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The reaction sequence of the Na^+/K^+-ATPase: rapid kinetic measurements distinguish between alternative schemes

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Conformational changes between E_1 and E_2 enzyme forms of a dog kidney Na^+/K^+-ATPase preparation labeled with 5-iodoacetamidofluorescein were followed with a stopped-flow fluorimeter, in terms of the rate constant, k_{obs}, and the steady-state magnitude, $\%\Delta F$ of fluorescence change. On rapid mixing of enzyme plus Mg^{2+} plus Na^+ with saturating (0.5 mM) ATP in the absence of K^+, k_{obs} varied with Na^+ concentration in the range 0–155 mM, with a $K_{1/2}$ of 10 mM, while $\%\Delta F$ was relatively insensitive to Na^+, with a $K_{1/2}$ of 0.5 mM. Oligomycin reduced k_{obs} by 98–99% for $Na^+ \geq 10$ mM, but only by 50% for $Na^+ = 1$ mM; $\%\Delta F$ was reduced at most by 20%. At 155 mM Na^+, both k_{obs} and $\%\Delta F$ changed if K^+ was present with the enzyme. k_{obs} decreased by 50% when K^+ was increased from 0 to 0.2 mM, but increased when K^+ was varied in the range 0.2–5 mM. K^+ increased $\%\Delta F$ by a factor of 3 with a $K_{1/2}$ of 0.3–0.5 mM as measured in both stopped-flow and steady-state experiments. These data are considered in terms of the derived presteady-state equations for two alternate schemes for the enzyme, with the E_1P to E_2P conformational change either preceding (Albers-Post) or following (Nørby-Yoda-Skou) Na^+ transport and release. The analysis indicates that: (i) Na^+ must be released before the conformational transition, from an E_1 form; (ii) the step in which the second and/or third Na^+ is released is rate-limiting, but this release is accelerated by Na^+; and (iii) the release is also accelerated by K^+ acting with low affinity (possibly at extracellular sites).

Introduction

How the chemical and conformational changes in the Na^+/K^+-ATPase drive cation transport is a central problem in relating this enzyme to its function as the Na^+/K^+-pump. For twenty years, the standard model for the reaction sequence has been the Albers-Post scheme [1–3]. Albers and co-workers demonstrated ADP-sensitive (E_1P) and K^+-sensitive (E_2P) phosphoenzymes, and subsequently proposed a four-step sequence:

$$E_1 \rightarrow E_1P \rightarrow E_2P \rightarrow E_2 \rightarrow E_1$$

with Na^+-activated phosphorylation and K^+-activated hydrolysis [4,5]. Post and co-workers [6] confirmed the sensitivity of the two phosphoenzyme forms and demonstrated their chemical identity. Both [5,6] proposed that E_1 conformations had cation sites facing the cytoplasm, whereas E_2 conformations had cation sites facing the extracellular medium, and Post [6] added intermediate stages where the cations were occluded; thus transport was specifically associated with the conformational transitions. Subsequently, Post et al. [7] indicated a role for ATP at low affinity sites accelerating the K^+-transport process, and Karlish et al. [8] provided an explicit, detailed scheme in which cation transport was directly tied to the E_1P to E_2P and E_2 to E_1 conformational changes. Release of Na^+ from E_2P was portrayed as a single step, but could occur as sequential steps without conceptual alterations in the scheme.

Direct evidence for Na^+- and K^+-induced enzyme conformations came from alternative patterns of tryptic digestion [9] and from fluorescence changes in native and labeled enzyme [10,11], and these Na^+- and K^+-forms were equated to E_1 and E_2 conformations, respectively. Moreover, such clear distinction between

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Abbreviations: BIPM, *N*-[*p*-(2-benzimidazolyl)phenyl]maleimide; E_1 and E_2, two conformations of Na^+/K^+-ATPase; E_1P and E_2P, phosphorylated forms of E_1 and E_2; IAF, 5-iodoacetamidofluorescein; Na^+/K^+-ATPase, sodium plus potassium-dependent adenosine triphosphatase (EC 3.6.1.3).

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the two conformational families gave credence to schemes in which the E_1P to E_2P and E_2 to E_1 transitions played fundamental roles.

However, Nørby et al. [12], studying ADP- and K^+-activated dephosphorylation as a function of Na^+ concentration, showed that at least three successive pools of EP, designated A, B, and C, were required for quantitative description of their data. Pool A had cytoplasmically-accessible Na^+ sites, reacted directly with ADP, and also was susceptible to hydrolysis. Pool B had extracellularly-accessible Na^+ sites and was directly susceptible only to hydrolysis; it was classified as an E_1P conformation. Pool C also had extracellularly accessible Na^+ sites and was directly susceptible to hydrolysis, but it was classified as an E_2P conformation. Nørby et al. [12] explicitly specified that transport preceded the conformational change.

Yoda and Yoda [13–16] proposed a similar model with three phosphoenzymes:

E_1P, E^*P, and E_2P

but with the stipulation that E^*P would be dephosphorylated by both ADP- and K^+-activated processes; however, the ability of their formulation to account quantitatively for the data of Nørby et al. was not examined. Yoda and Yoda specified that one Na^+ was transported during the E_1P to E^*P step, that E^*P was an E_1 conformation, and that the remaining two Na^+ were transported during the E^*P to E_2P step(s), possibly preceding, accompanying, or following the actual conformational change. Thus, transport was at least partially dissociated from the conformational change. A central feature of their formulation was the sequential release of transported Na^+. Subsequently, Skou [17] presented a more detailed sequence in which transport was completely dissociated from the conformational changes, which were related instead to transitions between enzyme conformations bearing three cation sites (E_1) or two cation sites (E_2).

Among the differences between the Albers-Post and Nørby-Yoda-Skou schemes is whether the conformational change from E_1P to E_2P precedes or follows Na^+ transport, or even occurs after one but before all three Na^+ are transported. If the conformational change does not precede or accompany transport of all three Na^+, then its role in the reaction mechanism is different from that in the standard Albers-Post model, and thus the difference between the schemes is far more than a disagreement between definitions and nomenclature.

Here we seek to establish the step in the reaction sequence at which the conformational change from E_1P to E_2P occurs, whether before or after the release of transported Na^+. In these experiments, the transient-state kinetics of the conformational change are followed by stopped-flow fluorescence measurements with enzyme labeled with IAF; this fluorescent probe binds to a specific cysteine on the enzyme [18], has negligible effect on enzyme activity, and exhibits large fluorescence changes that correspond to the standard criteria for the conformational states [18–23]. The rate constant of fluorescence change as a function of Na^+ (in the presence and absence of oligomycin) and K^+ are then compared with the qualitative predictions that distinguish between the Albers-Post and the Nørby-Yoda-Skou schemes.

