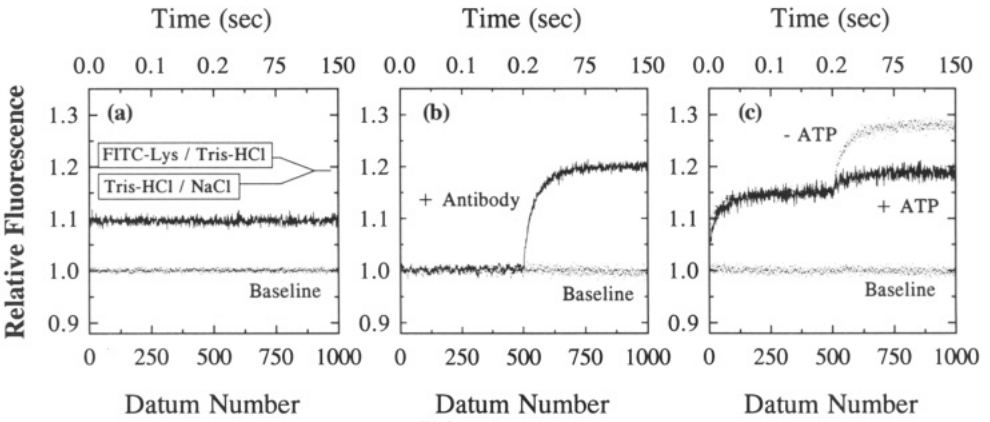

FIGURE 2: Characterization of empirical rate constants. This figure compares the fluorescence changes observed when NaCl is mixed with (a) FITC-lysine, (b) FITC-modified Na,K-ATPase in the presence of anti-FITC antibody, and (c) enzyme that was labeled with FITC in the presence of ATP. The stippled baselines were obtained by mixing each of the FITC-containing solutions with buffer. The protocols for obtaining the solid traces were as follows: (a) 0.16 μM FITC-lysine in 6.6 mM Tris-HCl at pH 7.4 was mixed with 200 mM NaCl in the same buffer. (b) After preincubation for 10 min at room temperature with excess anti-FITC antibody (18 units mL^{-1}), FITC-modified enzyme (80 μg mL^{-1}) in 6.6 mM Tris-HCl and 17.8 mM K^+ at pH 7.4 was mixed with 200 mM NaCl in the same buffer. (c) Na,K-ATPase-containing membranes reacted with FITC in the presence of ATP were suspended in 6.6 mM Tris-HCl at pH 7.4 and then mixed with 200 mM NaCl in the same buffer. The stippled upper trace was obtained by mixing NaCl with enzyme modified in the absence of ATP.

with intermediate time constant in Figure 2b, we draw the additional conclusion that the reaction in Figure 1 with millisecond half-time is an effect of the ionic strength jump on the emission intensity of fluorescein at antibody-accessible sites.

(*3*) *Second Half-Time.* Figure 2b also demonstrates that the reaction in Figure 1 with 20 s half-time is still observed when FITC-enzyme plus anti-fluorescein antibody is mixed with Na^+, so the reporter group must be fluorescein incorporated into antibody-inaccessible sites. This reaction is not caused by the ionic strength jump, because an enhancement with the same half-time is observed in stopped-flow experiments designed so that no change in ionic strength results from mixing (Faller *et al.*, 1991). Figure 2c is an experiment designed to test the hypothesis that the slowest fluorescence enhancement in Figure 1 reports the conformational change caused by Na^+ binding to Na,K-ATPase.

Abbott *et al.* (1991) were able to demonstrate two other differences between antibody-accessible and antibody-inaccessible sites. First, 5 mM ATP practically prevented FITC reaction with antibody-inaccessible sites, but preincubation with ATP did not noticeably affect the labeling of antibody-accessible sites. Second, the fluorescence intensity of membranes modified by FITC in the presence of ATP was unaffected by Na^+ or K^+, but addition of anti-fluorescein antibody approximately doubled the percentage change in fluorescence caused by Na^+ and K^+ in membranes modified by FITC in the absence of ATP. Therefore, the authors concluded that only FITC modification of antibody-inaccessible, ATP-protectable sites reports the conformational change between E_1 and E_2 forms of sodium pump.

