Mechanism of the Conformational Change in Sodium Pump Reported by Eosin†

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ABSTRACT: The conformational change in sodium pump reported by eosin has been reinvestigated because of apparent discrepancies between results obtained in other laboratories with this reversibly-bound, fluorescent reporter group and results obtained in our laboratory with covalently bound fluorescein. The study demonstrates that both probes report the same reaction. Data obtained by following the conformational change in unphosphorylated enzyme with either fluorescein or eosin can be superimposed and fit by an equivalent site mechanism [Smirnova, I. N., & Faller, L. D. (1995) *Biochemistry 34*, 8657−8667] with the same numerical values of the rate constants for the conformational change and of the dissociation constants for the transported ions. The result is synthesis of data from several laboratories obtained with different probes over more than a decade into a simple and quantitative interpretation of Na^+ and K^+ interaction with the enzyme and regulation of a protein conformational change that explains the stoichiometry of transport. Therefore, the study supports both the hypothesis that conformational changes function in transport and our proposal that the ion binding sites in one conformation of the enzyme are identical and independent. A corollary to be tested in the future is that ions are transported by a concerted mechanism.

The working hypothesis in the sodium pump field is that the energy released when the enzyme catalyzes adenosine 5′-triphosphate (ATP)[1] hydrolysis is coupled to the physical translocation of Na^+ and K^+ by a sequence of protein conformational changes. The conformational hypothesis has been tested by studying changes in the fluorescence of intrinsic and extrinsic probes that are sensitive to protein environment. This investigation focuses on evidence for conformational changes in the second half of the catalysis−transport cycle when the enzyme is unphosphorylated.

In a seminal paper, Post *et al.* (1972) predicted from different rates of rephosphorylation depending upon the K^+ congener used to accelerate dephosphorylation that there is a rate-limiting conformational change in unphosphorylated enzyme which is accelerated by ATP binding. The existence of a slow conformational change was confirmed by measurements of the intrinsic fluorescence. The tryptophans of the protein report a reaction with a half-time of several seconds that is induced by Na^+ and K^+ binding and accelerated by ATP binding (Karlish & Yates, 1978). A conformational change with the same slow rate is reported by chemical modification of Na,K-ATPase with FITC (Karlish, 1980).

The change in fluorescence intensity of fluorescein covalently attached to lysine 501 (Farley *et al.*, 1984) is large enough to study precisely as a function of ion concentrations, and we have recently shown (Smirnova *et al.*, 1995) that the concentration dependence of the reported conformational change can explain the stoichiometry of Na^+ and K^+ countertransport (Glynn, 1984). The sigmoidal dependence of the first-order rate constant for the reaction on $[K^+]$ implies that two K^+ must be bound for the conformational change reported by fluorescein to occur (Smirnova & Faller, 1993b), and the sigmoidal dependence of the magnitude of the observed fluorescence change on $[Na^+]$ indicates that at least two sites must be filled to explain reversal of the conformational change by Na^+ (Faller *et al.*, 1994).

An unexpected result was that the estimated dissociation constants for both K^+ and Na^+ are consistent with competitive binding to identical and independent sites. Therefore, we have proposed an equivalent site mechanism for control of the conformational change reported by the covalently bound, extrinsic fluorophore (Smirnova *et al.*, 1995). The purpose of this paper is to test the equivalent site mechanism by investigating whether the algebraic equations derived for the functional dependence of the fluorescein reaction on $[Na^+]$ and $[K^+]$ can also describe the reaction reported by a noncovalently bound extrinsic fluorophore.

Eosin reports a conformational change in sodium pump indirectly by binding specifically with a change in fluorescence to only one conformation of the enzyme (Skou & Esmann, 1981). Eosin binds competitively with ATP (Skou & Esmann, 1981), and ATP protects against fluorescein incorporation into the protein by reaction with FITC (Farley *et al.*, 1984). Therefore, both fluorophores are presumed to interact with the enzyme's active site. Most mechanisms that are written for sodium pump assume only two conformations of the unphosphorylated enzyme designated E_1 and E_2, but there is experimental evidence both for and against equating the conformational changes reported by eosin and fluorescein.

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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; eosin, eosin Y (2′,4′,5′,7′-tetrabromofluorescein); FITC, fluorescein 5′-isothiocyanate (isomer 1); Tris ($Tris^+$), tris(hydroxymethyl)aminomethane; RH421, *N*-(4-sulfobutyl)-4-[4-[(*p*-dipentylamino)phenyl]-butadienyl]pyridinium, inner salt; ChoCl, choline (Cho^+) chloride.

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In support of concluding that both extrinsic probes report the same reaction, the conformational change reported by eosin occurs with the same slow rate as the reactions reported by tryptophan and fluorescein (Skou & Esmann, 1983b). There are also qualitative parallels between the conformational changes reported by eosin and fluorescein that indicate the two fluorophores report the same protein rearrangement. With both probes, the magnitude of the fluorescence change observed at equilibrium depends sigmoidally on titrant concentration when enzyme in K^+ (or histidine) is titrated with Na^+ (Skou & Esmann, 1983b; Faller *et al.*, 1994) and when enzyme in Na^+ is titrated with K^+ (Skou & Esmann, 1983b; Smirnova *et al.*, 1995). The first-order rate constant measured with both fluorophores increases when enzyme in the Na^+ conformation is mixed with increasing concentrations of K^+ (Skou & Esmann, 1983b; Smirnova *et al.*, 1995), but the sigmoidal dependence of the empirical rate constant on $[K^+]$ has been demonstrated only for the reaction reported by fluorescein (Smirnova & Faller, 1993b). Increasing $[Na^+]$ decreases the rate of the change from Na^+ to K^+ conformation reported by both fluorophores (Skou & Esmann, 1983b; Smirnova *et al.*, 1995). Conversely, increasing $[K^+]$ increases the rate of the change in the opposite direction from K^+ to Na^+ conformation reported by both probes (Skou & Esmann, 1983b; Smirnova *et al.*, 1995).

However, other experimental observations suggest that eosin and fluorescein report different conformational changes. Diametrically different conclusions have been drawn about the functional dependence of the empirical first-order rate constant for the change in the direction from K^+ to Na^+ conformation on $[Na^+]$. Direct dependence of the measured first-order rate constant on $[Na^+]$ has been reported with eosin (Skou & Esmann, 1983b; Esmann, 1994), whereas the observed first-order rate constant depends inversely on $[Na^+]$ for the reaction reported by fluorescein (Faller *et al.*, 1991a; Smirnova *et al.*, 1995). Another difference between the results obtained with the two probes is that biexponential or triexponential time curves are sometimes observed with eosin (Skou & Esmann, 1983b; Esmann & Skou, 1983; Van Uem *et al.*, 1991; Esmann, 1994), whereas monoexponential time curves are seen with fluorescein (Faller *et al.*, 1991b; Sminova & Faller, 1993a; Smirnova *et al.*, 1995).

In this paper, we report experiments designed to quantitatively compare the reactions reported by eosin and fluorescein. We show that eosin as well as fluorescein reports sigmoidal dependence of the observed first-order rate constant for the conformational change on $[K^+]$. We demonstrate inverse dependence of the empirical first-order rate constant for the conformational change on $[Na^+]$ when the reaction is followed with eosin as well as when the reaction is followed with fluorescein. We show that data obtained with the two fluorophores can be superimposed and fit by the equivalent site mechanism with the same estimates of the experimental parameters. We explain why multiexponential time curves may be observed with eosin. We conclude that eosin and fluorescein report the same conformational change in sodium pump and that studies with eosin also support the conformational hypothesis and the equivalent site mechanism for regulation of the conformational change in unphosphorylated Na,K-ATPase by Na^+ and K^+.

EXPERIMENTAL PROCEDURES

Materials

Na,K-ATPase. Membrane fragments containing Na,K-ATPase were isolated from pig kidneys and purified by the procedure previously described (Faller *et al.*, 1991a). The results obtained with eosin are compared with data from an earlier publication in which different enzyme preparations were chemically modified with FITC (Smirnova *et al.*, 1995).

Reagents. Fresh pig kidneys were a gift from Farmer John Clougherty Packing Co. (Los Angeles, CA). Eosin, histidine, Tris, and 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. The specifications of our stopped-flow instrument were reported in earlier publications (Faller *et al.*, 1991a; Smirnova & Faller, 1993a). Eosin was excited with 495 nm light, and all of the emitted light that passed through a 3 mm thick OG 530 long-band-pass optical filter was detected. The methods we use to collect data, estimate empirical first-order rate constants, and analyze their concentration dependence have all been explained in detail previously (Smirnova *et al.*, 1995). The design of individual experiments is described in the figure legends.