Materials and Methods

Frozen dog kidneys were obtained from Pel-Freez Biologicals (Rogers AR). Na^+/K^+ATPase was isolated from the outer medulla of the kidney by a modification of method C of Jørgensen [24]; specific activity was in the range of 10–25 μmol P_i (mg protein)$^{-1}$ min^{-1} at 37 °C (except for this assay, all experiments described in this paper were done at 24–25 °C). Enzyme was labelled with IAF as described earlier [19,23].

Steady-state fluorescence experiments on IAF-enzyme were performed on a Perkin-Elmer fluorescence spectrophotometer (model MPF-66; Perkin-Elmer Corp., Oak Brook, IL) under the control of a Perkin-Elmer computer (model 7500). Enzyme suspensions were excited at 490 nm, and fluorescence was measured at 520 nm.

Steady-state enzyme activity was measured with a fluorescence assay [25] which couples the production of P_i to the conversion, by purine nucleotide phosphorylase, of the fluorescent substrate 7-methylguanosine to the nonfluorescent product, 7-methylguanine.

Stopped-flow experiments were performed on a stopped-flow fluorimeter (Kinetic Instruments, Inc., Ann Arbor, MI) interfaced with a Macintosh IIcx personal computer (Apple Computer, Inc., Cupertino, CA) through a MacADIOS interface board (GW Instruments, Somerville, MA). Equal aliquots from two syringes were rapidly combined in a mixing chamber and then introduced into an observation chamber. Mixing times were not measured, but processes with rate constants greater than 100 s^{-1} could be monitored easily with this system. Light from a 75-watt xenon lamp driven by a regulated current power supply (Hamamatsu Corp., Middlesex, NJ) passed through a monochromator (ISA Instruments, SA, Inc., Metuchen, NJ), and was used to excite the sample ($\lambda_{ex} = 492$ nm). Fluorescence was monitored at 90° with a photomultiplier tube (R928, Hamamatsu). Scattered light was filtered with a cutoff filter (3-69, Corning, NY; wavelength at half-maximum = 529 nm). The photomultiplier tube output was fed through a current-voltage converter to the MacADIOS board. Data were collected and saved as text files. Multiple shots (typically 8

or 9) were averaged to yield mean fluorescence and standard error. These data were fitted by one or two exponentials with a nonlinear least-squares fitting program [26]. Fits yielded model parameters with estimates of standard errors.

Results

Steady-state kinetics of ATPase activity

For comparison with stopped-flow kinetic data, steady-state rates of Na^+-ATPase activity were measured at 25 °C with and without oligomycin, using unlabeled enzyme and a coupled assay system to measure P_i released (Table I). The steady-state Na^+-ATPase rate was 0.14 μmol P_i (mg protein)$^{-1}$ min^{-1}. This level of activity was 15-fold less than the Na^+/K^+-ATPase activity measured at 25 °C and about 100-fold less than at 37 °C. Oligomycin reduced the Na^+-ATPase activity by half and the Na^+/K^+-ATPase activity by over 90% (Table I).

Effect of Na^+ on conformational transitions

Na^+ is required for enzyme phosphorylation and for the conformational change between E_1 and E_2P. When IAF-enzyme in K^+-free buffer containing saturating Na^+ was mixed with saturating ATP, its fluorescence decreased (Fig. 1). The change in fluorescence corresponds to a transition from the E_1 conformation to the E_2P form in the steady state [20,21]. The time-course of this fluorescence change could be fitted with a single exponential (solid line in Fig. 1). The rate constant of this exponential (k_{obs}, 81 s^{-1} for this trace) represents the rate of formation of E_2 conformations, and the magnitude (%ΔF, 5.6 %) reflects the steady-state distribution between the E_1 and E_2 conformations. Both

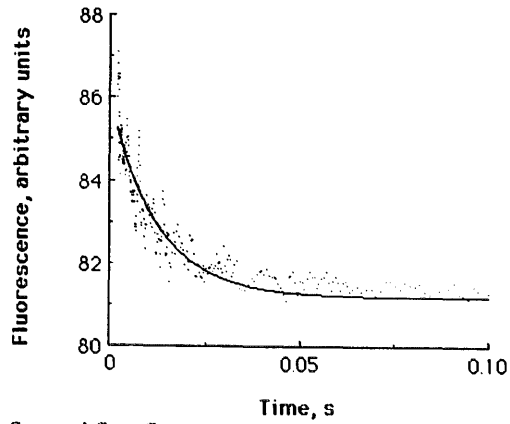


Fig. 1. Stopped-flow fluorescence response of IAF-enzyme to ATP. Syringe 1 contained enzyme in buffer (155 mM NaCl, 4 mM $MgCl_2$, 1 mM EDTA, 25 mM imidazole-HCl (pH 7.0), t = 24 °C). Syringe 2 contained 2 mM ATP in the same buffer. Approximately 90 μl from each syringe were mixed rapidly at time zero. Dots represent the measured fluorescence from 3 to 100 ms. The solid line is the nonlinear least-squares fit of the data to a single exponential, starting at 2 ms. The fit yielded a rate constant of 81 s^{-1} and a magnitude of 5.6 %ΔF.

k_{obs} and %ΔF were insensitive to the amount of enzyme used.

In order to examine the effect of Na^+ on the stopped-flow fluorescence change of the IAF-enzyme, the Na^+ concentration was varied at constant (saturating) ATP (0.5 mM) in two different concentration ranges: low (0.1 to 5 mM) and high (1 to 155 mM). In the high concentration range, Na^+ affected k_{obs} (Fig. 2A), but had only a marginal effect on %ΔF (Fig. 2B), while in the low range, increasing Na^+ increased both k_{obs} (Table II) and %ΔF (Fig. 3).

The variation of %ΔF at low Na^+ concentrations (Fig. 3) could not be fitted with a rectangular hyperbola, but only with the Hill equation. This fit yielded a $K_{1/2}$ of 0.5 mM, %ΔF_{max} of 4.6, and a Hill coefficient of 2.2.

Experiments were performed either under conditions where Na^+ concentration was varied reciprocally with choline to maintain ionic strength constant, or under conditions where Na^+ was varied alone. When ionic strength was maintained with choline chloride, k_{obs} increased monotonically with Na^+ concentration (Fig. 2A, open circles). This curve could not be fitted adequately by a rectangular hyperbola, but could be fitted by the Hill equation (not shown), with a Hill coefficient of 1.2; the maximal k_{obs} was about 80 s^{-1} and the $K_{1/2}$ for Na^+ was approx. 10 mM.