Figure 2c compares the fluorescence changes observed in the stopped-flow when Na^+ is mixed with membranes reacted with FITC plus ATP (solid middle trace) and minus ATP (stippled upper trace). The baseline shift and faster exponential enhancement are essentially unaffected by the presence of 3 mM ATP during the modification reaction. However, the amplitude of the slower exponential enhancement is markedly reduced by the presence of ATP during the reaction of FITC with the enzyme. The similarity of the empirical first-order rate constants estimated for the slowest fluorescence increase when Na^+ is mixed with Na,K-ATPase labeled plus ATP (0.044 ± 0.006 s^{-1}) and minus ATP (0.034 ± 0.004 s^{-1}) is kinetic evidence in support of the conclusion drawn by Abbott *et al.* (1991) from equilibrium measurements that it is FITC modification of ATP-protectable, antibody-inaccessible sites that reports the conformational change between E_1 and E_2 caused by Na^+ and K^+.

Selectivity of Monovalent Cations. Grell *et al.* (1991) have reported that "unselective" ions, such as choline, can enhance the fluorescence of FITC-modified Na,K-ATPase. Therefore, we repeated the experiment of Figure 1 substituting ChoCl for NaCl.

(*a*) *Unselective Cations.* Somewhat surprisingly, all three of the reaction phases shown in Figure 1 could be reproduced by mixing ChoCl with FITC-modified enzyme suspended in low ionic strength Tris-HCl (not shown). There is a positive displacement of the baseline within the dead time of the instrument followed by two exponential enhancements with estimated empirical first-order rate constants $k_1 = 57.0 \pm 3.8$ s^{-1} and $k_2 = 0.076 \pm 0.010$ s^{-1} that are comparable to the values estimated for reversal by NaCl. The implication of this result is that ChoCl can cause the slow protein conformational change reported by fluorescein as well as the two faster and relatively nonspecific effects of an ionic strength jump on free fluorophore and fluorescein bound to antibody-accessible protein sites.

Grell *et al.* (1991) reported Na^+-like titrations by a number of monovalent and divalent cations of FITC-modified Na,K-ATPase dissolved in histidine hydrochloride buffer, which would be understandable if histidine caused a K^+-like fluorescence quench. Figure 3 confirms that histidine quenches the fluorescence of FITC-enzyme suspended in 20 mM Tris-HCl buffer. Details of the quench experiment are given in the figure caption. Figure 4 demonstrates that the enhancement observed when ChoCl is mixed with FITC-enzyme suspended in 10 mM histidine includes a slow reaction with a several second half-time. The estimated empirical first-order rate constants are $k_1 = 211 \pm 42$ s^{-1} and $k_2 = 0.134 \pm 0.005$ s^{-1}.

(*b*) *Selective Cations.* The experiments summarized in Figure 5 were designed to learn whether Na^+ and K^+ are selective compared to choline and histidine in their ability to cause the conformational change in Na,K-ATPase. In Figure 5a the fluorescence of FITC-modified enzyme that had been

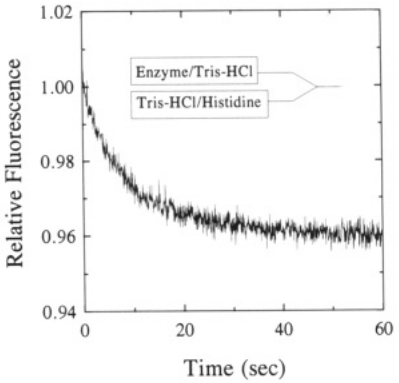

FIGURE 3: Histidine quench. A decrease in fluorescence is observed when 100 μL of 180 μg mL^{-1} FITC-enzyme in 0.13 mM EDTA and 20 mM Tris-HCl buffer adjusted to pH 7.4 at 15 °C is mixed with 100 μL of 200 mM histidine in the same buffer. Since the baseline was not determined in this experiment, the magnitude of the fluorescence quench is expressed relative to the initial fluorescence level. The estimated first-order rate constant for the change is 0.137 ± 0.008 s^{-1}, and the estimated amplitude of the quench is 3.7% ± 0.1%.

