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Rapid kinetic analyses of the Na^+/K^+-ATPase distinguish among different criteria for conformational change

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The Na^+/K^+-ATPase couples the hydrolysis of ATP to the transport of Na^+ and K^+ via a phosphorylated intermediate and conformational changes. In order to identify these conformational changes, we have probed the sequence of steps from $EP\{3Na^+_{in}\}$ to $EP + 3Na^+_{out}$ with three fluorescent probes (IAF: 5-iodoacetamidofluorescein; BIPM: *N*-[*p*-(2-benzimidazolyl) phenyl]maleimide; and RH421) and the sensitivity of their fluorescence changes to oligomycin and divalent cations (Ca^{2+} and Mn^{2+}). The magnitude ($\%\Delta F$) and rate constant (k_{obs}) of ATP-induced fluorescence changes were measured on a fluorescence stopped-flow apparatus, and yielded the following results. (a) With RH421, k_{obs} and $\%\Delta F$ varied with $[Na^+]$ (maximal $k_{obs} = 100\ s^{-1}$, $K_{1/2} = 6$ mM; $\%\Delta F_{max} = 6\%$, $K_{1/2} = 1$ mM); these values are comparable to those previously reported using IAF-labeled enzyme (Pratap, P.R., Robinson, J.D. and Steinberg, M.I. (1991) Biochim. Biophys. Acta 1069, 288–298). (b) With BIPM-labeled enzyme, k_{obs} did not vary with $[Na^+]$ over the range tested, and was twice as high as the maximum k_{obs} for RH421. (c) Treatment with oligomycin reduced k_{obs} for all three probes to about the same level ($\approx 1–2\ s^{-1}$) while $\%\Delta F_{max}$ was largely unaffected. (d) Replacing Mg^{2+} with Ca^{2+} had similar effects to treatment with oligomycin. (e) RH421 fluorescence change was completely abolished in the presence of oligomycin and Ca^{2+}. (f) Replacing Mg^{2+} with Mn^{2+} decreased IAF fluorescence, i.e., put the enzyme in an E_2-like form, reduced k_{obs}, and rendered oligomycin less effective in reducing k_{obs}. From these results, we conclude: (a) the release of the second/third Na^+ is the rate-limiting step for the conformational change measured by IAF and charge transfer measured with RH421; (b) BIPM indicates an earlier step, either the deocclusion of Na^+ and/or the release of the first Na^+; (c) oligomycin blocks Na^+ deocclusion, and this step is sensitive to the divalent cation used to activate enzyme phosphorylation; and (d) Ca^{2+} slows the step reported by IAF as well. These experiments indicate that a simple model with two conformations (E_1 and E_2) is insufficient to explain transient kinetic data.

Introduction

The Na^+/K^+-ATPase is the enzymatic equivalent of the sodium pump, utilizing energy derived from the hydrolysis of ATP to pump Na^+ and K^+ against their electrochemical gradients (for reviews, see Refs. 1–4). A fundamental question in the study of this pump is: how does the enzyme couple ATP hydrolysis with ion transport?

The standard model is the Albers-Post scheme [1–3]: the enzyme is thought to exist in two conformations, E_1 and E_2, each of which can be either phosphorylated or unphosphorylated, in the sequence $E_1 \rightarrow E_1P \rightarrow E_2P \rightarrow E_2 \rightarrow E_1$, with Na^+-activated phosphorylation of E_1, and K^+-activated hydrolysis of E_2P. Evidence for these two conformations include differences in: (a) affinity for Na^+ and K^+ [1–3], (b) tryptic digestion patterns [1,5], (c) fluorescence quantum affinities of intrinsic [1,6] and extrinsic probes [1,7–10], and (d) sensitivity to dephosphorylation by ADP (E_1P) and by K^+-stimulated hydrolysis (E_2P) [1,3,4,11].

Because of the distinct differences in affinity for Na^+ and K^+, ion transport was assumed to be coupled to conformational changes between E_1 and E_2 conformations, defined in terms of the properties listed earlier. These properties were assumed to change together with the conformations at the same points in the en-

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Abbreviations: BIPM, *N*-[*p*-(2-benzimidazolyl)phenyl]maleimide; DCCD, dicyclohexylcarbodiimide; 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 adenosinetriphosphatase (EC 3.6.1.3).

zyme cycle. Among the discrepancies with this model were measurements of the EP forms dephosphorylated by ADP ('E_1P') and by K^+ ('E_2P') that summed to more than 100% of total EP [12–16]. 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; they 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. This conclusion was thermodynamically unsatisfactory: in the presence of ADP, E*P may be dephosphorylated back to E_1, re-forming ATP and thus resulting in the transport of 1 Na^+ with no expense in energy.