When ionic strength was not maintained constant, the time-course of the fluorescence changes was biphasic at Na^+ concentrations below 5 mM (Fig. 4). This response consisted of a rapid initial increase in fluorescence followed by a slower decrease. These data were fitted by a sum of two exponentials (solid line in Fig. 4;

TABLE I

Steady-state Na^+- and Na^+/K^+-ATPase activity ± oligomycin

Steady-state activity, expressed as percentage of Na^+-ATPase activity in the absence of oligomycin, was measured with unlabeled enzyme in 4 mM $MgCl_2$, 1 mM EDTA, 25 mM imidazole (pH 7.0 at 25 °C), 45 μM 7-methylguanosine and 0.1 units/ml purine nucleotide phosphorylase. Total enzyme was adjusted so that the purine nucleotide phosphorylase activity was not limiting. Total monovalent cation concentration was maintained at 155 mM with choline chloride. The effect of oligomycin (10 μg/ml) is also presented (in parenthesis) as the percentage of uninhibited enzyme activity under the same conditions.

[Cation] (mM)	Relative activity (%)	
	−oligomycin	+oligomycin
Na^+-ATPase		
155 Na^+	100	47 (47%)
5 Na^+	37	24 (65%)
Na^+/K^+-ATPase		
155 Na^+, 5 K^+	1500	125 (8.3%)

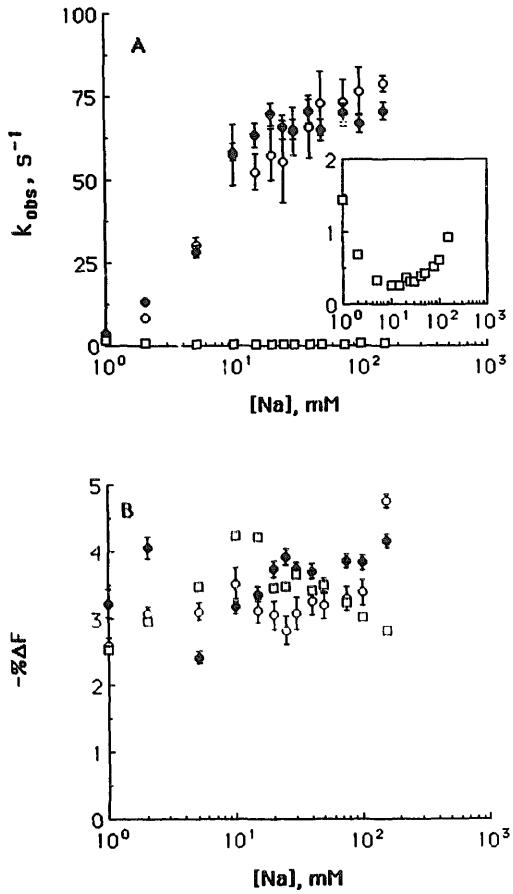


Fig. 2. Rate constant (A) and magnitude (B) of fluorescence change induced by rapid mixing of enzyme with ATP (0.5 mM final concentration) at different NaCl concentrations. Open circles represent experiments where the ionic strength was maintained with choline chloride. Filled circles represent experiments with no choline chloride. Open squares represent experiments with enzyme with 10 μg/ml oligomycin added just before loading the syringe. Buffers in both syringes were identical, with 4 mM $MgCl_2$, 1 mM EDTA, 25 mM imidazole-HCl (pH 7.0), $t = 24\,°C$. Inset: Expanded curve of k_{obs} as a function of Na^+ concentration in the presence of oligomycin. Experiments with oligomycin were performed in buffer where ionic strength was maintained with choline.

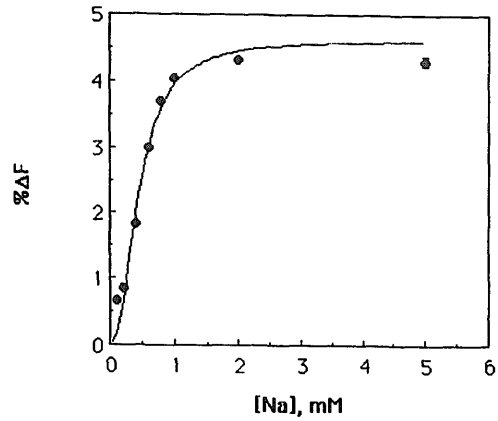


Fig. 3. Magnitude of stopped-flow fluorescence change vs. Na^+ at low concentrations of Na^+. Experimental conditions were the same as in Fig. 2. The solid line represents a fit of the data by the Hill equation: $\%\Delta F_{max} = 4.6$, $K_{1/2} = 0.46$ mM, n_H (Hill coefficient) = 2.2.

fit parameters in Table III). In the absence of choline chloride (when ionic strength was quite low) both the rate constant and magnitude of this rapid increase in fluorescence were 50 % greater with 2 mM Na^+ than with 0 Na^+.

On the other hand, k_{obs} and %ΔF for the slow decrease corresponded with k_{obs} for the fluorescence decrease in the presence of choline chloride at the same Na^+ concentration. $\%\Delta F$ and k_{obs} for this decrease (filled circles in Figs. 2A and 2B, respectively) were not affected appreciably by the absence of choline: for k_{obs}, $K_{1/2}$ for Na^+ was again about 10 mM, while $\%\Delta F$ varied little with Na^+ concentration; the Hill coefficient for k_{obs} was slightly higher (1.6).

Effects of oligomycin on conformational transitions

Oligomycin is an inhibitor of conformational change in the Na^+/K^+-ATPase [4,8,19]. In order to examine the effects of oligomycin on the variation of k_{obs} with Na^+, we measured k_{obs} and $\%\Delta F$ of enzyme incubated with 10 μg/ml oligomycin at different Na^+ concentrations. Preincubation with oligomycin reduced k_{obs} by 98–99% for Na^+ concentrations greater than 10 mM. However, at 1 mM Na^+, k_{obs} is reduced by only 50 % (open squares in the inset of Fig. 2A). In the presence of oligomycin, k_{obs} at 1 mM Na^+ was greater (by a factor of about 6) than k_{obs} at 10 mM Na^+. The $\%\Delta F$ (open squares, Fig. 2B) was approximately the

TABLE II

Effect of oligomycin on k_{obs} and $\%\Delta F$ for fluorescence change in low-Na^+ buffer

Enzyme in buffer with x mM NaCl ($x = 0.1$, 0.3, or 1), (155 − x) mM choline chloride, 4 mM $MgCl_2$, 1 mM EDTA, 25 mM imidazole-HCl (pH 7.0), was rapidly mixed with 1 mM ATP in the same buffer at 24 °C. The fluorescence as a function of time was fitted by a single exponential.