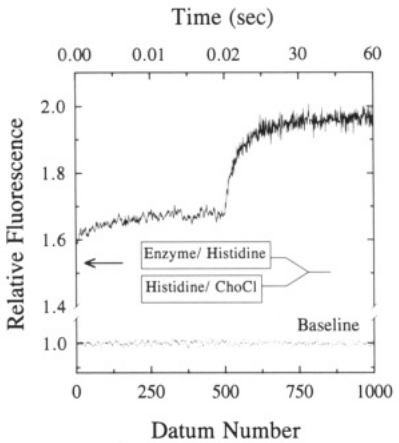

FIGURE 4: ChoCl reversal. The solid (upper) trace is a recording of the fluorescence changes that occur when 100 μL of 180 μg mL^{-1} FITC-modified Na,K-ATPase in 0.13 mM EDTA and 10 mM histidine adjusted to pH 7.4 with Tris base at 15 °C is mixed with 200 mM ChoCl in the same buffer. The first 500 data points were collected in 0.02 s, and the second 500 data points were collected in 60 s. The stippled (lower) trace was obtained at the same photomultiplier high voltage by mixing buffered enzyme with buffer. The arrow is the estimated relative fluorescence at the start of the reaction with 3.3-ms half-time. The amplitude of the reaction with 5.2-s half-time is 15.3% ± 0.3%.

quenched by 5 mM KCl was reversed with 100 mM NaCl. All three of the effects observed in the absence of K^+ are seen again. There is a positive displacement of the dilution baseline within the instrument dead time, followed by exponential enhancements with empirical first-order rate constants k_1 = 62.9 ± 7.6 s^{-1} and k_2 = 0.029 ± 0.001 s^{-1}. In sharp contrast, when labeled enzyme preincubated with 5 mM KCl was mixed with 200 mM ChoCl in Figure 5b, only the baseline shift and the faster of the two resolvable enhancements were observed. ChoCl is unable to cause the slowest enhancement, presumably by displacing K^+ from the enzyme and reversing the conformational change (Karlish, 1980). Conversely, in experiments that are not shown, 100 mM histidine was unable to quench the fluorescence of FITC-enzyme preincubated with 2 mM NaCl, but 100 mM KCl completely quenched the fluorescence of labeled enzyme incubated in 100 mM NaCl. These results suggest that the selectivity of Na^+ and K^+ compared to choline and histidine results from tighter binding of the ions that are transported physiologically.

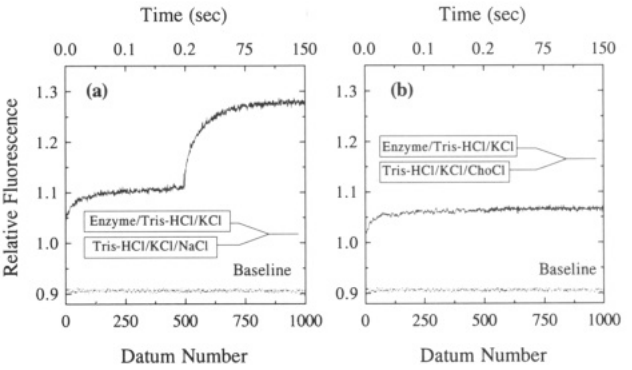

FIGURE 5: Selectivity of cation binding. This figure compares the fluorescence changes observed when FITC-modified Na,K-ATPase preincubated with K^+ is mixed with (a) NaCl and (b) ChoCl. The stippled baselines were obtained by mixing FITC-enzyme in K^+ with buffer plus K^+. The protocols for obtaining the solid (upper) traces were as follows: (a) FITC-enzyme in 6.6 mM Tris-HCl at pH 7.4 containing 5 mM KCl was mixed with 200-mM NaCl and 5 mM KCl in the same buffer. (b) 200 mM ChoCl was substituted for 200 mM NaCl in the titrant syringe.