In both those schemes, transport was at least partially dissociated from the conformational change. Skou [17] presented a more detailed scheme, completely dissociating transport from the conformational change and redefining the E_1 and E_2 conformations in terms of the number of cation binding sites on each, with E_1 having three and E_2 two. This implied that the conformational change must occur after transport of each cation species. Earlier work from this laboratory [18] was consistent with this model: those data indicated that the fluorescence of IAF-labeled enzyme changed at or after the release of the second and/or third Na^+ from the enzyme to the extracellular side; however, this conclusion was based on equating the conformational change to the fluorescence change in the IAF-enzyme.

This paper aims to test the underlying assumption in the Albers-Post model that a single conformational change mediates in the transport of Na^+ across the membrane. We have used enzyme labeled with extrinsic probes of conformational change (BIPM and IAF) and of charge transfer across the membrane (RH421). In addition, we have probed the enzyme cycle with oligomycin, an inhibitor of ATPase activity that favors the Na^+-sensitive conformation; we have also examined the effects of divalent cations on the fluorescence signals. From these data, we put forward a scheme for the transition mediating Na^+ transport that departs from the traditional concept of two conformations ('E_1' and 'E_2'): we propose that each step in the transition involves a conformational change, and that the various indicators of conformational change examined here indicate different steps in the transition.

Materials and methods

Frozen dog kidneys were obtained from Pel-Freez Biologicals (Rogers, AR), IAF and RH421 from Molecular Probes (Eugene, OR), and BIPM from Wako Chemicals USA (Dallas, TX). Low-fluorescence imidazole, EDTA and ATP were from Sigma (St. Louis, MO); reagent-grade salts were either from Sigma or from Mallinckrodt (Paris, KY).

Na^+/K^+-ATPase was isolated from the outer medulla of the kidney by a modification of method C of Jørgensen [19]; 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 [10,20] and with BIPM as described by Taniguchi and co-workers [21].

For experiments with RH421, IAF enzyme in the appropriate buffer was mixed with RH421 in ethanol; the final RH421 concentration was 2 μM.

Steady-state experiments were performed as described before [18] on a Perkin-Elmer fluorescence spectrophotometer (model MPF-66; Perkin-Elmer, Oak Brook, IL). Stopped-flow experiments were performed on a stopped-flow fluorimeter (Kinetic Instruments, Ann Arbor, MI) interfaced with a Macintosh IIcx personal computer (Apple Computer, Cupertino, CA) through a MacADIOS interface board (GW Instruments, Somerville, MA). as described earlier [18,22]. For IAF-enzyme, fluorescence excitation wavelength (λ_{ex}) was 492 nm; emission was measured with a photomultiplier tube using a cutoff filter (Corning 3-69) with the wavelength of 50% transmission ($\lambda_{1/2}$) = 529 nm. Similarly, for BIPM-enzyme, λ_{ex} = 308, cutoff filter was Corning O-52 ($\lambda_{1/2}$ = 360 nm); for RH421, λ_{ex} = 530 nm, cutoff filter was Corning 2-58 ($\lambda_{1/2}$ = 646 nm).

In experiments where oligomycin was used, oligomycin in ethanol was added to the enzyme at the time when the enzyme suspension was prepared. An equal volume of ethanol was added to the control enzyme (−oligomycin). This resulted in the enzyme being incubated with oligomycin for 2–4 h prior to the experiment.

Concentrations of free Mg^{2+} and MgATP (in Fig. 6) were calculated using dissociation constants presented by Fabiato and Fabiato [23].

Results

Kinetics of charge transfer

RH421 belongs to a class of styryl dyes that were developed as reporters of membrane potential changes

[24]. These dyes are believed to work via an electrochromic mechanism: the fluorescence quantum efficiency of these dyes is sensitive to changes in electric field. Recently, Klodos and Forbush [25], Bühler et al. [26], and Stürmer et al. [27] reported ATP-induced fluorescence changes in enzyme (in broken membrane preparations) labelled with RH421 and the related dye RH160. These fluorescence changes were interpreted by Bühler et al. [26] and by Stürmer et al. [27] as indications of charge transport through the Na^+/K^+-ATPase. As charge moves into and out of the membrane during the enzyme cycle, the dye was thought to sense changes in the electric field within the membrane.