[Na] (mM)	−Oligomycin		+Oligomycin		Effect of oligomycin (% of control)	
	k_{obs} (s^{-1})	%ΔF	k_{obs} (s^{-1})	%ΔF	k_{obs}	%ΔF
0.1	1.0 ± 0.4	0.41 ± 0.06	1.6 ± 0.4	0.63 ± 0.05	162 ± 79	154 ± 26
0.3	0.8 ± 0.1	1.33 ± 0.04	0.9 ± 0.1	1.24 ± 0.04	112 ± 17	93 ± 4
1	3.0 ± 0.1	3.99 ± 0.03	1.7 ± 0.1	3.07 ± 0.04	57 ± 3	77 ± 1

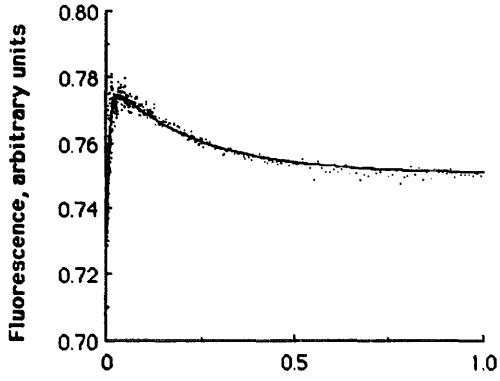


Fig. 4. ATP-induced stopped-flow response of enzyme in low-Na^+ buffer: 1 mM NaCl, 4 mM $MgCl_2$, 1 mM EDTA, 25 mM imidazole-HCl (pH 7.0), $t = 24\,^\circ C$. Final ATP concentration was 1 mM. Dots represent experimental points, while the solid line is a fit of two exponentials to the data. For the initial rise, $k_{obs} = 124\ s^{-1}$, $\%\Delta F = +7.3$; for the subsequent decrease, $k_{obs} = 4.2\ s^{-1}$, $\%\Delta F = -3.5$.

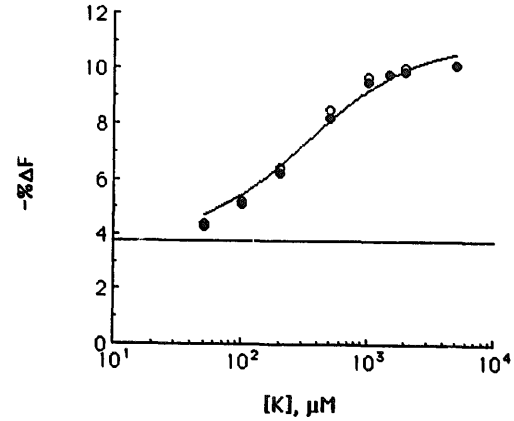


Fig. 5. Steady-state fluorescence change of IAF-enzyme with varying amounts of K^+. ATP (100 μM) was added to enzyme (20 μg/ml) in K^+-free buffer (155 mM NaCl, 4 mM $MgCl_2$, 1 mM EDTA, 25 mM imidazole-HCl (pH 7.0), $t = 25\,^\circ C$), and varying amounts of KCl were added after the fluorescence reached a steady level. The subsequent K^+-induced fluorescence change was plotted as a function of the K^+ concentration. The solid line is a nonlinear least-squares fit of the data by a rectangular hyperbola.

same whether or not the enzyme was treated with oligomycin.

Effects of K^+ on conformational transitions

In both schemes for this enzyme presented in the Introduction, K^+ interacts with E_2P to promote dephosphorylation and form the $E_2(2K^+)$ complex. The rate for the conformational change from $E_2(2K^+)$ to E_1 plus $2K^+$ is very slow, and is accelerated by the binding of ATP to a low-affinity binding site ($K_{1/2} \approx 0.2$ mM) [7,22,23,27].

To determine the steady-state fluorescence changes that occurred when K^+ interacted with E_2P, the enzyme was first mixed with Na^+ and 0.1 mM ATP. This concentration of ATP provided large responses, but was not expected to accelerate optimally the conformational change from $E_2(2K^+)$ to E_1. When a fluorescence plateau was attained, varying amounts of K^+ were then added, resulting in a further decrease in fluorescence. The magnitude of this K^+-induced steady-state fluorescence change varied as a function of KCl concentration (Fig. 5). These data were fitted by a rectangular hyperbola: the total maximal magnitude (sum of fluorescence changes induced by ATP and K^+) estimated from this fit was about 11 $\%\Delta F$, with a $K_{1/2}$ for K^+ of 325 μM.

Stopped-flow fluorescence changes were also measured under similar conditions. However, for technical reasons the IAF-enzyme could not be incubated in 100 μM ATP and then mixed with buffer containing various amounts of K^+ (as in the steady-state case) because significant amounts of the ATP would be consumed during the time required for the complete run (9 shots). Instead, IAF-enzyme was incubated in buffer

TABLE III

$\%\Delta F$ and k of fluorescence change in choline-free media at low Na^+

Enzyme in buffer with 0, 1 or 2 mM NaCl, 4 mM $MgCl_2$, 1 mM EDTA, 25 mM imidazole-HCl (pH 7.0), was rapidly mixed with 2 mM ATP in the same buffer at 24 °C. The fluorescence as a function of time was fitted by a sum of two exponentials.