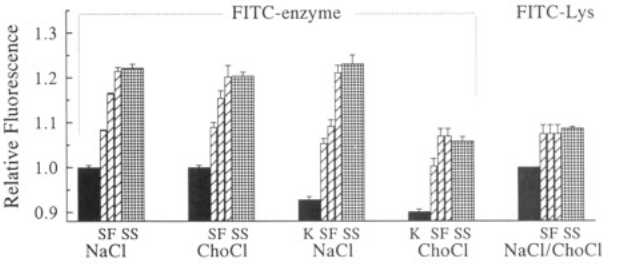

FIGURE 6: Comparison of equilibrium and kinetic experiments. A bar graph is used to compare fluorometric titrations of FITC-modified Na,K-ATPase in a stationary-state instrument with the results of the stopped-flow, "kinetic" titrations like those in Figures 1, 2a, and 5. The final experimental conditions in paired experiments were identical except for the FITC-enzyme concentration, which was lower by about half in the stationary-state experiments. The magnitude of the fluorescence change expressed as relative fluorescence is plotted on the vertical axis. That is, the fluorescence intensity after adding salt or mixing with buffer was divided by the fluorescence intensity of FITC-modified enzyme at the final concentration in 6.6 mM Tris-HCl buffer at pH 7.4 to permit quantitative comparison of the equilibrium and kinetic data. The solid bar is the baseline minus or plus K^+ (K). From left to right the fastest, intermediate and slowest fluorescence enhancements observed in stopped-flow (SF) experiments are represented by narrow diagonally hatched bars. The rectangularly cross-hatched bar is the result of the stationary-state (SS) control experiment. The set of bars on the right shows the result of mixing FITC-lysine with either NaCl or ChoCl.

Comparison of Equilibrium and Kinetic Fluorescence Changes. The magnitude of the fluorescence change observed in an equilibrium titration should be the resultant of the time-resolved amplitude changes seen in stopped-flow experiments. Therefore, measurements paralleling the stopped-flow experiments reported in Figures 1, 2a, and 5, except for the FITC-X concentration, were made in a conventional fluorometer in which only stationary-state measurements of reactions occurring in less than a minute or so are possible. Figure 6 is a bar graph summarizing the equilibrium measurements and comparing them to averaged kinetic results. To facilitate the comparison, fluorescence intensity divided by the fluorescence intensity of the same concentration of FITC-modified enzyme or lysine in low ionic strength Tris-HCl buffer is plotted. The filled bar is the baseline, that is, the relative fluorescence level of FITC-X minus K^+ or plus K^+ (K) before titration with NaCl or ChoCl. The narrow, diagonally hatched bars represent from left to right the amplitude of the enhancements observed in the stopped-flow

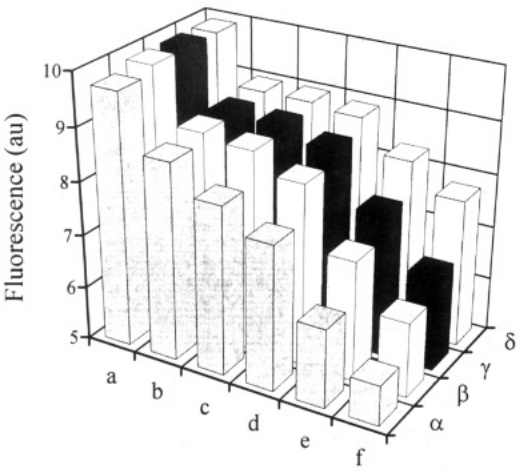

FIGURE 7: Distribution of enzyme between conformations. A three-dimensional bar graph summarizes stopped-flow "kinetic" titrations of FITC-enzyme plus or minus K^+ with 100 mM NaCl in which the ionic strength and/or concentration of Tris-HCl buffer was varied. Fluorescence in arbitrary units (au) is plotted along the vertical axis. The cumulative amplitudes of the baseline (α) plus enhancements with microsecond (β), millisecond (γ), and second (δ) half-times are plotted from front to rear along one horizontal axis. The other horizontal axis indicates the ion composition of the enzyme solution. Two hundred millimolar NaCl in the Tris-HCl buffer at pH 7.4 and 15 °C corresponding to a–f was mixed with 180 $\mu g\ mL^{-1}$ FITC-enzyme in the following solutions: (a) 50 mM Tris-HCl plus 200 mM ChoCl, (b) 50 mM Tris-HCl, (c) 20 mM Tris-HCl, (d) 10 mM Tris-HCl, (e) 6.6 mM Tris-HCl, and (f) 6.6 mM Tris-HCl plus 5 mM KCl. ChoCl was omitted from the titrant syringe in *a*, but K^+ was included in *f*.