In order to compare the kinetics of charge transfer (as measured by RH421) with that of conformational change (as reported by IAF [18]), ATP-induced RH421 fluorescence changes (Fig. 1) were measured as a function of sodium concentration (Fig. 2) under conditions identical to those previously used for measuring conformational changes with IAF, including the use here of IAF-labeled enzyme [18].

The rate constant of the fluorescence change (k_{obs}) rose from 1 s^{-1} at 1 mM Na^+ to about 100 s^{-1} at 155 mM Na^+ (Fig. 2A). These data (Fig. 2A) were fitted with the Hill equation, which yielded a $K_{1/2}$ of 6 mM and a Hill coefficient of 1.7. The saturation value for k_{obs} (100 s^{-1}) was (consistently) about 25% higher than that observed with the IAF enzyme under the same conditions [18], but the values for $K_{1/2}$ was comparable to that for the IAF fluorescence change [18]. The

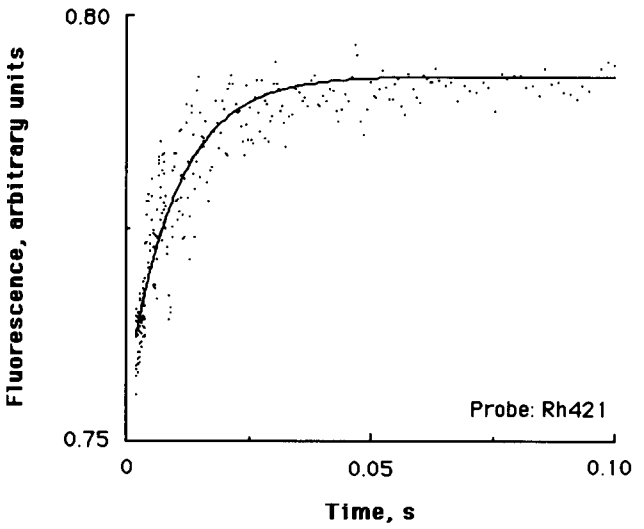

Fig. 1. Stopped-flow response of RH421-enzyme to ATP. Syringe 1: 100 μg/ml enzyme with 2 μM RH421 in buffer (50 mM NaCl, 105 mM choline chloride, 4 mM $MgCl_2$, 1 mM EDTA, 25 mM imidazole-HCl (pH 7.0)). Syringe 2: 1 mM ATP in the same buffer. Approx. 90 μl from each syringe was rapidly mixed, and the fluorescence was followed as a function of time (t = 24°C). The solid line is a fit of the data (2 ms to 1 s) by a single exponential. This fit yielded a k_{obs} of 98 s^{-1} and a $\%\Delta F$ of 4.9.