$[Na^+]$ (mM)	k_{fast} (s^{-1})	$\%\Delta F_{fast}$	k_{slow} (s^{-1})	$\%\Delta F_{slow}$
0	83.0±2.4	5.44±0.09	–	–
1	109.2±8.1	7.20±0.26	3.79±0.30	−3.58±0.12
2	126.0±7.4	7.48±0.21	13.4 ±0.6	−4.78±0.19

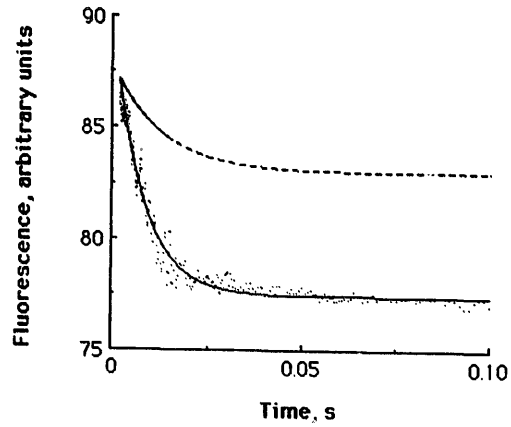


Fig. 6. Typical fluorescence response of enzyme in buffer with saturating Na^+ and K^+. Enzyme, in the same buffer as in Fig. 1 except that 5 mM Na^+ was replaced with an equal amount of K^+, was mixed with 200 μM ATP in the same buffer. Dots represent the measured fluorescence, the solid line represents a fit to a single exponential ($k_{obs} = 112\ s^{-1}$, $\%\Delta F = 13.9$). The dashed line is the fit to the fluorescence response of the enzyme in K^+-free buffer. This particular curve is the same as that shown in Fig. 1, but scaled so that the fitted values with or without K^+ are the same at 2 ms (the first data point shown).

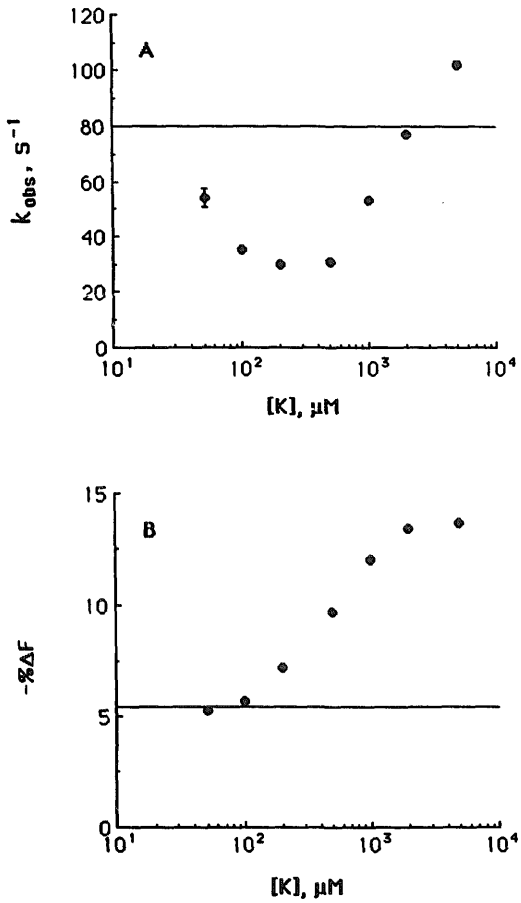


Fig. 7. Rate constant (A) and magnitude (B) of the ATP-induced stopped-flow fluorescence response of IAF-enzyme in buffers with varying K^+ concentrations. The solid lines represent these parameters in the absence of K^+. ATP in syringe 2 was 200 μM. Buffers contained 155 mM NaCl, 4 mM $MgCl_2$, 1 mM EDTA, 25 mM imidazole (pH 7.0), $t = 24\,°C$, and the concentrations of KCl indicated.

with varying amounts of K^+ and 155 mM Na^+ and rapidly mixed with ATP in the same buffer (final concentration 100 μM). For the fluorescence change at 5 mM K^+ (points and solid line in Fig. 6), $\%\Delta F$ was about three times that in the absence of K^+ (dashed line in Fig. 6), and k_{obs} was about 25% higher. Steady-state controls (not shown) indicated that the same $\%\Delta F$ is obtained if the enzyme is incubated in buffer containing 5 mM K^+ and then mixed with 0.1 mM ATP as when K^+ is added after ATP (as in Fig. 5).

The rate constant of the fluorescence change (k_{obs}) decreased between 0 and 0.2 mM K^+, and then increased as K^+ was increased (Fig. 7A). At 5 mM K^+, k_{obs} was about 25% higher than k_{obs} in the absence of K^+. The magnitude of fluorescence change under these conditions increased monotonically from approximately $5\%\Delta F$ in the absence of K^+ to about $15\%\Delta F$ at saturating (5 mM) K^+ (Fig. 7B). The half-maximal K^+ concentration was about 0.5 mM. Both the saturating fluorescence and $K_{1/2}$ are consistent with the results obtained under steady-state conditions (Fig. 5).

Discussion

The Albers-Post and Nørby-Yoda-Skou sequences are given here as Schemes I and II, respectively. In both schemes, the subscripts 'in' and 'out' represent Na^+ sites accessible from either the intracellular or the extracellular side of the membrane; enclosure of ions in parentheses implies occlusion. The k's represent pseudo-first order reaction rate constants; if a particular step involves ligands, the k for that step is the product of the actual rate constant and the concentrations of the ligands involved. The primed rate constants represent steps in the reaction sequence when K^+ is present. In this and subsequent discussions we will adopt the convention that the step represented by the rate constants k_i will be termed step i; for example, step 4 is represented by the rate constant k_4, and step 6′ is represented by the constant k_6'.

For each scheme we have calculated the overall rate constant for the system by letting each step in turn be rate-limiting. The resulting expressions are given in Table IV. These calculations assume that: (a) the reaction schemes do not contain branches; (b) fluorescence decreases represent transitions from the E_1 conformation to E_2, and increases represent transitions from E_2 to E_1 (an implication of this assumption is that fluorescence changes are not affected by steps which occur after the conformational change, and although the overall rate constant of the system is given in Table IV when these steps are rate-limiting, such rate constants will not be observed); (c) in the absence of K^+ the hydrolysis of E_2P is very slow compared to the other rates; (d) in the presence of K^+ and 0.1 mM ATP, the conformational change from $E_2(2K^+)$ to E_1 is very slow compared to other rates; (e) in the absence of ADP and P_i, steps 2 and 6 in Scheme I, and 2 and 7 in Scheme II are irreversible; and (f) since the $K_{1/2}$ for the dissociation of the first Na^+ is 0.4 M [15], step 4 is also irreversible.

The salient observations and considerations then are:

(i) The increase in the rate constant and magnitude of fluorescence decrease at low Na^+ concentrations (0.1 to 5 mM), in the absence or presence of choline chloride, is attributable to E_1P formation (step 1) as the rate-limiting step. The $K_{1/2}$ for Na^+ of 0.5 mM is comparable to the K_d for Na^+ binding to the enzyme (0.34 mM [28]) and the $K_{1/2}$ for the rate of enzyme phosphorylation (0.2 mM [29]). These observations are accommodated by both schemes.