(SF) following the reactions with microsecond, millisecond, and second half-times. The rectangularly cross-hatched bar is the magnitude of the fluorescence change measured in the stationary-state (SS) instrument. In every case, the resultant of the enhancements time-resolved by the stopped-flow instrument is equal within experimental error to the relative fluorescence change measured in an equilibrium titration.

Distribution of Enzyme between Conformations. The larger baseline shift and the larger amplitude of the slowest enhancement seen in the third set of bars from the left in Figure 6, when NaCl was reacted with FITC-enzyme preincubated with K^+, would be understandable if the enzyme as we prepare and label it with FITC is distributed between the conformations with high and low fluorescence quantum yield in low ionic strength buffer. To test this explanation, we mixed FITC-enzyme in solutions of varying buffer concentration and ion composition with 200 mM NaCl. The three-dimensional bar graph in Figure 7 summarizes our results. From left to right along one horizontal axis the ionic strength and/or Tris-HCl buffer concentration decreases. In f, K^+ was added. From front to back along the other horizontal axis the bars represent the cumulative sum of the baseline (α) plus the enhancements with microsecond (β), millisecond (γ), and second (δ) half-times. Fluorescence in arbitrary units is plotted along the vertical axis. The systematic changes in baseline fluorescence level and in magnitude of the three kinetic phases confirm that the distribution of FITC-enzyme between different conformers depends on ionic strength and on the concentrations of choline and protonated Tris, which may have Na^+-like effects (Skou & Esmann, 1980).

DISCUSSION

In their paper demonstrating two classes of modification sites that can be distinguished by anti-fluorescein antibody, Abbott *et al.* (1991) caution that the labeling of multiple sites by FITC "add(s) another level of complexity to the interpretations of previous studies". Grell *et al.* (1991) conclude that "unselective, electrostatic binding of numerous mono- and divalent cations including buffer ions...induces the $E_2 \rightarrow E_1$ conformational transition" in the summary of their paper on equilibrium titrations of FITC-modified Na,K-ATPase. Our results confirm the suspicions of these authors and prove that free fluorophore and fluorescein incorporated at sites other than lysine 501, which is protectable by ATP, may contribute to the fluorescence change observed in stationary-state titrations by demonstrating three distinct fluorescence changes in stopped-flow experiments that are on order-of-magnitude different time scales (Figure 1).

First, there is a baseline shift that we attribute to free fluorophore, because it is also observed with FITC-lysine (Figure 2a). Second, there is a protein-dependent reaction with millisecond half-time that is quenched by anti-fluorescein antibody and therefore reported by fluorescein at antibody-accessible sites (Figure 2b). The first two fluorescence increases can be eliminated by keeping ionic strength constant. Finally, there is a slower enhancement with a half-time of several seconds that is not caused by an ionic strength jump and is reported by fluorescein at antibody-inaccessible, ATP-protectable sites (Figure 2c). Abbott *et al.* (1991) concluded that only fluorescein at antibody-inaccessible, ATP-protectable sites reports the conformational change between E_1 and E_2 forms of the enzyme. Figure 2b,c provides kinetic confirmation that only fluorescein incorporated at antibody-inaccessible, ATP-protectable sites reports the conformational change implicated in transport.