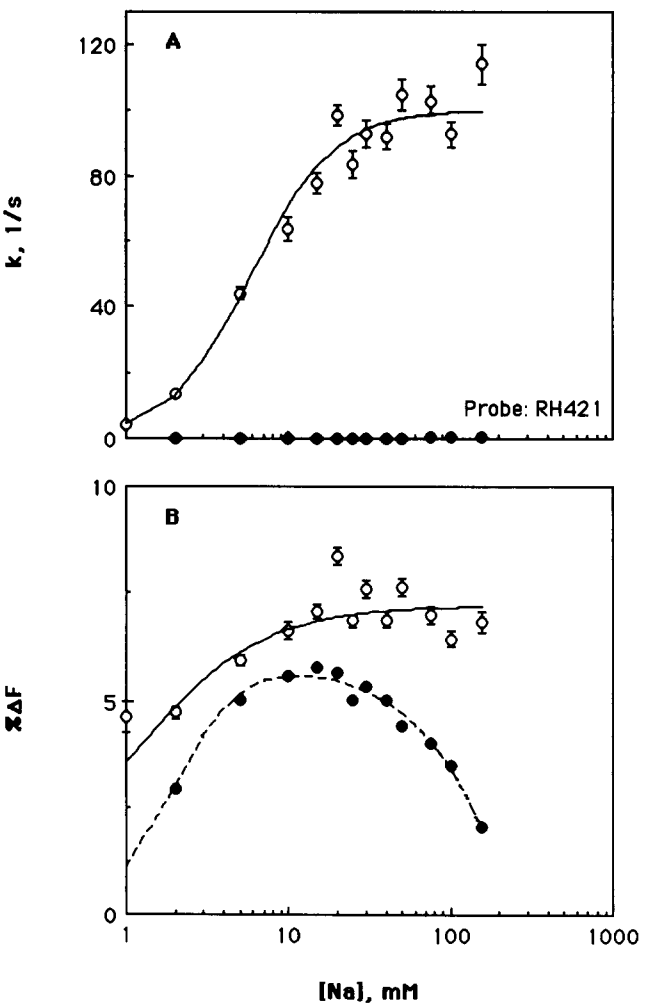

Fig. 2. Rate constant (A) and magnitude (B) of RH421 fluorescence change as a function of $[Na^+]$ in the absence (○) and presence (●) of oligomycin (when present added only in the syringe with the enzyme, at a concentration of 10 μg/ml). Ionic strength was maintained constant with choline chloride. Error bars represent the standard error of the mean. In panel A, the line represents a fit of k_{obs} (−oligomycin) to the Hill equation ($k_{max} = 100 \pm 2$ s^{-1}, $K_{1/2} = 6.0 \pm 0.3$ mM, and $n_H = 1.70 \pm 0.07$). In panel B, the solid line represents a fit of $\%\Delta F$ (−oligomycin) to the Hill equation ($\%\Delta F_{max} = 7.2 \pm 0.1$, $K_{1/2} = 1.0 \pm 0.1$ mM, and $n_H = 1.1 \pm 0.2$). For $\%\Delta F$ (+oligomycin), the rising portion was fitted with a Hill equation ($\%\Delta F_{max} = 6.03 \pm 0.03$, $K_{1/2} = 2.02 \pm 0.03$ mM, $n_H = 2.1 \pm 0.1$), and the decrease at higher Na^+ was simultaneously fitted with a straight line (slope = -0.0261 ± 0.0004).

Hill coefficient was higher than that seen with the IAF enzyme, indicating that the RH421 signal may be due to the release of the second and third Na^+ from the extracellular side.

Apell and co-workers [26,27] have shown that the disappearance of a positive charge (or the appearance of a negative charge) within membrane labeled with RH421 results in the increase of RH421 fluorescence. They interpreted this fluorescence change as a reflection of charge transported across the membrane. Consistent with this interpretation, the magnitude of the

fluorescence change ($\%\Delta F$) represents the steady-state distribution of states with different quantum efficiencies (Fig. 2B), reflecting EP forms prior to charge transfer (low fluorescence) and EP forms after charge is transferred across the membrane (high fluorescence). This parameter also changed with Na^+ concentration, but the $K_{1/2}$ was significantly lower, 1 mM; this $K_{1/2}$ was again comparable to that of the IAF fluorescence change [18].

In the presence of oligomycin, k_{obs} was reduced by two orders of magnitude at 155 mM Na^+, and no Na^+ dependence could be detected within the range of concentration used. However, $\%\Delta F$ still varied with Na^+, increasing from 2.9% at 2 mM [Na^+] to 5.8% at 20 mM [Na^+] (Fig. 2). Thereafter, it decreased, so that at 155 mM Na^+ $\%\Delta F$ was approximately the same as at 1 mM Na^+ (Fig. 2B). Such a decrease was not observed with IAF fluorescence over this Na^+ concentration range (1–155 mM) [18]. The maximal $\%\Delta F$ was only about 20% lower than in the absence of oligomycin (6% vs. 7.2%).