(ii) The increase in k_{obs} at higher Na^+ (1 to 155 mM), on the other hand, specifically discriminates between the two schemes. For the fluorescence change to be sensitive to Na^+ requires that the Na^+-sensitive step precede or be identical to the step represented by the fluorescence change. The variation of k_{obs} with

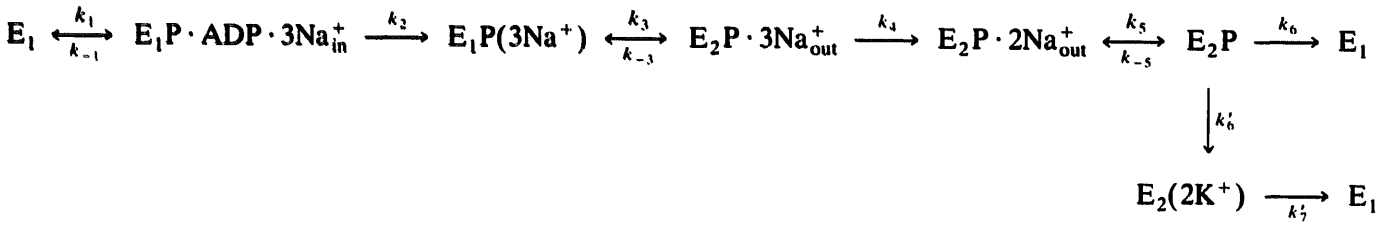
TABLE IV

Overall rate constant, calculated for Schemes I and II assuming different rate-limiting steps, expressed in terms of the pseudo-first-order rate constants of each step

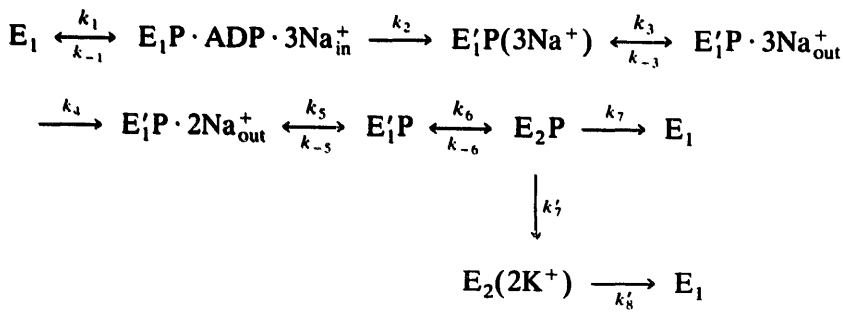
Rate-limiting step	Scheme I		Scheme II	
	$k_{\text{overall, }-K^+}$	$k_{\text{overall, }+K^+}$	$k_{\text{overall, }-K^+}$	$k_{\text{overall, }+K^+}$
1	k_1	k_1	k_1	k_1
2	$\frac{k_1k_2}{k_1+k_{-1}}$	$\frac{k_1k_2}{k_1+k_{-1}}$	$\frac{k_1k_2}{k_1+k_{-1}}$	$\frac{k_1k_2}{k_1+k_{-1}}$
3	k_3	k_3	k_3	k_3
4	$\frac{k_4k_3}{k_3+k_{-3}}$	$\frac{k_4k_3}{k_3+k_{-3}}$	$\frac{k_4k_3}{k_3+k_{-3}}$	$\frac{k_4k_3}{k_3+k_{-3}}$
5	k_5+k_{-5}	k_5	$k_5+\frac{k_{-5}k_{-6}}{k_6+k_{-6}}$	k_5
6,6'	-	$\frac{k_6'k_5}{k_5+k_{-5}}$	$\frac{k_6k_5}{k_5+k_{-5}}+k_{-6}$	$\frac{k_6'k_5}{k_5+k_{-5}}$
7'	-	-	-	$\frac{k_5k_6k_7'}{k_5(k_6+k_{-6})+k_{-5}k_{-6}}$

Na^+ concentration therefore supports Scheme II and is incompatible with Scheme I. The $K_{1/2}$ for this dependence (10 mM) is in accordance with the K_d for the release of the second and/or third Na^+ to the extracellular side. The Na^+-dependence of k_{obs} is attributable therefore to step 5 in Scheme II, implying that this step is rate-limiting. Then, k_{obs} (in the absence of K^+) is $k_5+k_{-5}k_{-6}/(k_6+k_{-6})$ (Table IV).

Note that k_{obs} increases by two orders of magnitude over the range of Na^+ used (1 to 155 mM); with this expression for k_{obs}, such variation can be accounted for by one of two possibilities: (a) only k_{-5} depends on Na^+, and $k_5 \ll k_{-5}$ at 155 mM Na^+, or (b) both k_5 and k_{-5} depend on Na^+. A still further implication of possibility (a) is that k_6 must be either comparable to or less than k_{-6}: if k_6 were much greater than k_{-6},



Scheme I.



Scheme II.

the second term in the expression for k_{obs} would be small, and the dependence on Na^+ concentration would be weakened.

Yoda and Yoda [13] specifically proposed that the transition from E*P to E_2P is rate-limiting; their E*P is equivalent to $E_1'P \cdot 2Na^+$ in Scheme II, and our data are therefore in accord with their analyses. This separation of transport steps from conformational changes is also in accord with proposals by Jencks and coworkers for the related Ca^{2+} ATPase from the sarcoplasmic reticulum [30,31].

The variation of k_{obs} with Na^+ supports Scheme II over Scheme I, but Scheme I could be modified to include low-affinity sites at step 3 that would act to accelerate the conformational/fluorescence change, and thus avoid the criticism in this section. Although this might seem to be an ad hoc solution, Karlish and Stein [32] demonstrated in steady-state experiments extracellular allosteric sites for Na^+ that modify Na^+-activated transport. However, they found that Na^+ at these sites increased $K_{1/2}$ without increasing V_{max}: that effect thus would not increase the velocity at higher Na^+ concentrations as needed to make Scheme I accommodate the data.