Mechanism. The chemical equation for the equivalent site mechanism, the assumptions implicit in the derivation of algebraic equations from the scheme, and the predictions of the mechanism have been discussed in detail elsewhere (Smirnova *et al.*, 1995). Therefore, only the algebraic equations needed to describe the experiments reported in this paper are repeated here. There are two measurable dependent variables: the first-order rate constant and the magnitude of the fluorescence change. For two identical and independent sites, the observed first-order rate constant (k_{obs}), or reciprocal relaxation time, is given by eq 1:

$$k_{obs} = \frac{1}{\tau} = k_f\left[\frac{[K^+]}{[K^+] + K_K(1 + [Na^+]/K_{Na})}\right]^2 + k_r \quad (1)$$

The relaxation time (τ) can be thought of practically as the time required for observation of approximately two-thirds of the fluorescence change. The independent variables in eq 1 are $[Na^+]$ and $[K^+]$. Following the convention of denoting the Na^+ conformation E_1 and the K^+ conformation E_2, the four experimentally determinable parameters are the rate constants for the conformational change in the E_1 to E_2 (k_f) and E_2 to E_1 (k_r) directions, the intrinsic (microscopic) Na^+ dissociation constant (K_{Na}), and the intrinsic K^+ dissociation constant (K_K). Equation 2 is the expression for the corrected (for instrument dead-time) magnitude of the observed fluorescence change (ΔF_o), if all of the enzyme is initially in the E_1 conformation:

$$-\Delta F_o = \left\{\frac{[K^+]^2}{[K^+]^2 + 2[K_K(app)/(1 + K_c)][K^+] + K_K(app)^2/(1 + K_c)} \times \frac{K_c}{1 + K_c}\right\}\Delta F_{max} \quad (2)$$

The apparent K^+ dissociation constant [K_K(app)] is given

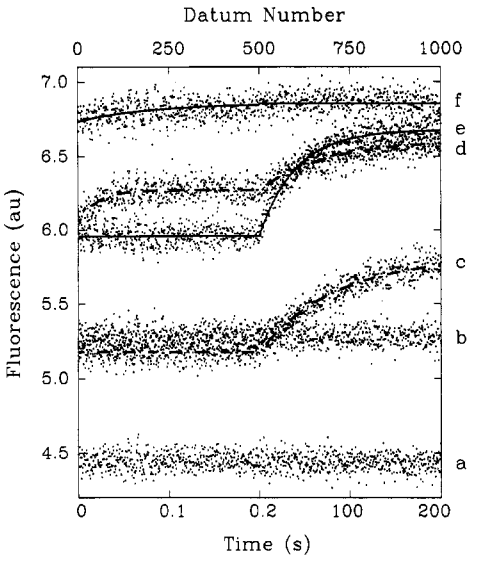

FIGURE 1: Reactions reported by eosin. Fluorescence intensity in arbitrary units (au) is plotted against datum number (upper axis), or time in seconds (lower axis). The first 500 points were collected in 0.2 s, and the second 500 points were collected in 200 s. The points are displayed as dots, and each trace is a single experiment. A constant high voltage of 630 V was applied to the photodetector, and the instrument time constant was set at 0.05 ms. Equal volumes (0.1 mL) from the enzyme and reagent syringes, both of which contained 0.1 mM KCl and 0.5 μM eosin in 10 mM histidine hydrochloride buffer at pH 7.4 and 15 °C, were mixed. The enzyme syringe also contained 0.10 mg mL^{-1} protein. The following additions were made to the solutions in the enzyme and/or reagent syringes in the individual experiments: (a) nothing; (b) 4 mM NaCl both solutions; (c) 50 mM NaCl reagent solution; (d) 4 mM NaCl enzyme solution and 50 mM NaCl reagent solution; (e) 50 mM ChoCl enzyme solution and 50 mM NaCl reagent solution; (f) 46 mM ChoCl + 4 mM NaCl enzyme solution and 50 mM NaCl reagent solution. The lines are fitted curves that are explained in the text. The parameters estimated from the experimental traces are summarized in Table 1.

by the second term in the denominator of eq 1. The difference in fluorescence intensity between E_1 and E_2 conformations is ΔF_{max}, and $K_c = k_f/k_r$. Percentage changes in fluorescence ($100\Delta F_o/F$), obtained by dividing both sides of eq 2 by the estimated final fluorescence intensity, are reported.

RESULTS

The interpretation of eosin results is complicated by the reversibility of eosin binding. Differences in experimental design could also help to account for discrepancies between results obtained with eosin and fluorescein. Therefore, before attempting to compare the eosin and fluorescein reactions, we investigated possible causes of the multiexponential time curves that have been observed in stopped-flow studies of sodium pump with eosin.

Multiple Exponentials. Complex kinetic curves from which more than one rate constant could be estimated have been observed when enzyme in histidine buffer and varying [K^+] was mixed with Na^+ (Esmann & Skou, 1983), when enzyme in 1 mM imidazole was mixed with a higher concentration of imidazole (Van Uem *et al.*, 1991), and when enzyme in Rb^+ and varying [Na^+] was mixed with a higher [Na^+] (Esmann, 1994). In all of these experiments, the ionic strength (μ) changed when the solutions in the enzyme and reagent syringes were mixed. Figure 1 compares experiments designed like those of Esmann (1994) with experiments in which ChoCl was added to the enzyme syringe to avoid a jump in μ. We also substituted K^+ for its congener Rb^+ in order to take advantage of the K^+ dissociation constants that have been estimated from studies with fluorescein (Smirnova *et al.*, 1995). All of the measurements shown in the figure were made without changing the amplification of the fluorescence signal, so that the fluorescence levels in different experiments can be directly compared. In order to observe two reactions with τ values differing by more than 3 orders of magnitude in a single experiment, half of the data points were collected in 0.2 s, and the other half of the data points were collected in 200 s. The lines are fits of the equation for a monoexponential or biexponential increase to the data. Table 1 summarizes the parameters estimated from the data in Figure 1.

Traces a and b (scatter plots without lines) in Figure 1 are base lines for the kinetic experiments. Trace a is the "K^+ baseline". In other words, it is the fluorescence intensity in arbitrary units (au) of eosin in the presence of the K^+ conformation of the enzyme, obtained by mixing equal volumes of identical (except for enzyme in one of the syringes) buffered solutions containing eosin and K^+ but no other added salts. A 19% increase in fluorescence intensity (Table 1) is observed when 4 mM Na^+ is added to the solutions in both syringes (trace b) that is primarily[2] attributable either to specific eosin binding only to the Na^+ conformation of the enzyme (Skou & Esmann, 1981) or to different fluorescence quantum yields when eosin is bound to the Na^+ and K^+ conformations. Therefore, trace b is the "Na^+ baseline".

Traces c and d (dashed lines) show experiments in which mixing the enzyme and reagent solutions changed μ. Adding 50 mM Na^+ only to the solution in the reagent syringe has two effects (trace c). There is a time-unresolved shift in the K^+ base line (trace a) to trace c (shorter time scale) followed by another fluorescence enhancement that occurs gradually on the longer time scale. The τ for the time-resolved fluorescence change in trace c (88 s) falls within the range of measured τ values for the conformational change reported by fluorescein (Smirnova *et al.*, 1995). Assuming the slow reaction reported by eosin is the conformational change between the E_1 and E_2 conformations of the enzyme, the fluorescence increase (17%) that occurs within the dead-time of the instrument (2.2 ± 0.7 ms) must be an effect of the jump in μ (25 mM) on the fluorescence of free eosin or eosin bound to the K^+ conformation. Three effects are observed when enzyme in 4 mM Na^+ is mixed with 50 mM Na^+ (trace d). The unresolved increase in fluorescence intensity from the K^+ base line (trace a) to trace d at $t = 0$ (37%) approximately equals the sum of the contributions of eosin binding/the conformational change before mixing (19%, trace b) and the 25 mM jump in μ (17%, trace c shorter time scale) to the observed fluorescence intensity. In other words, when the enzyme is preincubated in 4 mM Na^+, the base line is trace b, which is displaced to trace d ($t = 0$) within the dead-time of the instrument because of the jump

[2] There may also be contributions from specific effects of Na^+ on the fluorescence quantum yield of eosin bound to E_2 and/or E_1. We have observed disproportionate increases in the fluorescence intensity of bound eosin with Na^+ additions below 1 mM (not shown), and continued addition of Na^+ eventually decreases the fluorescence intensity (Skou & Esmann, 1983a).