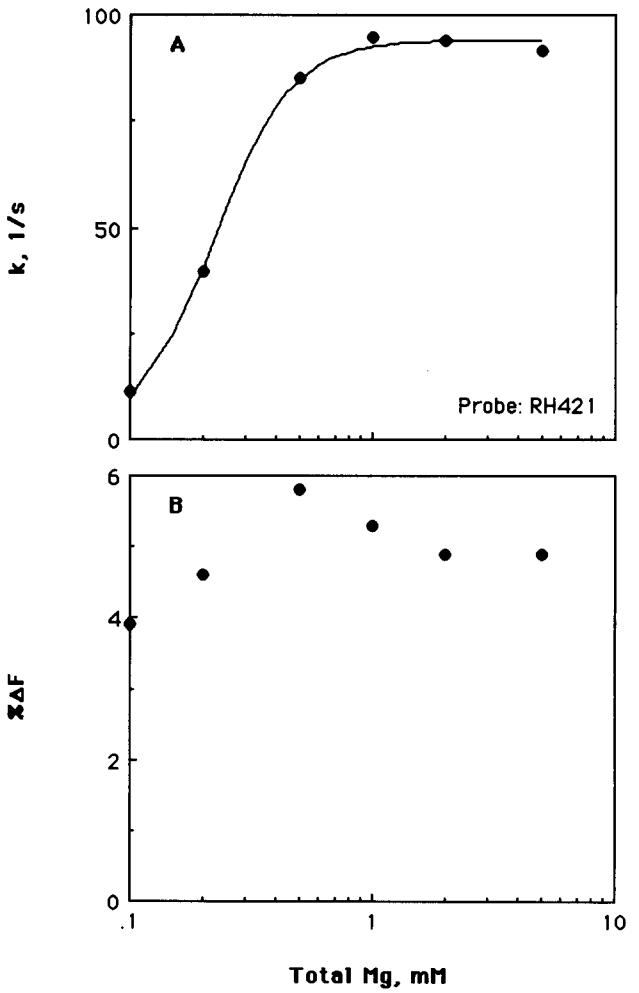

Fig. 3. Rate constant (A) and magnitude (B) of RH421-enzyme fluorescent change as a function of total [$MgCl_2$]. All experimental conditions are as in Fig. 1, except that the EDTA concentration was reduced to 0.1 mM and the [$MgCl_2$] changed as indicated. The solid line in A represents a fit to the Hill equation, with parameters: $k_{max} = 94 \pm 1\ s^{-1}$, $K_{1/2} = 0.221 \pm 0.008$ mM, and $n_H = 2.7 \pm 0.3$.

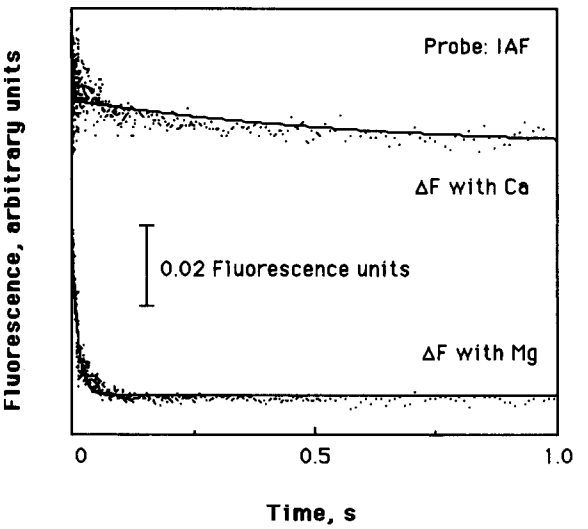

Fig. 4. Stopped-flow fluorescence response of IAF-enzyme with Mg^{2+} (lower trace) and with Ca^{2+} (upper trace). Syringe 1: 100 μg/ml IAF-enzyme in buffer (25 mM imidazole-HCl (pH 7.0), 1 mM EDTA, 4 mM divalent cation, 20 mM NaCl, 135 mM choline-Cl); syringe 2, 1 mM ATP in the same buffer. After rapid mixing of 90 μl from each syringe, fluorescence was recorded for 16 s. Only the first second of the fluorescence change is shown here, to show the rapid fluorescence change observed. In order to separate further the points associated with the two traces, the upper trace has been shifted up by 0.04 fluorescence units. Solid lines represent fits of data (2 ms to 16 s) to a single exponential: for enzyme with Mg^{2+}, $k_{obs} = 82\ s^{-1}$, $\%\Delta F = 5.9$; for enzyme with Ca^{2+}, $k_{obs} = 1.5\ s^{-1}$, $\%\Delta F = 2.8$. (In experiments with Ca^{2+} there is considerable scatter of the data during the first 50 ms, above and below the fitted line; however, the fit over the full 16 s was better with a monoexponential than a biexponential curve).

In order to estimate a lower limit for the $K_{1/2}$ for the fluorescence increase, these data were fitted to a Hill equation for the rising phase plus a straight line for the declining phase of the curve (the straight line introduced ad hoc to account for the decrease in fluorescence at higher [Na^+]). This fit yielded a lower limit for $K_{1/2}$ of 2 mM (Fig. 2).

Effects of Mg^{2+}

In the presence of 1 mM ATP and 0.1 mM EDTA, RH421 fluorescence changes (Fig. 3) varied with the total Mg^{2+} concentration, k_{obs} increasing by a factor of approx. 9 from 0.1 to 5 mM [Mg^{2+}]. The $K_{1/2}$ for this increase was 0.22 mM total Mg^{2+}. Over this range, $\%\Delta F$ was between 4 and 6%.