(iii) The magnitude of the fluorescence change ($\%\Delta F$), which reflects the steady-state levels of the E_1 and E_2 forms of the enzyme, is little affected by oligomycin or by changes in Na^+ concentration. The insensitivity to oligomycin is explainable by oligomycin acting before the rate-limiting step. The insensitivity to Na^+, however, presents difficulties for both Schemes I and II. For Scheme II, $\%\Delta F$ (in the absence of Na^+) can be calculated under the assumptions that (a) step 4 is fast and irreversible; and (b) step 6 is much slower than all other steps in the reaction sequence. Thus, under steady-state conditions, all the enzyme is in one of the forms $E_1'P \cdot 2Na^+$, $E_1'P$, or E_2P. Then,

$$\%\Delta F = \frac{(\phi_2 - \phi_1)/\phi_1}{1 + (k_{-6}/k_6)[1 + (k_{-5}/k_5)]} \qquad (1)$$

where ϕ_1 and ϕ_2 are the fluorescence quantum yields of the E_1 and E_2 families of conformations, respectively. Note that Eqn. 1 is a function of the *ratios* of rate constants, whereas the expressions for k_{obs} (Table IV) are functions of *sums* of rate constants. Of the rate constants involved, k_6 and k_{-6} describe the conformational change, and are not dependent on Na^+. If only k_{-5} depends on Na^+, then from possibility (a) presented in (ii) above, $k_{-5} \gg k_5$ (and $k_{-5}/k_5 \gg 1$), and Eqn. 1 will reduce to

$$\%\Delta F \approx \frac{(\phi_2 - \phi_1)/\phi_1}{k_{-5}k_{-6}/k_5k_6} \qquad (2)$$

Eqn. 2 implies that $\%\Delta F$ should decrease with increasing Na^+. If $\%\Delta F$ is to be largely independent of Na^+ concentrations above 1 mM, both k_5 and k_{-5} must respond similarly to changes in Na^+ concentrations above 1 mM. The insensitivity of $\%\Delta F$ to Na^+ concentration thus supports possibility (b) described in (ii): both k_5 and k_{-5} vary similarly with Na^+ concentration.

Such dependence of k_5 on Na^+ could be due to an allosteric interaction of Na^+ with the enzyme. However, k_5 must not reduce to zero at low Na^+ (1 mM), for if it did the enzyme would not be able to transport Na^+ at these concentrations. For Scheme I, if Na^+ could accelerate both k_3 and k_{-3} comparably, then $\%\Delta F$ would be insensitive to Na^+ concentration and k_{obs} would increase with Na^+ concentration. However, we know of no precedent for an allosteric site that accelerates equally both the forward and backward rates of an enzymatic step.

(iv) Bühler et al. [33] found that the rate of conformational change (measured with the IAF-enzyme) is comparable to the rate of charge movement through the enzyme. The $3Na^+$ to $2K^+$ stoichiometry of the pump is associated with an electrogenic movement of one net positive charge localized to the Na^+-transporting phase of the enzyme cycle [34]. It is tempting to imagine that two of the three Na^+ are transported out of the cell in an electroneutral fashion, as are the two K^+ transported inwardly; then, is the Na^+ transported as an uncompensated net positive charge the first, second, or third Na^+ discharged? By Scheme I, the release of the uncompensated charge follows the conformational change, and could be any one of the three. By Scheme II, the rate of charge transfer must not be appreciably faster than the conformational change: according to the argument in (ii), this must be step 5 or 6. In accord with this analysis, Yoda and Yoda [14] found that ionophores accelerated the transition from E*P to E_2P (steps 5 and 6 in Scheme II), and attributed this acceleration to the ionophore reducing an electrostatic barrier. These considerations thus argue for the uncompensated Na^+ being the second or third released. Moreover, if the electrostatic signal does not occur until step 5, then the transport of charge cannot occur until after the first Na^+ is released. Such sequential transport could occur if the transition from E_1P to $E_1'P$ forms (Scheme II) involves little transmembrane movement of ions, but rather reorientation of protein barriers; the transmembrane movements could then occur sequentially by successive openings of the outer barrier. Actual translocation occurs then by diffusion through an intra-enzyme transmembrane channel.

(v) The biphasic fluorescence response at very low Na^+ concentrations (Fig. 4 and Table III) in the absence of choline is attributable to the rapid transition to an E_1 conformation followed by the slower forma-

tion of E_2P. In the absence of choline, in low ionic strength media, the enzyme will exist to a considerable extent in an E_2 conformation [35]. Adding ATP will then cause a rapid increase in fluorescence with the transition to E_1, followed by the slower Na^+-activated transition to E_2P.

(vi) Oligomycin decreased k_{obs} by 98–99% at Na^+ concentrations greater than 10 mM, but only by 50% at 1 mM Na^+. Oligomycin promotes Na^+occlusion by E_1 [36] and presumably E_1P, but inhibits E_2P hydrolysis: it promotes ADP/ATP exchange and inhibits ATPase activity [4]. According to both schemes, oligomycin could inhibit step 3 to such an extent that it becomes rate-limiting at high Na^+. In the presence of oligomycin and for Na^+ concentrations greater than 10 mM, k_{obs} is low and does not vary much with Na^+. This may be because k_5 and k_{-5} are much larger than k_3 under these conditions. At an Na^+ concentration of 1 mM, k_{obs} is dominated by k_5 (as k_{-5} is low; see (iii) above), so the reduction of k_3 does not have as great an effect on k_{obs}.

(vii) $\%\Delta F$ increases as K^+ is raised from 0 to 5 mM. Similar results were obtained by Steinberg and Karlish under identical conditions [23]. They attributed this increase to K^+ activating E_2P hydrolysis. In both schemes presented above, since E_2P hydrolysis is very fast, none of the back reactions is significant and the enzyme conformations are pulled toward the E_2 form, resulting in a large increase in $\%\Delta F$.

(viii) k_{obs} decreases when K^+ is increased from 0 to 0.2 mM. The decrease cannot be explained in the context of Scheme I, because the rate constant of conformational change (k_3) is independent of K^+. In Scheme II, the initial decrease is attributable to the increase in the rate of hydrolysis of E_2P. From Table IV, we see that the overall rate constant of the system decreases from $k_5 + k_{-5}k_{-6}/(k_6 + k_{-6})$ to k_5 in the presence of K^+ (Fig. 7). At 0.2 mM K^+ k_{obs} is half that in the absence of K^+. This K^+ concentration is comparable to the $K_{1/2}$ for the increase in $\%\Delta F$ (see Fig. 7b). Both these changes (decrease in k_{obs} and increase in $\%\Delta F$) are attributable to the increase in the hydrolysis of E_2P; thus, the decrease in k_{obs} has a $K_{1/2}$ of 0.2 mM, implying that k_5 is small (at saturating Na^+). The $K_{1/2}$ values for the increase in $\%\Delta F$ and the decrease in k_{obs} are comparable with the $K_{1/2}$ for the effects of K^+ on nucleotide-binding by the enzyme [37,38] and for K^+-activation of ATPase activity [39], but are several times higher than the $K_{1/2}$ for K^+ binding to E_2 in the absence of ATP [40]. The data also suggest that under steady-state conditions in the absence of K^+, the enzyme exists primarily in the E_1 forms; this is also in accord with the $\%\Delta F$ of enzyme in ($Na^+ + K^+$) media being three-fold higher than in Na^+ media [19–21].