Resolution of the fluorescence into three phases explains the wide variation in maximum percentage change that has been reported in the literature for titrations of FITC-enzyme and helps to explain why fluorescence changes are also observed in titrations with ions other than K^+ and Na^+. Figure 6 shows that the fluorescence change measured in an equilibrium titration is the resultant of the fluorescence increases seen in a stopped-flow experiment. This explains why fluorescence changes have been reported ranging from 10% to 15% in stopped-flow experiments designed so that only the slowest enhancement is observed (Faller *et al.*, 1991) to over 100% in equilibrium titrations of FITC-enzyme suspended in low ionic strength buffer where all three time-resolved effects are occurring (Grell *et al.*, 1991). In the latter type of experiment, two-thirds (Figure 4) or more of the observed fluorescence change may be reported by free fluorescein and/or fluorescein at antibody-accessible sites. The observation that these nonspecific effects are caused by an ionic strength jump with a variety of salts partly explains why the stability constants estimated from equilibrium titrations depended only on the charge of the ion (Grell *et al.*, 1991) and could be calculated from a theoretical equation for ion pair formation (Fuoss, 1958). Qualitatively, the different collection times needed to resolve the enhancement reported by fluorescein at antibody-accessible sites when FITC-enzyme was mixed with divalent cations (Smirnova & Faller, 1993a), or with monovalent cations in different buffers (Figures 1 and 4), can be explained by the complex dependence of the rate of ion pair formation on the concentration, mobility, and charge of the ions and on the stability of their coordination spheres (Eigen, 1959). We have not investigated the mechanism of the reaction reported by fluorescein bound to antibody-accessible sites quantitatively, because this reaction, as well as the baseline shift, is an artifact that can be avoided in studies of the conformational change either by equilibrium titrations in the presence of anti-

fluorescein antibody (Figure 2b) or by stopped-flow measurements designed so that the ionic strength is the same before and after mixing (Faller *et al.*, 1991).

Figures 3 and 4 demonstrate that ions other than K^+ and Na^+ can cause the conformational change reported by fluorescein at antibody-inaccessible, ATP-protectable sites. The long half-time (5 s) of the quench by histidine (Figure 3), compared to the half-times of quenches by K^+ (Figure 1, Smirnova & Faller, 1993b), and the somewhat larger empirical first-order rate constants measured when ChoCl was mixed with FITC-enzyme in Tris-HCl ($0.076 \pm 010\ s^{-1}$, not shown) or in histidine ($0.134 \pm 0.005\ s^{-1}$, Figure 4) than when the titrant was 200 mM NaCl ($0.034 \pm 004\ s^{-1}$, Figure 1), do not mean that histidine and choline cause a different conformational change than K^+ and Na^+, because the rate measured for the reaction $E_1X \rightleftharpoons E_2X$, where X is the ion that causes the conformational change, depends on the concentrations of X, its dissociation constant, and the numerical values of the forward and reverse rate constants for the conformational change with X bound (eq 4, Faller *et al.*, 1991).

Grell *et al.* (1992) have introduced a conformation F_1 characteristic of unselective cation binding, instead of the "selective Na^+ binding...implied so far in the literature by denoting this state as E_1", because their equilibrium titrations with Na^+ gave an estimate of the Na^+ dissociation constant approximately 2 orders of magnitude larger than published estimates obtained from direct binding measurements. However, we have previously shown that the apparent dissociation constant, or half-maximum concentration ($K_{0.5}$), expected in an equilibrium titration with a cation that shifts the conformational equilibrium toward E_1 depends on the $K_{0.5}$ and concentration of the cation that caused the conformational change to E_2 (eqs 5 and 6, Faller *et al.*, 1991). The actual constant for dissociation of Na^+ from the E_1 conformation to the enzyme (K_{Na}) can be obtained by extrapolating a plot of the empirical first-order rate constant estimated from Na^+ reversal experiments, which we have shown is a reciprocal relaxation time, versus $[Na^+]_0$ to infinite sodium concentration (eq 4, Faller *et al.*, 1991). Our estimate of $K_{Na} = 0.19$ mM is in excellent numerical agreement with the results of direct binding measurements, for example, 0.34 mM (Matsui & Homareda, 1982), and with the value (0.19 mM) estimated from a transport assay (Garay & Garrahan, 1973). Substituting the kinetic estimates of K_{Na}, the K^+ dissociation constant from E_1 (K_K), and the equilibrium constant between E_2K and E_1K conformations (K_c) at 22 °C into eqs 5 and 6 (Faller *et al.*, 1991) gave a value of $K_{0.5}$ for Na^+ (69 mM) that agrees well with the value estimated from measurements of the stopped-flow amplitude for Na^+ reversal of the K^+ quench as a function of Na^+ concentration (57 mM). The value of $K_{0.5}$ for Na^+ (12.6 mM) estimated by Grell *et al.* (1992) is not too different from our estimate, considering the different experimental conditions used in their measurements (10 mM imidazole hydrochloride buffer, no added K^+). Interpretation of the equilibrium titrations of Grell and coworkers is further complicated by the fact that ionic strength was not controlled. For example, an earlier titration of FITC-enzyme in 10 mM histidine hydrochloride (Grell *et al.*, 1991) was interpreted as evidence that 2 Na^+ ions bind with dissociation constants of 0.67 mM and 8.9 mM. Since the dissociation constant predicted by Grell *et al.* (1991) for ion pair formation (5 mM) and the $K_{0.5}$ expected for Na^+ binding coupled to the $E_1 \rightleftharpoons E_2$ conformational change (69 mM) differ only by a factor of about 10, an alternative possibility is that the broadening of equilibrium titration curves reported by Grell and co-workers resulted from superposition of ion pair formation, reported by free fluorescein and fluorescein at antibody-accessible sites, and binding coupled to the conformational change reported by fluorescein at antibody-accessible, ATP-protectable sites. What is clear from our studies is that failure to observe tight binding of Na^+ in an equilibrium titration with Na^+ does not mean that Na^+ does not bind tightly, because Na^+ binding is coupled to the equilibrium between E_1 and E_2 conformations. Conversely, K^+ binding appears to be tighter than it actually is because of coupling to the $E_1 \rightleftharpoons E_2$ conformational change (Smirnova & Faller, 1993b).