When expressed in terms of free [Mg^{2+}] or [MgATP], the data were satisfactorily fitted with a Michaelis-Menten equation (when fitted with a Hill equation, the resultant Hill coefficient was not significantly different from 1). For k_{obs} vs. [free Mg^{2+}], the maximal k_{obs} was $95 \pm 3\ s^{-1}$, with a $K_{1/2}$ of 13.2 ± 3.3

μM. For k_{obs} as a function of [MgATP], the fits yielded a maximal k_{obs} of 132 ± 15, with a $K_{1/2}$ of 200 ± 70 μM.

Effects of Ca^{2+}

The Na^+/K^+-ATPase requires a divalent cation for activity. The optimal cation is Mg^{2+}, but the enzyme can hydrolyze ATP in the presence of a range of alternative divalent cations [28]. When Mg^{2+} is replaced by Ca^{2+}, the enzyme is phosphorylated very slowly [29], while ATP hydrolysis is almost abolished.

When Mg^{2+} was replaced by Ca^+, the IAF-enzyme showed a much reduced fluorescence change (Fig. 4): k_{obs} was 1.9% that with Mg^{2+}, and $\%\Delta F$ was about half that with Mg^{2+}.

These results could mean either (a) Ca^{2+} slowed the step reported by IAF so that it now became the rate-limiting step (thus affecting both k_{obs} and $\%\Delta F$), or (b) Ca^{2+} made an earlier step rate-limiting and simultaneously affected the step reported by IAF. If possibility (a) were true, then Ca^{2+} would have no effect on the RH421 fluorescence change, since this occurs prior to the IAF signal. If (b) were true, Ca^{2+} would slow k_{obs} for the RH421 signal, while not affecting $\%\Delta F$.

In order to test these two possibilities, RH421 fluorescence changes were measured with Ca^{2+}. In these experiments, k_{obs} was reduced by two orders of magnitude (Fig. 5, upper trace), to approximately the same level as with IAF-enzyme (Fig. 4). But unlike experiments with the IAF fluorescence, the $\%\Delta F$ was essentially the same as with Mg^{2+}. The RH421 response

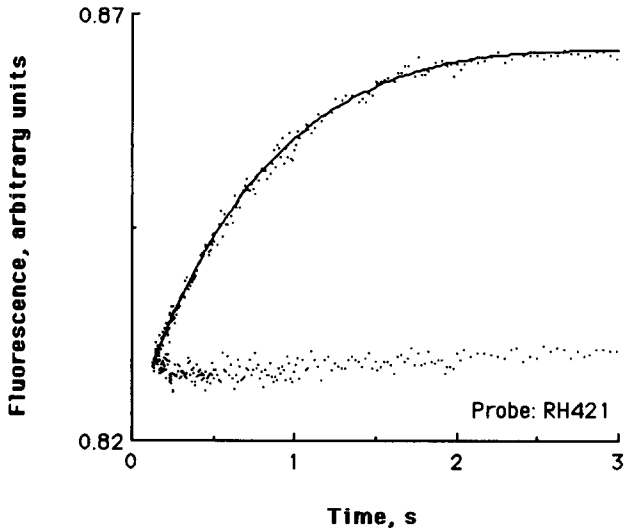

Fig. 5. Stopped-flow fluorescence response of RH421-enzyme when Mg^{2+} is replaced with Ca^{2+}, in the absence (upper trace) and presence (lower trace, dots) of oligomycin. Experimental conditions were the same as in Fig. 2, except that: syringe 1 contains, in addition, 2 μM RH421 and 4 mM $CaCl_2$ was substituted for 4 mM $MgCl_2$ for both the traces. Solid line is a fit to a single exponential: $k_{obs} = 1.5\ s^{-1}$; $\%\Delta F = 4.6$.