Table 1: Summary of Parameter Estimates from Data in Figure 1

trace	[NaCl] (mM)[a] enzyme	[NaCl] (mM)[a] reagent	final[b] μ (mM)	F (au) at $t = 0$ ($n = 3$)[c]	$1/\tau$ (s^{-1}) fast ($n = 9$)	$1/\tau$ (s^{-1}) slow ($n = 3$)	$\Delta F_{max}/F$ (%) fast ($n = 9$)	$\Delta F_{max}/F$ (%) slow ($n = 3$)
a	0	0	0	4.43 ± 0.09				
b	4	4	4	5.28 ± 0.07				
c	0	50	25	5.17 ± 0.07		0.0113 ± 0.0001		11.0 ± 0.2
d	4	50	27	6.08 ± 0.11	37.0 ± 6.5	0.0126 ± 0.0021	3.9 ± 0.3	5.4 ± 0.3
e	0	50	50	5.97 ± 0.10		0.0238 ± 0.0004		10.6 ± 0.1
f	4	50	50	6.73 ± 0.08	12.8 ± 6.8		1.4 ± 0.5	

[a] Both syringes also contained KCl and eosin, and in e and f the enzyme syringe also contained ChoCl. The details of the design of each experiment are given in the legend of Figure 1. [b] After mixing from the added salts NaCl and ChoCl, since the contributions of KCl and buffer to μ are negligible. [c] n is the number of parameter estimates that were averaged.

in μ when the solutions in the enzyme and reagent syringes are mixed. The τ of the faster time-resolved enhancement in trace d (27 ms) corresponds to the measured τ ($1/k_{obs}$) = 20 ms for eosin binding under comparable conditions (Esmann, 1994), whereas the slower time-resolved enhancement occurs with a τ (79 s) that is characteristic of the protein conformational change.

Traces e and f (solid lines) show experiments designed so that μ does not change when the enzyme and reagent solutions are mixed. Enzyme in 50 mM Cho^+ was mixed with 50 mM Na^+ to obtain trace e. The base line when μ = 50 mM (trace e shorter time scale) is 35% above the K^+ base line (trace a), or approximately twice the increase in fluorescence caused by a 25 mM jump in μ (17%, trace c shorter time scale). Only one kinetic effect is observed: a slow increase in fluorescence on the same time scale as the conformational change reported by fluorescein. The amplitude (10.6%) of the time-resolved fluorescence enhancement in trace e is approximately the same as $\Delta F_{max}/F$ for the analogous experiment in which μ changed (trace c). The τ estimated from trace e (42 s) is shorter than the τ estimated from trace c (88 s), but the final μ is higher (50 mM in trace e compared with 25 mM in trace c). Enzyme in 4 mM Na^+ and 46 mM Cho^+ was mixed with 50 mM Na^+ in the experiment shown by trace f. The fluorescence intensity at $t = 0$ is 52% above the K^+ base line (trace a), or approximately the sum of the increases caused by eosin binding/protein conformational change before mixing (19%, trace b) and the effect of 50 mM μ on eosin fluorescence (35%, trace e shorter time scale). Comparing trace f to trace d for the analogous experiment in which enzyme was preincubated with 4 mM Na^+ but μ changed, a smaller fluorescence enhancement (1.4 ± 0.5%) with τ (78 ± 42 ms) corresponding roughly to the τ for eosin binding is still observed, but there is no evidence for a slower enhancement on the time scale of the conformational change in the protein.

Why there is still evidence for eosin binding, but no indication of a conformational change, when μ does not change (trace f) and why the conformational change is faster when μ is higher (trace e compared with the longer time scale in traces c and d) will be explained in the Discussion. The important conclusion from the observations in Figure 1 for the design of experiments that we have carried out to compare eosin with fluorescein results is that eosin, like fluorescein (Lin & Faller, 1993), reports at least three reactions. In Figure 1, the effects of (1) μ on the fluorescence quantum yield of eosin, (2) eosin binding, and (3) the conformational change are on different time scales. However, different reactant concentrations could result in poorly resolved rates, and explain the multiexponential time curves that have been reported.

Single Exponentials. The effects of μ and eosin reequilibration on the observed fluorescence intensity are potential artifacts in a study of the protein rearrangement that can be avoided by changing the design of experiments with eosin. First, a jump in μ can be eliminated from stopped-flow experiments by adding appropriate amounts of ChoCl to the solutions in the reagent and/or enzyme syringes. A total added salt concentration of 200 mM in each syringe was chosen to approximate μ in cells and to permit variation of [Na^+] and [K^+] over a wide range without changing μ. Second, choosing a high, near-physiological μ also minimizes any contribution of eosin binding to the observed fluorescence response because the amplitude of the faster enhancement is inversely related to μ (compare final μ and fast $\Delta F_{max}/F$ of traces d and f in Table 1). In addition, we have chosen to avoid the problems encountered following the conformational change from E_2 to E_1 with eosin (Figure 1) by starting each experiment in sufficient Na^+ and/or Cho^+ and $Tris^+$, which have "Na^+-like" effects on eosin fluorescence (Skou & Esmann, 1980) and affect the structural equilibrium (Lin & Faller, 1993), to shift the protein entirely into the E_1 conformation. When enzyme in Na^+ is mixed with increasingly higher K^+ concentrations, half-times shorter than the half-time for eosin dissociation are eventually observed (Skou & Esmann, 1983b). This result was interpreted by Skou and Esmann (1983b) to mean that eosin remains bound while the conformation changes but dissociates more rapidly from E_2 than from E_1. However, the alternative possibility that the fluorescence change is caused by different microenvironments of the probe in the E_1 and E_2 conformations, instead of tight eosin binding only when Na^+ is bound, cannot be excluded. In either case, eosin reports the conformational change.

The experiments illustrated by the traces in Figure 2 demonstrate that the conformational change can be studied kinetically without interference from the eosin binding reaction. The difference between Figure 2a and trace d in Figure 1 is that there is no fluorescence enhancement on the shorter time scale when enzyme covalently labeled with fluorescein and preincubated with 4 mM Na^+ is mixed with 50 mM Na^+ (Figure 2a), confirming that the shorter time-resolved τ in trace d resulted from eosin binding. Figure 2b shows that there is a rapid fluorescence quench with τ = 8.7 ± 0.6 ms when enzyme plus 0.1 μM eosin in 200 mM Cho^+ is mixed with 200 mM K^+. The rate constants for eosin binding to the Na^+ conformation of Na,K-ATPase [k_{on} = (19.5 ± 0.7) × 10^6 M^{-1} s^{-1}] and dissociation from the

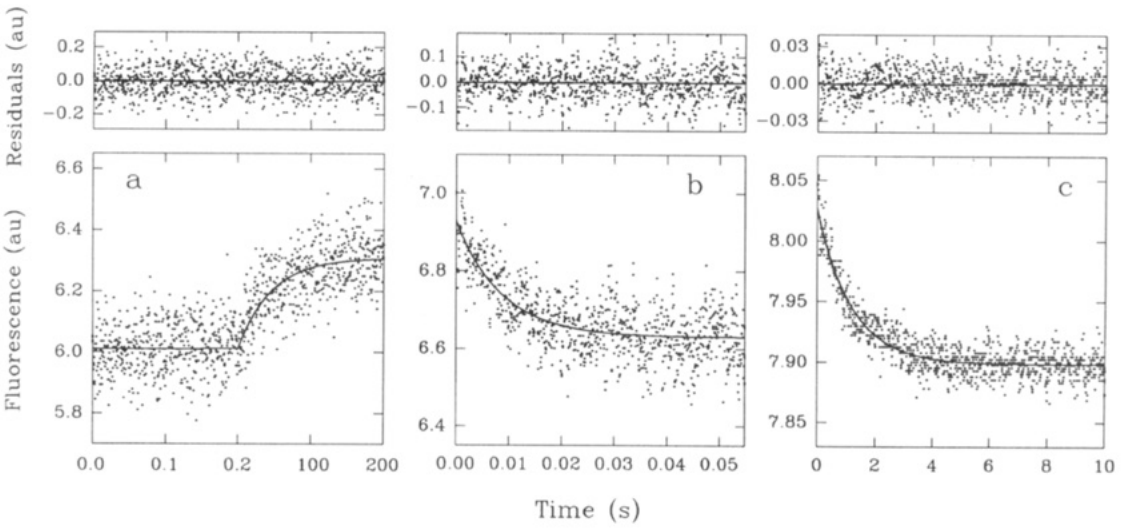

FIGURE 2: Monoexponential dependence of eosin fluorescence change on time. Fluorescence intensity is plotted against time in the lower graphs. The lines are fits of the equation for a single exponential to the data, and the upper graphs are plots of the residuals about the theoretical curves (lines). The measurements shown were made at 15 °C in buffer adjusted to pH 7.4 with HCl. (a) The experimental conditions were exactly the same as for trace d in Figure 1, except that 0.16 mg ml^{-1} FITC-labeled enzyme was substituted for unlabeled Na,K-ATPase in the enzyme syringe and eosin was omitted from the solutions in both syringes. A single trace is shown that was collected on the two time scales described in the legend of Figure 1. The parameter estimates are $1/\tau = 0.0219 \pm 0.0020$ s^{-1} and $\Delta F_{max}/F = 4.7 \pm 0.1\%$. (b) Protein (0.10 mg mL^{-1}) in 0.1 μM eosin, 200 mM ChoCl, and 50 mM Tris was mixed with an equal volume of 0.1 μM eosin and 200 mM KCl in the same buffer. The signal-to-noise ratio in this experiment is small, so the average of six individual traces is shown. The parameter estimates are $1/\tau = 115.0 \pm 7.5$ s^{-1} and $\Delta F_{max}/F = -5.9 \pm 0.9\%$. (c) The enzyme syringe contained 0.10 mg mL^{-1} protein in 50 mM Tris-HCl buffer containing 1 μM eosin, 5 mM NaCl, and 195 mM ChoCl. The same concentrations of eosin, buffer, and NaCl, 6 mM KCl, and 189 mM ChoCl were in the reagent syringe. A single trace is shown. The parameter estimates are $1/\tau = 0.834 \pm 0.023$ s^{-1} and $\Delta F_{max}/F = -1.61 \pm 0.03\%$.