Comparison of panels a and b in Figure 5 shows that when FITC-enzyme is quenched with 5 mM K^+, the slowest reversal with a half-time of several seconds is not observed if 200 mM NaCl is replaced by 200 mM ChoCl as the titrant. Conversely, 100 mM histidine does not quench the fluorescence of FITC-enzyme preincubated with 2 mM Na^+, which is a saturating amount since we have seen in the preceding paragraph that K_{Na} is approximately 0.19 mM. On the other hand, in another experiment that is not shown, 100 mM K^+ completely quenched the fluorescence of FITC-enzyme preequilibrated with 100 mM Na^+. The simplest explanation of these results is that choline and Na^+ bind competitively and that histidine competes with K^+. The implication of this conclusion is that Na^+ and K^+ binding is selective compared to the binding of choline and histidine to the extent that the physiologically transported ions bind tighter.

The effect of unselective ions on the equilibrium between E_1 and E_2 forms of sodium pump demonstrated in this paper is not new. Skou and Esmann (1980) showed some time ago that Mg^{2+} ions and both choline and protonated Tris have Na^+-like effects on the intrinsic fluorescence of Na,K-ATPase and on eosin acting as a reversibly bound extrinsic fluorescent probe. Abbott *et al.* (1991) were the first to realize that the fluorescence signal from FITC-modified enzyme comes from fluorescein bound to two classes of sites. What we have done is time-resolve the fluorescence signals from fluorescein at the two classes of modified sites and show that the low affinity for Na^+ observed in equilibrium titrations is consistent with tight binding of Na^+ coupled to the $E_1 \rightleftharpoons E_2$ conformational change. The conclusion that ions like choline can also shift the equilibrium between E_1 and E_2 conformations of FITC-modified Na,K-ATPase means that equilibrium titrations cannot be easily corrected for the effects of ionic strength by subtracting a control in which FITC-enzyme is titrated with ChoCl (Rabon *et al.*, 1990).

This study demonstrates three ways in which artifacts can be avoided when studying the conformational change reported by FITC modification of Na,K-ATPase. First, equilibrium titrations can be performed in the presence of anti-fluorescein antibody (Figure 2b). The problem with this approach is that antibody bound to antibody-accessible sites may affect the affinity for monovalent cations of the sites that cause the conformational reported by fluorescein at antibody-inaccessible, ATP-protectable sites. Second, stopped-flow experiments can usually be designed so that there is no change in ionic strength and the only reaction observed is the conformational change of interest (Faller *et al.*, 1991). Finally, if an ionic strength jump cannot be avoided, time resolution of the observed fluorescence change by stopped-flow measurements can be used to isolate the enhancement reporting the conformational change. Figure 7 summarizes the fluoresence changes resolved by stopped-flow fluorometry when 200 mM