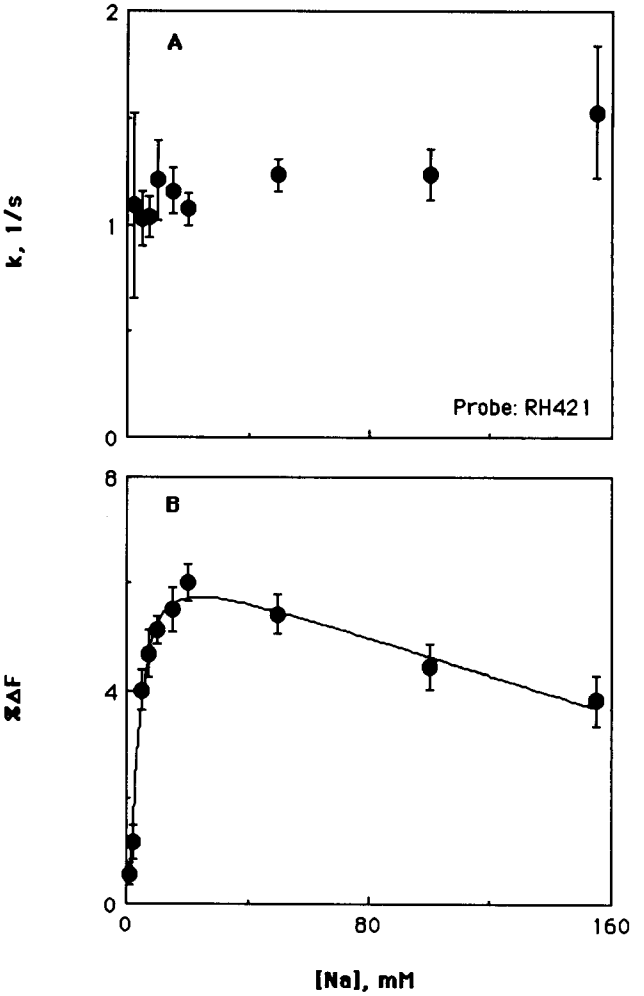

Fig. 6. Rate constant (A) and magnitude (B) of RH421-enzyme fluorescence change as a function of $[Na^+]$ when Mg^{2+} is replaced by Ca^{2+}. Experiments were performed as in Fig. 2, except that Mg^{2+} was replaced with 4 mM Ca^{2+}. The solid line is a fit to the Hill equation for the rising part and a straight line for the falling part of the data ($\%\Delta F_{max} = 6.4$; $K_{1/2} = 4$ mM; $n_H = 1.8$).

with Ca^{2+} was similar to that of RH421 with Mg^{2+} in the presence of oligomycin. Further, unlike the case with Mg^{2+}, with Ca^{2+} oligomycin abolished the RH421 response to ATP (Fig. 5, lower trace). These results support possibility (b) above.

In the presence of Ca^{2+}, k_{obs} for the RH421 fluorescence change did not vary with Na^+ over the concentration range examined (1–155 mM; Fig. 6A). The magnitude of fluorescence change (Fig. 6B) varied in approximately the same manner as with Mg^{2+} in the presence of oligomycin (Fig. 2B, filled symbols): $\%\Delta F$ increased at low $[Na^+]$, to a maximum at about 20 mM $[Na^+]$, and then decreased at higher $[Na^+]$. As with RH421 enzyme with Mg^{2+} + oligomycin, the data were fitted to a Hill equation for the rising phase plus a straight line for the declining phase; a minimum value for $K_{1/2}$ (Fig. 6B, line), so estimated, was 4 mM.

Effects of Mn^{2+}

Mn^{2+} is partially effective in activating the Na^+/K^+-ATPase [30,31], and also favors E_2 conformations [31]. Thus, in the presence of Mn^{2+} and in low [Na^+] (< 50 mM), IAF-enzyme fluorescence corresponded to that of the E_2 conformation. When enzyme under these conditions was mixed rapidly with 1 mM ATP, an initial increase in fluorescence was observed (Fig. 7A and Table I), attributable to the $E_2 \rightarrow E_1 \cdot ATP$ transition. The observed rate-constant for this fluorescent increase (7.7 s^{-1}; Table I) was consistent with the rate-constant reported for the $E_2(K) \rightarrow E_1$ transition [20]. (Under the conditions of this experiment, the enzyme can undergo a further transition from E_2 to E_1P; however, since the transition from E_1 to E_1P is 'fluorescently silent', rate constants associated with this transition will not be reflected in the observed fluorescence change.)