enzyme ($k_{off} = 25.9 \pm 0.7$ s^{-1}) when $\mu \approx 250$ mM and $T =$ 15 °C were determined (data not shown) from the slope and ordinate intercept, respectively, of a plot of k_{obs} versus eosin concentration (Skou & Esmann, 1983b; Esmann, 1992, 1994). Therefore, τ for reequilibration of 0.1 μM eosin with the enzyme after mixing with K^+ in the experiment shown in Figure 2b is about 36 ms, or roughly 4 times the τ of the observed fluorescence change, confirming that eosin does not have to dissociate before the enzyme can change conformation. Since the fluorescence change reports the protein rearrangement, the data in Figure 2b also confirm that Na^+ is not required for eosin binding. Figure 2c demonstrates that the observed decrease in eosin fluorescence with time is monoexponential, if a stopped-flow experiment is designed so that $\mu \approx 250$ mM does not change and K^+ is mixed with the Na^+ conformation of sodium pump. The residual plot in the upper graph visually demonstrates that the data points scatter randomly about the theoretical line calculated with the parameters estimated by fitting the equation for a single exponential decay (solid line in lower graph) to the data points. A quantitative measure of the "goodness of fit" is the Durbin−Watson value (Draper & Smith, 1981), which is 1.52 for the fit shown in Figure 2c compared with the theoretical value for a single exponential of 1.60. The protocol of the experiment in Figure 2c was followed in the measurements comparing the $[Na^+]$ and $[K^+]$ dependence of the reactions reported by eosin and fluorescein that are described next.

K^+ Dependence of Reaction: $1/\tau$, $\Delta F_o = f([K^+])_{[Na^+]}$. The $[K^+]$ dependence of $1/\tau$ for the reaction reported by eosin (filled circles) is shown in Figure 3. The fixed $[Na^+]$ was 5 mM. To facilitate comparison with the reaction reported by fluorescein, data for an identical experiment with FITC-modified enzyme from Figure 2a in Smirnova *et al.* (1995) are also plotted (open circles). The reciprocal relaxation time

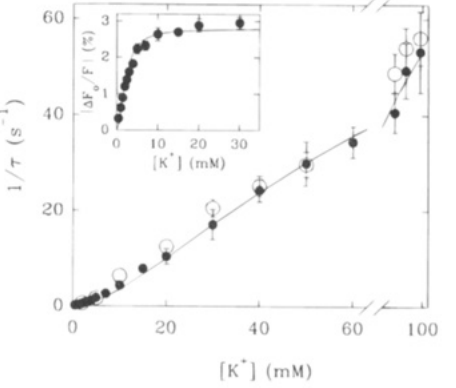

FIGURE 3: Comparison of K^+ titration curves reported by eosin (●) and fluorescein (○). Reciprocal relaxation time is plotted against final (after mixing) $[K^+]$ with the concentration axis broken to show the inflection point below 20 mM K^+ more clearly. The inset is the corresponding plot of the absolute value of the percentage change in fluorescence observed in the eosin experiments. The standard deviation in the estimates of the dependent variable from individual experimental traces ($n = 8-10$) is indicated by error bars when the uncertainty is bigger than the symbol size. The fluorescein data are taken from Figure 2 in Smirnova *et al.* (1995). The theoretical curves were drawn with eqs 1 and 2 (inset) and the parameter estimates quoted in the text. The [KCl] in the reagent syringe was twice the value shown on the abscissa, and ChoCl was added so that the total added salt concentration ([NaCl] + [KCl] + [ChoCl]) was 200 mM. Other details of the experimental design are given in the legend of Figure 2c.

for the conformational change reported by eosin also depends sigmoidally on $[K^+]$. The theoretical curve is a fit of eq 1 to the eosin data that gave parameter estimates $k_f = 118 \pm 6$ s^{-1} and $K_K = 3.6 \pm 0.2$ mM with $k_r = 0.1$ s^{-1} and $K_{Na} =$ 0.4 mM. The amplitudes measured in the eosin experiments are shown plotted against $[K^+]$ in the inset. The same parameter estimates that fit the $1/\tau$ data and the additional parameter $|\Delta F_{max}/F| = 2.8\%$ were used to draw the theoreti-

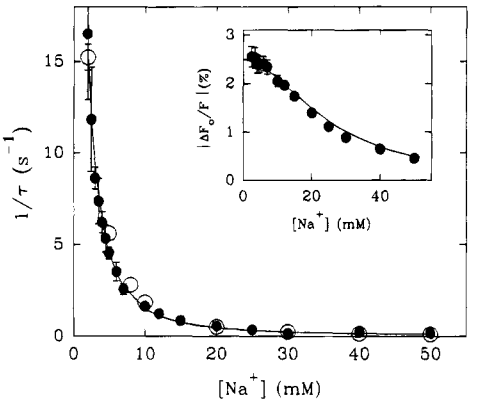

FIGURE 4: Comparison of Na^+ titration curves reported by eosin (●) and fluorescein (○). Reciprocal relaxation time is plotted against [Na^+]. The inset is the corresponding plot of the absolute value of the percentage change in fluorescence observed in the eosin experiments. The error bars indicate the standard deviations in the estimates of the dependent variable from individual experimental traces ($n = 8-10$). The theoretical curves were drawn with eqs 1 and 2 (inset) and the parameter estimates quoted in the text. The [NaCl] in both syringes was the same and varied from 2 to 50 mM. The reagent syringe contained 20 mM KCl. Other details of the experimental design are given in the legends of Figures 2c and 3.

cal curve in the inset with eq 2. The amplitudes corresponding to the fluorescein $1/\tau$ values are not shown because the fluorescence changes observed with fluorescein were significantly larger ($\Delta F_{max}/F \approx 12\%$) than the changes seen with eosin and could not be plotted on the same ordinate scale without introducing an additional normalization factor.

Na^+ Dependence of Reaction: $1/\tau$, $\Delta F_o = f([Na^+])_{[K^+]}$. Since stopped-flow studies of the conformational change in sodium pump can be analyzed as relaxation effects (Faller *et al.*, 1991b; Smirnova & Faller, 1993a), the same information about the [Na^+] dependence of $1/\tau$ and ΔF_o can be obtained either by mixing enzyme preincubated in fixed [K^+] with varying [Na^+] or by mixing fixed [K^+] with enzyme preincubated in varying [Na^+]. Choosing [Na^+] as the independent variable for experiments in which the fluorescence of eosin is quenched by mixing with fixed [K^+] (conversion of E_1 to E_2) avoids the uncertainties inherent in analysis of biexponential time curves (Figure 2c) and has the additional advantage of permitting variation of [Na^+] over a wider range (Smirnova *et al.*, 1995).

The [Na^+] dependence of $1/\tau$ for the reaction reported by eosin (filled circles) is shown in Figure 4. At every [Na^+], the fluorescence was quenched by 10 mM K^+ (after mixing). The titration was not extended below 2 mM Na^+ because the uncertainty in the estimate of $1/\tau$ becomes too great with eosin. Data for the same [Na^+] range from an identical experiment with FITC-modified enzyme reported in Figure 4a of Smirnova *et al.* (1995) are plotted on the same graph (open circles) for comparison. The theoretical curve is a fit of eq 1 to the eosin data that gave parameter estimates $K_{Na} = 0.41 \pm 0.01$ mM and $k_r = 0.10 \pm 0.13$ s^{-1} with $k_f = 150$ s^{-1} and $K_K = 3.6$ mM (Figure 3). In the inset, the amplitudes of the kinetic traces observed in the experiments with eosin are plotted versus [Na^+]. The fluorescence changes measured with fluorescein and shown in Figure 4b of Smirnova *et al.* (1995) were significantly larger than the changes seen with eosin, so the fluorescein amplitudes could not be plotted on the same ordinate scale without normalization. However, the reversed S-shape of the $\Delta F_o/F$ vs [Na^+] plot is the same with both reporter groups. The parameter estimates substituted into eq 2 to calculate the theoretical curve drawn through the eosin points in the inset were $k_f = 150$ s^{-1}, $K_K = 4.8$ mM, $K_{Na} = 0.32$ mM, $k_r = 0.1$ s^{-1}, and $|\Delta F_{max}/F| = 2.5\%$.