Na^+ was used to titrate FITC-enzyme as a function of Tris-HCl, ChoCl, and K^+ concentrations. A plot like Figure 7 can be used either to predict, or explain, the fluorescence change observed in an equilibrium titration. For example, the decrease in baseline fluorescence intensity with Tris-HCl concentration (from b,α to e,α) means that a different E_1Na conformation of the enzyme is not necessary to explain an enhancement when Na^+ is added to FITC-enzyme in 50 mM Tris-HCl (Hegyvary & Jorgensen, 1981). We have previously shown that a different conformation (MgE_2K) of metalloenzyme (Karlish, 1980; Hegyvary & Jorgensen, 1981) is not needed to explain the effect of Mg^{2+} on titrations of FITC-enzyme with K^+ and Na^+ (Smirnova & Faller, 1993a).

REFERENCES

Abbott, A. J., Amler, E., & Ball, W. J., Jr. (1991) *Biochemistry 30*, 1692–1701.

Eigen, M. (1959) in *Preceedings of The Robert A. Welch Foundation Conferences on Chemical Research III. Molecular Structure*, pp 161–189, The Robert A. Welch Foundation, Houston, TX.

Faller, L. D. (1990) *Biochemistry 29*, 3179–3186.

Faller, L. D., Diaz, R. A., Scheiner-Bobis, G., & Farley, R. A. (1991) *Biochemistry 30*, 3503–3510.

Farley, R. A., Tran, C. M., Carilli, C. T., Hawke, D., & Shively, J. E. (1984) *J. Biol. Chem. 259*, 9532–9535.

Fuoss, R. M. (1958) *J. Am. Chem. Soc. 80*, 5059–5061.

Garay, R. P., & Garrahan, P. J. (1973) *J. Physiol. 231*, 297–325.

Grell, E., Warmuth, R., Lewitzki, E., & Ruf, H. (1991) in *The Sodium Pump: Recent Developments* (Kaplan, J. H., & DeWeer, P., Eds.) pp 441–445, The Rockefeller University Press, New York.

Grell, E., Warmuth, R., Lewitzki, E., & Ruf, H. (1992) *Acta Physiol. Scand. 146*, 213–221.

Hegyvary, C., & Jorgensen, P. L. (1981) *J. Biol. Chem. 256*, 6296–6303.

Jorgensen, P. L. (1974) *Biochim. Biophys. Acta 356*, 36–52.

Jorgensen, P. L. (1975) *Biochim. Biophys. Acta 401*, 399–415.

Karlish, S. J. D. (1979) in *(Na,K) APTase Structure and Function* (Skou, J. C., & Norby, J., Eds.) pp 115–128, Academic Press, New York.

Karlish, S. J. D. (1980) *J. Bioenerg. Biomembr. 12*, 111–135.

Karlish, S. J. D., & Yates, D. W. (1978) *Biochim. Biophys. Acta 527*, 115–130.

Kyte, J. (1975) *J. Biol. Chem. 250*, 7443–7449.

Lin, S.-H., & Faller, L. D. (1993) *FASEB J. 7*, A1145.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Matsui, H., & Homareda, H. (1982) *J. Biochem. (Tokyo) 92*, 193–217.

Post, R. L., Kume, S., Tobin, T., Orcutt, B., & Sen, A. K. (1969) *J. Gen. Physiol. 54*, 306s–326s.

Rabon, E. C., Bassilian, S., Sachs, G., & Karlish, S. J. D. (1990) *J. Biol. Chem. 265*, 19594–19599.

Skou, J. C., & Esmann, M. (1980) *Biochim. Biophys. Acta 601*, 386–402.

Smirnova, I. N., & Faller, L. D. (1993a) *Biochemistry 32*, 5967–5977.

Smirnova, I. N., & Faller, L. D. (1993b) *J. Biol. Chem. 268*, 16120–16123.

Watt, R. M., & Voss, E. W., Jr. (1978) *Immunochemistry 15*, 875–882.

Xu, K. (1989) *Biochemistry 28*, 5764–5772.