(ix) k_{obs} increases when K^+ is raised from 0.2 to 5 mM. This increase cannot be explained by either scheme as written, for it requires that K^+ bind at or before the conformational/fluorescence change. In the presence of K^+, k_{obs} according to both schemes is k_5. From arguments presented in (ii) and (viii) above, k_5 will be quite small in the absence of K^+ and at saturating Na^+. If the effect of K^+ was just to accelerate the rate of E_2P hydrolysis, then the rate of Na^+/K^+ transport (which is proportional to k_5) would be small under optimal conditions (high Na^+ inside, high K^+ outside, and millimolar ATP) because k_5 would be small (according to arguments presented in (ii) and (vii) above). For rapid transport in the presence of K^+, K^+ must also increase k_5. Although this increase may be related to the stimulation of ATPase activity by cytoplasmic K^+ observed by Yoda and Yoda [16], an effect of extracellular K^+ seems to be necessary, otherwise the rapid transport seen with K^+ solely in the extracellular medium [32] could not occur. This increase in k_5 is probably not related to the putative Na^+-induced increase postulated in (ii) and (iii), because all experiments with K^+ were done in the presence of saturating (155 mM) Na^+.

(x) The experiments described here do not address the ADP- and K^+-sensitivity of the intermediate EP forms. There are enzymological considerations that militate against a form sensitive to both ADP and K^+. Moreover, it is possible to account alternatively for the experimentally defined ADP- and K^+-sensitive groups of EP summing to more than 100% of the total EP, for example, by schemes with rapid transitions to terminal ADP- or K^+-sensitive forms, without invoking ADP-*and* K^+-sensitive forms [41].

Conclusions

We have attempted to distinguish experimentally between two proposed reaction schemes for the Na^+/K^+-ATPase: (a) the traditional Albers-Post scheme (Scheme I in the discussion) where the release of Na^+ occurs along with or following the conformational change from E_1P to E_2P; and (b) the scheme proposed by Nørby, Yoda, and Skou (Scheme II) where Na^+ is released prior to the conformational change. Our data support Scheme II, and indicate that the rate-limiting step during Na^+ translocation is the release of the second and/or third Na^+ from an E_1 form of the enzyme.

Our experiments were designed to specifically distinguish between the Albers-Post and the Nørby-Yoda-Skou schemes. Both these schemes depict two distinct conformations (E_1 and E_2), but the actual reaction cycle of the enzyme may be more complicated in that it may involve additional conformations. Even if this were the case, we can conclude from the mono-exponential traces we obtain that only two of these

conformations are distinguishable with the technique used here. Taniguchi and co-workers [43–45], working with BIPM-labeled enzyme, have been able to detect subconformations in addition to the classical E_1 and E_2. Since the definition of the two conformations is largely empirical, it is possible that more conformations may be defined as experimental techniques are refined.

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Appendix

For each of the schemes presented in the discussion, we wish to determine the rate constant of the overall reaction sequence if each step in the sequence is taken in turn to be rate limiting; the technique used here has been discussed in detail by Hammes and Schimmel [42]. Consider an enzymatic reaction which involves n steps:

$$X_1 \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} X_2 \underset{k_{-2}}{\overset{k_2}{\longleftrightarrow}} \cdots \underset{k_{-(n-1)}}{\overset{k_{n-1}}{\longleftrightarrow}} X_n \qquad \text{(A-1)}$$

where X_1, etc. are the various enzyme forms, and the k's are pseudo-first order rate constants for the transition from one form of the enzyme to the next. If this transition involves ligands, the concentrations of these ligands are included in the k's. The underlying assumption is that the concentrations of the ligands do not change with time.

The following square array, $\boldsymbol{b}$, can then be defined:

$$\boldsymbol{b} = \begin{pmatrix} (k_1+k_{-1}) & -k_{-1} & 0 & 0 & \cdots & 0 & 0 \\ -k_2 & (k_2+k_{-2}) & -k_{-2} & 0 & \cdots & 0 & 0 \\ 0 & -k_3 & (k_3+k_{-3}) & -k_{-3} & \cdots & 0 & 0 \\ \cdot & \cdot & \cdot & \cdot & \cdot & \cdot & \cdot \\ 0 & 0 & 0 & 0 & \cdots & -k_{-(n-1)} & \cdot \\ 0 & 0 & 0 & 0 & \cdots & -k_n & (k_n+k_{-n}) \end{pmatrix} \qquad \text{(A-2)}$$

If the ith step in the reaction sequence (A-1) is rate limiting then the overall time-constant for A-1 (τ_i) is given by

$$1/\tau_i = [\det(\boldsymbol{b})]/[\det(\boldsymbol{b}_{ii})] \qquad \text{(A-3)}$$

where $\boldsymbol{b}_{ii}$ is the array obtained by removing the ith row and ith column of $\boldsymbol{b}$, and $\det(\boldsymbol{b})$ and $\det(\boldsymbol{b}_{ii})$ are the determinants of $\boldsymbol{b}$ and $\boldsymbol{b}_{ii}$.

Eqn. A-3 holds only if the X_i's in Eqn. A-1 are thermodynamically independent, i.e., no cycles are present in the reaction sequence A-1. In order to apply this equation to Schemes and II in the Discussion, we assume that the rate at which the enzyme returns to the E_1 conformation is very slow and can be assumed to be zero. In the absence of K^+, the hydrolysis rate is determined by the rate of dephosphorylation of E_2P, which is less than 0.5 s^{-1}. In the presence of K^+, the conformational change from $E_2(K^+)$ to E_1 is the slowest step in the reaction sequence (≈ 1 s^{-1}). Under either condition, the rate of the final step in the cycle is thus slow enough that the cycling of the enzyme can be neglected for the calculation of k_{obs}, provided the analysis is applied only to the initial rate of fluorescence change.

A general point to note is that if a step in the sequence is fast (not rate-limiting), irreversible, and occurs after the rate-limiting step, then this step decouples the rate-limiting step from steps which follow. For example, in the analysis of Scheme I, step 3 (the conformational change) was assumed to be rate-limiting, and step 4 (the release of the first Na^+ to the extracellular side) was assumed to be irreversible. These assumptions made the overall rate constant of the system independent of Na^+ release from the extracellular sites and of K^+. Scheme I would predict that the only way K^+ could change k_{obs} would be to act at an allosteric site to affect steps 1, 2, or 3.

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