Requirements for Eosin Binding. The requirements for eosin binding with high affinity inferred from published studies of the interaction between eosin and sodium pump are (1) that the enzyme is in the E_1 conformation and (2) that more than one Na^+ is bound to E_1. The conclusion that eosin binds tighter to E_1 than to E_2 is based on evidence from observations like the rapid fluorescence quench in Figure 2b that eosin reports the conformational change from E_1 to E_2 in "K^+-quench" experiments. The conclusion that more than one Na^+ site must be filled for tight eosin binding is based on evidence from observations like the faster of the two fluorescence enhancements in trace d of Figure 1 that some eosin binds in "Na^+-jump" experiments before the enzyme conformation can change. We have tested the assumptions that are implicit in the proposed interpretations of Na^+-jump and K^+-quench experiments, and our results are summarized in Figure 5.

The published interpretation of Na^+-jump experiments assumes that rapid eosin binding is observed because of the [Na^+] perturbation (Esmann, 1994). However, μ and the enzyme concentration also change when the protocol used to obtain trace d in Figure 1 is followed. Eosin binding is not a consequence of the jump in μ because a fluorescence enhancement on the millisecond time scale is also observed when the experiment is redesigned so that μ does not change (Figure 1, trace f). Figure 5a shows an experiment designed so that mixing the enzyme and reagent solutions does not change either μ or [Na^+]. There is no evidence for a fluorescence enhancement on the slower time scale that corresponds to the conformational change because neither ion concentration changes. However, a fluorescence enhancement with the τ characteristic of eosin reequilibration is still observed. The only difference between the solutions that were mixed was the presence of enzyme in one of them. Therefore, the only possible explanation of the fluorescence enhancement with $\tau = 52$ ms in Figure 5a is that more eosin binds when the enzyme is diluted.

The published interpretation of K^+-quench experiments assumes that eosin reports the conformational change because the fluorophore dissociates more rapidly from E_2 than from E_1 (Skou & Esmann, 1983b). This interpretation cannot be tested directly by measuring the rate of eosin equilibration with E_2 because there is no fluorescence change when eosin is added to the K^+ conformation of the enzyme (Skou & Esmann, 1981). However, assuming eosin binding to E_2 is diffusion-controlled like eosin binding to E_1 ($k_{on} > 10^7$ M^{-1} s^{-1}), a faster off-rate implies lower affinity of the E_2 conformation for eosin. If eosin does bind significantly tighter to E_1 than to E_2 (or visa versa), then changing the eosin concentration should affect the magnitude of the faster fluorescence enhancement compared to the sum of the faster and slower fluorescence increases by shifting the equilibrium between the enzyme conformations. The fractional contribution of the faster fluorescence enhancement to the observed fluorescence change depends on the initial distribution of enzyme between conformations because ΔF_{max} for the faster change is a function of E_1 and ΔF_{max} for the slower change

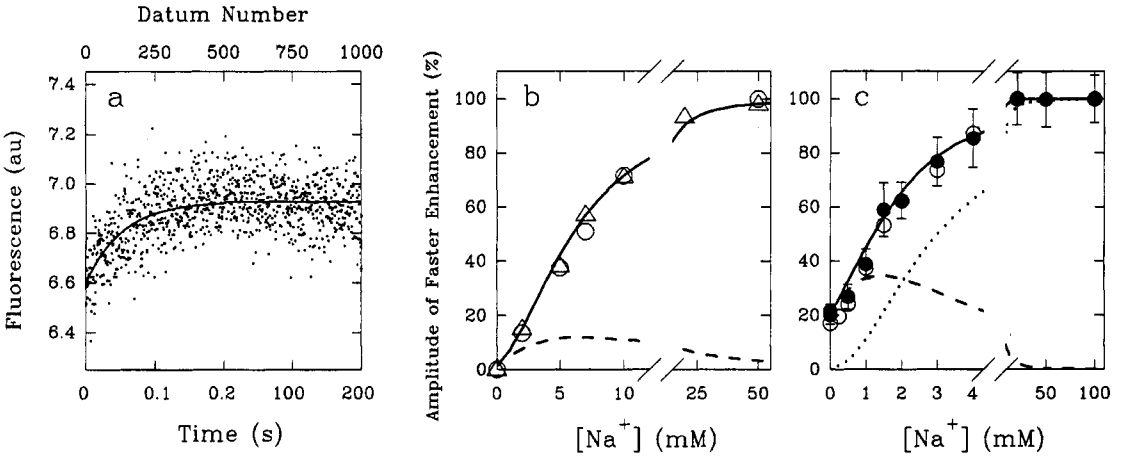

FIGURE 5: Requirements for eosin binding. (a) Fluorescence intensity in arbitrary units is plotted against time in seconds. Data were collected on two time scales as described in the legend of Figure 1. The enzyme syringe contained 0.10 mg mL^{-1} protein and 0.1 μM eosin in 0.1 mM KCl, 25 mM NaCl, and 10 mM histidine hydrochloride at pH 7.4 and 15 °C. The reagent syringe contained no enzyme, but otherwise the reagent solution was identical to the enzyme solution. Fitting the equation for a single exponential to the data (line) gave parameter estimates $1/\tau = 19.2 \pm 1.6\ s^{-1}$ and $\Delta F_{max}/F = 5.0 \pm 0.3\%$. (b) Amplitude of the faster time-resolved fluorescence enhancement, expressed as a percentage of the total fluorescence increase (faster plus slower change), is plotted against $[Na^+]$ in the enzyme syringe. Error bars are omitted for clarity. The solution in the enzyme syringe contained 0.10 mg mL^{-1} protein, 0.1 (○) or 0.5 (△) μM eosin, 1.1 mM KCl, the [NaCl] shown on the abscissa, and (200 − [NaCl]) mM ChoCl in 50 mM Tris-HCl at pH 7.4 and 15 °C. The reagent solution contained 200 mM NaCl in the same buffer with the same concentrations of eosin and KCl, but no enzyme. The theoretical curves were calculated for the two identical and independent sites mechanism assuming all E_1 forms bind eosin (solid line), or only E_1Na_2 binds eosin. See Discussion for details of the computation and parameter values. (c) The enzyme and reagent solutions contained 0.3 μM eosin and 0.3 mM KCl. Otherwise the enzyme solution was identical to the enzyme solution in panel b. In addition to the same buffer, the reagent solution either contained 100 mM NaCl and 100 mM ChoCl (●) or the $[Na^+]$ recorded on the abscissa and (200 − [NaCl]) mM ChoCl (○). The uncertainties in the estimates of the percentage of the total fluorescence change that occurs on the faster time scale have been calculated for the former data set and are shown by error bars. The theoretical curves were calculated for a three identical and independent site mechanism assuming all E_1 forms (solid line) bind eosin after mixing, or all E_1 forms except E_1KNa_2, E_1Na_2, and E_1Na_3 (dashed line) bind eosin in the Na^+-jump experiment and only E_1KNa_2, E_1Na_2, and E_1Na_3 (dotted line) reequilibrate with eosin in the enzyme dilution experiment. Details of the computation and parameter values are given in the text.

is proportional to E_2. Figure 5b shows that there is no perceptible difference between the relative amplitudes of the faster and slower fluorescence changes measured in Na^+-jump experiments when the eosin concentration is changed 5-fold.

Since eosin does not affect either the equilibrium between enzyme conformations (Figure 5b) or the parameter values estimated with the equivalent site mechanism (Figures 3 and 4), the published argument for high-affinity eosin binding only to E_1 with more than one Na^+ site filled can be checked by comparing experimental data points from Na^+-jump experiments like the measurements shown in Figure 5b with predicted values. The theoretical curves in Figure 5b were calculated with the two-equivalent-site mechanism for rapid eosin binding (after mixing) to all enzyme that was in the E_1 conformation before mixing (solid line) or only to E_1 that had fewer than two Na^+ bound before mixing (dashed line). Esmann (1994) did not observe the faster enhancement corresponding to eosin binding unless the enzyme was preincubated with Na^+ before the Na^+-jump. Figure 5c compares data from a Na^+-jump experiment (filled circles) and a control experiment in which mixing did not change $[Na^+]$ (open circles) that were purposely designed so that the enzyme was distributed between the E_1 and E_2 conformations before addition of Na^+. The theoretical lines are predictions of a three-equivalent-site mechanism for rapid, additional eosin binding after mixing to all, or only part, of E_1 present before mixing. The rationale for these comparisons and details of the computations are given under Discussion.

DISCUSSION

The chemical equations that have been written for the reaction reported by eosin (Skou & Esmann, 1983b; Esmann, 1994) are formally similar to the equivalent site mechanism we have proposed for ion binding and control of the conformational change reported by fluorescein (Smirnova *et al.*, 1995). However, algebraic equations were never derived for the eosin mechanisms, and many more than four parameters are proposed to explain the experimental observations. For example, in the earlier schemes enzyme forms with different numbers of K^+ bound are assumed to interconvert with different rate constants, and a site with lower affinity for K^+ than sites that become "occluded" is postulated to explain the observed increase in the rate of the E_2 to E_1 transition with $[K^+]$ (Skou & Esmann, 1983b). In the most recent mechanism proposed for eosin, Na^+ sites "other than those shown" in the chemical equation are postulated to explain an increase in k_{obs} with $[Na^+]$, and biexponental time curves when enzyme in Na^+ is mixed with more Na^+ are explained by postulating that eosin binds to E_1 with high affinity only when more than one Na^+ is bound (Esmann, 1994). The results obtained in this study using eosin as the reporter group are evidence for a single, unifying interpretation of direct studies of the conformational change in unphosphorylated sodium pump with fluorescent probes.

$1/\tau$ Depends Sigmoidally on $[K^+]$. Figure 3 demonstrates that $1/\tau$ for the reaction reported by eosin depends sigmoidally on $[K^+]$ as predicted by eq 1 derived from the equivalent site mechanism. The $[K^+]$ corresponding to half-maximum $\Delta F_o/F$ predicted with eq 8 in Smirnova *et al.* (1995) from the rate and dissociation constants used to fit

the kinetic data is 2 mM, in excellent agreement with the value observed experimentally (inset). The fixed [Na^+] used in this experiment is not high enough to visually demonstrate the sigmoidal dependence of $\Delta F_o/F$ on [K^+] predicted by eq 2, but an S-shaped K^+ titration curve with eosin in 30 mM Na^+ has already been published by Skou and Esmann (1983b).

$1/\tau$ Depends Inversely on [Na^+]. Figure 4 shows that $1/\tau$ for the reaction reported by eosin depends inversely on [Na^+] as predicted by eq 1. The magnitude of the fluorescence change (inset) depends sigmoidally on [Na^+] as predicted by eq 2 and previously demonstrated in studies with eosin (Skou & Esmann, 1983b). The empirical half-maximum [Na^+] for the amplitude titration is visually about 24 mM (inset), in approximate agreement with the value (43 mM) predicted by substituting the constants used to draw the theoretical $1/\tau$ curve into eq 9 in Smirnova *et al.* (1995) for the theoretical half-maximum [Na^+].

Eosin and Fluorescein Data Are Superimposable. Comparison of the closed and open circles in Figures 3 and 4 demonstrates that the values of $1/\tau$ measured as a function of [K^+] or [Na^+] by following the reaction with either eosin or fluorescein can be superimposed. The values of k_f (118 $\pm$ 6 s^{-1}) and K_K (3.6 $\pm$ 0.2 mM) estimated by fitting eq 1 to the $1/\tau$ versus [K^+] data in Figure 3 for eosin are in good agreement with published estimates (126 $\pm$ 3 s^{-1} and 3.4 $\pm$ 0.1 mM, respectively) from fits of fluorescein data, including the titration curve reproduced in the figure, with the same fixed values of K_{Na} and k_r (Smirnova *et al.*, 1995). Conversely, the estimates of K_{Na} (0.41 $\pm$ 0.01 mM) and k_r (0.1 $\pm$ 0.13 s^{-1}) obtained by fitting eq 1 to the $1/\tau$ versus [Na^+] data for eosin in Figure 4 with k_f and K_K fixed are in good agreement with published estimates of K_{Na} (0.47 $\pm$ 0.02 mM) and k_r (0.13 $\pm$ 0.03 s^{-1}) from fluorescein data. Therefore, eosin data are experimentally indistinguishable quantitatively as well as qualitatively from fluorescein data, justifying our use of averaged parameter estimates from more extensive studies of FITC-labeled enzyme to make predictions and interpret the experiments in Figures 1 and 5.

Explanation of Complex Time Curves. The importance of keeping μ constant and the protocol used to avoid a μ jump in mixing experiments were explained under Results. We inferred the existence of a base line displacement (Figure 1, trace a to trace c shorter time scale, for example) because of a jump in μ from a separate experiment in which NaCl was omitted from the reagent syringe (trace a). However, if mixing of solutions with different ionic strengths is incomplete, spurious signals may be observed, especially as τ of the reaction under investigation approaches the instrument dead-time. Examples of aberrations at the start of stopped-flow traces can be seen in some of the raw data curves that have been published for experiments with eosin in which μ was not controlled (Skou & Esmann, 1983a,b; Esmann & Skou, 1983).

The exponential enhancements on the shorter time scale in Figure 1 are real. Keeping μ constant reduces the magnitude of the effect (trace f compared with trace d), but does not eliminate the fluorescence increase on the 0−0.2 s time scale. Esmann (1994) attributed the enhancement with τ between 50 and 80 ms in his experiments to eosin binding because k_{obs} was proportional to eosin concentration when [Na^+] was fixed and fluorophore concentration was varied. Figure 2a confirms Esmann's interpretation by showing that no enhancement on the faster time scale is observed when the reporter group is covalently bound.

However, the [Na^+] perturbation is not necessarily the cause of the additional eosin binding observed in stopped-flow experiments designed like those of Esmann (1994). Figure 5a demonstrates that the faster fluorescence enhancement may also be observed in a mixing experiment designed so that there is no change in [Na^+]. The reason eosin binds when the enzyme is diluted is that the eosin concentration in the experiment shown is comparable to both the eosin dissociation constant and the enzyme concentration. Therefore, only a fraction of the eosin was bound initially, and more eosin binds because of the greater ratio of free eosin to eosin sites on the macromolecule after the enzyme is diluted. The amount of additional eosin binding can be estimated with the quadratic equation for ligand binding to a protein site (Faller, 1990). Assuming that the molecular weight of Na,K-ATPase is 1.47×10^5 and the eosin dissociation constant when μ = 25 mM is 0.26 μM (Esmann, 1994), an increase in the fraction of enzyme sites filled with eosin from 10.3% before mixing to 15.4% after halving the enzyme concentration is predicted for the experiment shown in Figure 5a. In experiments that are not shown, we confirmed that enzyme dilution is the cause of the fluorescence enhancement in Figure 5a by omitting eosin from the reagent syringe and experimentally demonstrating the predicted fluoresence quench when both enzyme and eosin concentrations are halved and by incrementing the eosin concentration in the reagent syringe until no fluorescence change was observed.

In general, both rate and equilibrium constants for both ligand binding and protein rearrangements depend upon μ. As a consequence, the degree of eosin binding to E_1 and/or the distribution of the enzyme between the E_1 and E_2 conformations differed at the beginning and/or at the end of the experiments reported in Figure 1. There is the additional complication that Na^+ may have unexplained specific effects on the fluorescence quantum yield of bound eosin.[2] Therefore, we will not attempt to quantitatively explain every nuance of the time curves in Figure 1. However, two observations have a common explanation that is germane to an understanding of apparent discrepancies between the reactions reported by fluorescein and eosin. The explanation of both the shorter τ of the slower fluorescence enhancement in trace e (42 s) than in traces c and d (~84 s) and the absence of a fluorescence change on the longer time scale in trace f is that both the rate constants for the conformational change and the dissociation constants for K^+ and Na^+ are affected by μ.

One effect of increasing μ is to shift the equilibrium between enzyme conformations toward E_1. A paper that tests the equivalent site mechanism by investigating whether μ affects the ratio of the constants for K^+ and Na^+ dissociation from two (or three) sites is in preparation. The estimates of k_f, K_K, K_{Na}, and k_r for μ = 20 mM and μ = 65 mM from that manuscript can be used to calculate the approximate fraction of the enzyme in the E_1 conformation before mixing in the experiments depicted in trace d (initial μ = 4 mM) and trace f (initial μ = 50 mM). Assuming two identical and independent sites, the quantity in braces in eq 2 is the fraction of the enzyme in the E_2 conformation (χ_2). Substituting the reactant concentrations before mixing and the parameter estimates for μ = 20 mM and μ = 65 mM into

the equation for $\chi_1 (1 - \chi_2)$ gives estimates of the percentage of the enzyme already in the E_1 conformation before mixing of 75% and 93%, respectively, for the experiments in traces d and f of Figure 1. Therefore, no evidence of a conformational change is seen in trace f because virtually all of the enzyme was already in the E_1 conformation before the $[Na^+]$ jump.

The second effect of increasing μ is to increase $1/\tau$ (k_{obs}). A 3-fold increase in $1/\tau$ when μ increases from 20 to 65 mM is predicted by substituting the parameter estimates for those μ values into eq 1, compared with the 2-fold increase recorded in Table 1 between $\mu = 25$ mM (trace c) and $\mu = 50$ mM (trace e). The direct dependence of k_{obs} on $[Na^+]$ observed by Esmann (1994) was for Na^+-jump experiments in which enzyme in a solution that included eosin, Rb^+, and buffer was mixed with an equal volume of the same solution containing from 50 to 900 mM NaCl without compensating for the difference in μ of the two solutions. The μ also changed in all of the mixing experiments in an earlier publication that claims direct dependence of k_{obs} on $[Na^+]$ for the conformational change reported by eosin (Skou & Esmann, 1983b). However, in two Na^+-jump experiments in which the increase in μ was constant because lower [NaCl] was compensated by ChoCl, k_{obs} either did not change or was inversely related to $[Na^+]$ as predicted by eq 1.

Requirements for Eosin Binding. Published studies of sodium pump with eosin have been interpreted to mean that eosin binds tightly only to the E_1 conformation of the enzyme and only when more than one Na^+ is bound. The experimental observations on which these conclusions are based and the assumptions underlying the published interpretations of the data were discussed under Results. Those assumptions were tested with the results shown in Figure 5, beginning with a demonstration (Figure 5a) that a $[Na^+]$ perturbation is not a necessary condition for observing eosin binding in a stopped-flow experiment.

There is evidence for different conformations of phosphorylated E_1 depending upon the number of Na^+ bound (e.g., Pratap & Robinson, 1993). Therefore, the conclusion that there are "several kinetically distinguishable E_1 states" (Esmann, 1994) is a potentially important corollary of the conclusion that eosin binds tighter to E_1 when more than one Na^+ site is filled. The argument in support of the proposal that eosin binds with high affinity only to E_1Na_3 and/or E_1Na_2 can be tested by comparing the relative amplitudes of the faster and slower fluorescence changes with calculated values, if eosin reports the conformational change in sodium pump because the microenvironment of the fluorophore is different in the two enzyme conformations. The alternative explanation that eosin dissociates faster from E_2 than from E_1, and by implication binds tighter to E_1, was tested by varying the eosin concentration. Figure 5b shows that changing the eosin concentration does not cause the change in the relative magnitudes of the faster and slower fluorescence enhancements that would be expected if eosin shifted the equilibrium between E_1 and E_2 by binding significantly tighter to one of the conformations. Therefore, parameters estimated from experiments in the absence of eosin can be substituted into algebraic equations derived assuming two or three Na^+ sites to calculate the distribution of the enzyme among the possible species in the equivalent site model.

To explain rapid eosin binding in Na^+-jump experiments when the enzyme is preincubated with Na^+, Esmann (1994) proposed that some forms of E_1 do not bind eosin tightly. Since Na^+ binding is diffusion-controlled, immediately after mixing with a higher $[Na^+]$ those forms are converted to the forms that do bind eosin with high affinity (E_1Na_3 and/or E_1Na_2), accounting for the fluorescence enhancement that precedes the conformational change. According to Esmann's interpretation, the fraction of the observed fluorescence change occurring on the faster time scale should be proportional to the fraction of the enzyme present before mixing with fewer than two Na^+ bound. The alternative possibility we propose is that after enzyme dilution eosin simply reequilibrates on the shorter time scale with all the enzyme present before mixing in the E_1 conformation, so that the relative amplitude of the faster enhancement should be proportional to the fraction of the enzyme present in any E_1 conformation $(1 - \chi_2)$. Assuming two Na^+ sites, the solid line in Figure 5b is the fit of the equation for χ_1 to the data with the constraints $k_f = 169\ s^{-1}$, $K_K = 4.5$ mM, and $k_r = 0.1\ s^{-1}$. The estimate of K_{Na} is 0.7 ± 0.5 mM. The same parameter values (including K_{Na}) were used to calculate the fraction of the enzyme in each of the other microscopic enzyme forms, and the dashed line is the fraction of the enzyme present as E_1K_2, E_1K, E_1, KE_1Na, or E_1Na before mixing. In this case, there is a peak that is not observed experimentally in the plot of percentage faster enhancement versus $[Na^+]$ because preincubating the enzyme with more Na^+ progressively converts any enzyme in the E_1 conformation before mixing to E_1Na_2 with eosin bound. Comparing the predictions of the two theories with the data in Figure 5b demonstrates that Esmann's interpretation of the faster fluorescence enhancement in Na^+-jump experiments is incorrect, but it does not prove that eosin binds tightly to E_1 conformations with fewer than two Na^+ bound because the large Na^+ perturbation saturates the enzyme with Na^+ within the stopped-flow dead-time.

Esmann (1994) did not observe any contribution of eosin binding to the fluorescence change seen in Na^+-jump experiments unless Na^+ was present in the enzyme syringe before mixing. However, Figure 5c shows that if a Na^+-jump experiment is designed so that the enzyme is initially distributed between conformations, then an enhancement on the faster time scale is observed even when $[Na^+] = 0$. Although there is no evidence from experiments with eosin for more than two Na^+ binding sites, three Na^+ sites have generally been assumed in the interpretation of eosin data because of the stoichiometry of Na^+ transport (Skou & Esmann, 1991). The solid line in Figure 5c is a plot of χ_1 versus $[Na^+]$, assuming three identical and independent Na^+ or K^+ sites and parameter estimates $k_f = 160\ s^{-1}$, $K_K = 10$ mM, $K_{Na} = 2$ mM, and $k_r = 0.1\ s^{-1}$. The ranges of estimates for these parameters found by fitting three-equivalent-site mechanisms to fluorescein data (Smirnova *et al.*, 1995) with k_r constrained ($0.1\ s^{-1}$) were $133 \leq k_f \leq 153\ s^{-1}$, $10 \leq K_K \leq 13$ mM, and $1.7 \leq K_{Na} \leq 2.0$ mM. The data for Na^+-jump experiments (filled circles) are consistent with eosin binding to the fraction of the enzyme in the E_1 conformation before mixing. On the other hand, the three-equivalent-site mechanism predicts a dependence of the relative fluorescence change occurring in the faster reaction on $[Na^+]$ that is not observed experimentally if eosin binds only to enzyme present as E_1K_3, E_1K_2, E_1K, E_1, E_1KNa, E_1K_2Na, or E_1Na

before mixing (dashed curve). Therefore, assuming three sites does not change the conclusion that eosin reequilibrates with all enzyme present in the E_1 conformation before the Na^+ perturbation.

The only difference between the experiments denoted by filled and open circles in Figure 5c is that in the latter there was no perturbation of [Na^+]. When there is no change in [Na^+], there is no slower fluorescence enhancement reporting the conformational change, so the amplitudes of the slower enhancement in the corresponding Na^+-jump experiments (filled circles) were used to express the observed fluorescence enhancements in the control experiments as fractions of the total fluorescence change that would be observed in Na^+-jump experiments. Superposition of the actual Na^+-jump (filled circles) and control (open circles, no jump) data is consistent with the conclusion that the faster fluorescence enhancement seen in Na^+-jump experiments results entirely from enzyme dilution. The dotted line is the calculated fraction of the enzyme present before and after mixing as E_1Na_2, E_1KNa_2, or E_1Na_3. Enzyme dilution does not affect the distribution of microscopic species because in these experiments [Na^+] and/or [K^+] is much higher than the enzyme concentration. The good agreement of the open circles with the theoretical curves for reequilibration with all E_1 conformers (solid line) and the poor agreement with the theoretical curve for reequilibration with the three species with two or three Na^+ bound (dotted line) further strengthen the conclusion that eosin reequilibrates with all E_1 conformers after enzyme dilution.

Caveats. The equivalent site mechanism contains four experimental parameters that appear together in the coefficients of the concentration terms, so that in general they cannot be estimated independently. Fortuitously, $k_f \gg k_r$ for the conformational change in sodium pump, and covalent incorporation of fluorescein into the protein reports the reaction in both directions over a wide range of Na^+ and K^+ concentrations as a monoexponential change in fluorescence intensity with high signal-to-noise ratio. Therefore, we were able to devise experiments that effectively isolated all of the parameters except K_{Na}, for which we could only obtain a semi-independent estimate (Smirnova *et al.*, 1995).

Eosin has severe experimental limitations as a probe of the conformational change in sodium pump by comparison. The underlying problem is that eosin binds reversibly, but this disadvantage is exacerbated by the high absorptivity of the probe and a dissociation rate that is not diffusion-controlled (Eigen, 1960). The high absorptivity makes it necessary to work at eosin concentrations that do not saturate the protein sites. The relatively slow off-rate restricts the number and type of stopped-flow experiments that can be run without interference from eosin reequilibration with the enzyme. The combined effect of these constraints is lower signal-to-noise ratios in many of the experiments reported in this paper than those with which we normally work.

The data and theoretical curves in Figures 3, 4, 5b, and 5c demonstrate that the equivalent site mechanism satisfactorily describes the reaction reported by eosin with the same parameter values that fit fluorescein data without postulating kinetically distinguishable E_1 species. An implicit assumption is that eosin does not affect the ion dissociation constants. In support of concluding that eosin does not affect the affinity of the enzyme for the transported ions, the estimates of K_K and K_{Na} from studies with FITC-modified enzyme are in agreement (Smirnova et al., 1995) with directly measured values for ion binding to unmodified enzyme (Yamaguchi & Tonomura, 1979), and fluorescein also interacts with the active site of sodium pump (Farley *et al.*, 1984). Finally, experiments in which the conformational change is followed with eosin cannot distinguish between two- and three-equivalent-site mechanisms, or rule out the possibility of anticooperative binding to a single site with dissociation constants coincidentally differing by a factor of 4, anymore than kinetic studies with FITC-modified enzyme could exclude these alternative interpretations of the data (Smirnova *et al.*, 1995).

Addendum. In a paper that appeared while this manuscript was being written, changes in fluorescence of the styryl dye RH421 when Na^+ and K^+ bind to sodium pump are interpreted as unambiguous evidence for three Na^+ sites, and the possibility of binding to identical and independent sites is ruled out (Schulz & Apell, 1995). These conclusions are based on quantitative arguments. An initial enhancement before the overall quench of RH421 fluorescence observed when the membrane fragment−dye complex was titrated with Na^+ permitted "unequivocal" determination of three fluorescence levels corresponding to enzyme with from one to three Na^+ bound, and the estimated ratios of the second to first macroscopic constant for dissociation of both Na^+ and K^+ were less than 2 compared with statistical ratios of 3 and 4, respectively, for binding to three or two equivalent, noninteracting sites. The problems with these arguments are that a "phenomenological correction factor" was applied to the K^+ data and the equations used to estimate the fluorescence intensities and ion dissociation constants are third-degree equations containing 7 (Na^+ titration) or 12 (K^+ titration with Na^+ present) parameters only 3 of which could be isolated and evaluated experimentally, so the numerical estimates the authors report are not unique.

For example, we obtained better[3] (lower residual sum of squares) five-parameter fits of their third-degree equation to the data in their Figure 3 with estimates of individual dissociation constants differing as much as 25-fold from the values reported in their Table 1. The parameter estimates depend on the initial guesses because all five of the undetermined parameters in the equation for the Na^+ titration are strongly interdependent (greater than 0.996 out of 1.000). The coefficients of the [Na^+] terms are products of a fluorescence level, and as many as three Na^+ dissociation constants divided by a third-degree polynomial in [Na^+] containing all of the Na^+ dissociation constants. We also obtained satisfactory fits with the relative values of the dissociation constants in the third-degree equation constrained to the ratios expected statistically for binding to three identical and independent sites and with a second-degree equation for Na^+ binding to only two sites.

Schulz and Apell (1995) support their conclusions by multiplying their estimate of the second K^+ dissociation constant, which in their scheme incorporates K_c, by a

[3] The data values were estimated from Figure 3 (Schultz & Apell, 1995) by enlarging the graph approximately 3-fold, measuring distances in millimeters, and converting to $\Delta F/F_o$ or [Na^+] with scale factors estimated from the original graph. Photographic distortion might explain better fits with parameter estimates differing from the published values, but should not influence relative fits of equations for two- and three-site mechanisms, for example, to the approximated data points with the nonlinear, least-squares algorithm used by SigmaPlot 5.1.

literature value of K_c (Heyse *et al.*, 1994), giving an estimated constant for dissociation of K^+ from E_1 close to twice the value reported by Karlish (1980) and a ratio of the second to first K^+ dissociation constants from E_1 (1500) consistent with the *ad hoc* assumption introduced by Karlish and Stein (1985) to rationalize hyperbolic K^+ binding curves with a K^+ stoichiometry of 2. However, we have shown that the half-maximum [K^+] observed in equilibrium titrations with K^+ in the presence of Na^+ is related to the true K^+ dissociation constant by a quadratic equation and that there is no experimental evidence for intrinsic K^+ dissociation constants differing in magnitude by several log orders (Smirnova *et al.*, 1995).

Schulz and Apell (1995) also cite evidence for ordered release of Rb^+ from sodium pump (Glynn *et al.*, 1985; Forbush, 1987) which they believe "excludes the idea of independent binding sites". However, we have pointed out that ordered release has been observed only from phosphorylated E_2 and does not preclude the possibility of identical and independent sites on the unphosphorylated enzyme (Smirnova *et al.*, 1995). The true K^+ dissociation constant from E_1 can only be estimated indirectly from kinetic measurements of the protein conformational change because K^+ binding is coupled to the equilibrium between E_1 and E_2. The conclusion drawn from following the reaction with both eosin and fluorescein is that Na^+ binds more than an order of magnitude tighter to E_1 than K^+. Consequently, there is no reason to reject the hypothesis that preferential binding of Na^+ to E_1 plays a role in determing the direction of physiological ion transport and normal enzymatic function of the sodium pump as an ATP hydrolase (Läuger, 1991).

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REFERENCES

Draper, N. R., & Smith, H. (1981) *Applied Regression Analysis*, pp 162−169, John Wiley & Sons, New York.

Eigen, M. (1960) *Z. Elektrochem. Ber. Bunsen-Ges. Phys. Chem. 64*, 115−123.

Esmann, M. (1992) *Biochim. Biophys. Acta 1110*, 20−28.

Esmann, M. (1994) *Biochemistry 33*, 8558−8565.

Esmann, M., & Skou, J. C. (1983) *Biochim. Biophys. Acta 748*, 413−417.

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

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

Faller, L. D., Scheiner-Bobis, G., & Farley, R. A. (1991b) in *The Sodium Pump: Recent Developments* (Kaplan, J. H., & De Weer, P., Eds.) pp 357−361, The Rockefeller University Press, New York.

Faller, L. D., Smirnova, I. N., Lin, S.-H., & Stengelin, M. (1994) in *The Sodium Pump: Structure, Mechanism, Hormonal Control and its Role in Disease* (Bamberg, E., & Schoner, W., Eds.) pp 583−604, Steinkopff, Darmstadt.

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

Forbush, B. (1987) *J. Biol. Chem. 262*, 11116−11127.

Glynn, I. M. (1984) in *Electrogenic Transport: Fundamental Principles and Physiological Implications* (Blaustein, M. P., & Lieberman, M., Eds.) pp 39−45, Raven Press, New York.

Glynn, I. M., Howland, J. L., & Richards, D. E. (1985) *J. Physiol. 368*, 453−469.

Heyse, S., Wuddel, I., Apell, H.-J., & Sturmer, W. (1994) *J. Gen. Physiol. 104*, 197−249.

Karlish, S. J. D. (1980) *J. Bioenerg. Biomembr. 12*, 111−136.

Karlish, S. J. D., & Yates, D. W. (1978) *Biochim. Biophys. Acta 525*, 252−264.

Karlish, S. J. D., & Stein, W. D. (1985) *J. Physiol. 359*, 119−149.

Läuger, P. (1991) *Electrogenic Ion Pumps*, pp 186−187, Sinauer Associates, Sunderland, MA.

Lin, S.-H., & Faller, L. D. (1993) *Biochemistry 32*, 13917−13924.

Post, R. L., Hegyvary, C., & Kume, S. (1972) *J. Biol. Chem. 247*, 6530−6540.

Pratap, P. R., & Robinson, J. D. (1993) *Biochim. Biophys. Acta 1151*, 89−98.

Schulz, S., & Apell, H.-J. (1995) *Eur. Biophys. J. 23*, 413−421.

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

Skou, J. C., & Esmann, M. (1981) *Biochim. Biophys. Acta 647*, 232−240.

Skou, J. C., & Esmann, M. (1983a) *Biochim. Biophys. Acta 727*, 101−107.

Skou, J. C., & Esmann, M. (1983b) *Biochim. Biophys. Acta 746*, 101−113.

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

Smirnova, I. N., & Faller, L. D. (1993b) *J. Biol. Chem. 256*, 16120−16123.

Smirnova, I. N., Lin, S.-H., & Faller, L. D. (1995) *Biochemistry 34*, 8657−8667.

Van Uem, T. J. F., Schuurmans Stekhoven, F. M. A. H., Borm, G. F., Theeuwes, A. G. M., Swarts, H. G. P., & De Pont, J. J. H. H. M. (1991) in *The Sodium Pump: Recent Developments* (Kaplan, J. H., & De Weer, P., Eds.) pp 369−373, The Rockefeller University Press, New York.

Yamaguchi, M., & Tonomura, Y. (1979) *J. Biochem. 86*, 509−523.

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