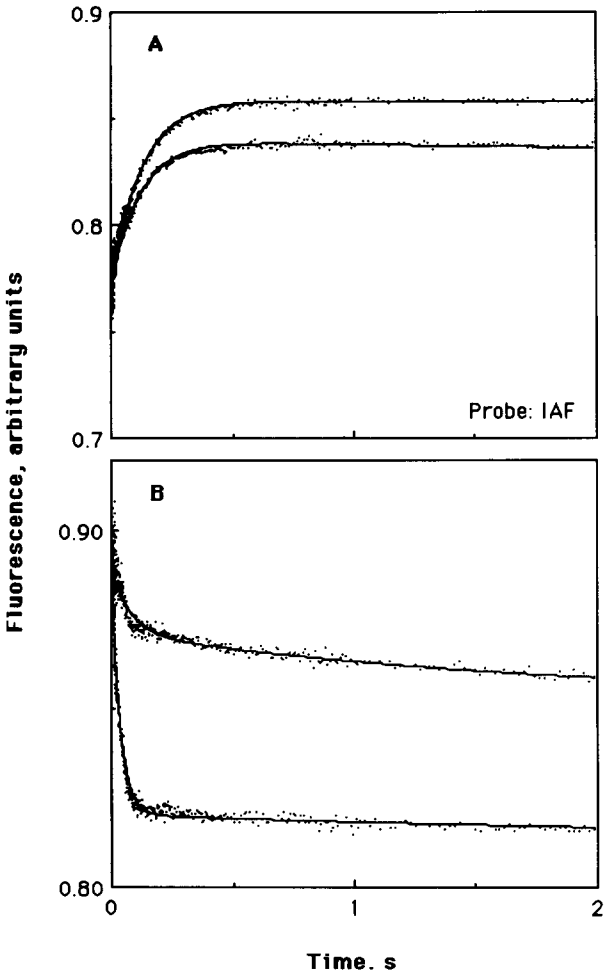

Fig. 7. Stopped-flow fluorescence response of IAF-enzyme when Mg^{2+} is replaced with Mn^{2+}. A. 100 μg/ml enzyme, either untreated or with 10 μg/ml oligomycin in buffer (25 mM imidazole-HCl (pH 7), 1 mM NaCl, 154 mM choline-Cl, 1 mM EDTA, 4 mM $MnCl_2$) is rapidly mixed with 1 mM ATP in the same buffer. Untreated, upper trace; oligomycin-treated, lower trace. B. Same as in A, except that [NaCl] = 155 mM, [choline-Cl] = 0. Solid lines are fits to two exponentials. Untreated, lower trace; oligomycin-treated, upper trace. Fit parameters are given in Table I.

TABLE I

Parameters of IAF fluorescence change when Mg^{2+} is replaced by Mn^{2+}

Enzyme (100 μg/ml) in x mM Na^+ (where $x = 1$ or 155); $155 - x$ mM choline$^+$; 4 mM Mn^{2+}; 1 mM EDTA; 25 mM imidazole-HCl (pH 7)), was rapidly mixed with 1 mM ATP in the same buffer (final concentrations: 50 μg/ml enzyme, 0.5 mM ATP). In experiments with oligomycin, the enzyme was preincubated with 10 μg/ml oligomycin. For each experiment, 7–9 shots were averaged under each condition; the averages were fitted to two exponentials. Traces of the experiments are shown in Fig. 7. The parameters reported are the averages of three experiments.

Condition	k_1	$\%\Delta F_1$ *	k_2	$\%\Delta F_2$ *
1 mM Na	7.7±0.3	10.6±1.0	0.33±0.06	−0.5±0.2
1 mM Na, +oligomycin	8.0±0.3	8.6±1.4	0.39±0.03	−0.9±0.2
155 mM Na	29.8±0.8	−7.9±0.3	0.33±0.03	−0.3±0.3
155 mM Na, +oligomycin	12.4±1.0	−2.0±0.1	0.64±0.7	−2.0±0.2

* A positive $\%\Delta F$ signifies an increase in fluorescence; a negative $\%\Delta F$ signifies a decrease.

With 1 mM Na^+, this initial increase in fluorescence (associated with the formation of E_1 and E_1P species) was followed by a small decrease, attributable to the transition to E_2P. As the Na^+ concentration was raised, both the magnitude and rate constant for this transition increased; however, with these intermediate concentrations, both the initial increase and the subsequent decrease in fluorescence occur, and it was thus difficult to evaluate each change separately.

At high [Na^+] (> 50 mM), the initial fluorescence increase disappeared, indicating that at these [Na^+] the enzyme was initially in the E_1 conformation even in the presence of Mn^{2+}; then only a decrease in fluorescence was observed after mixing with ATP, reflecting a transition to E_2P (Fig. 7B). The rate-constant for this transition at 155 mM Na^+ (Table I) was lower than that observed with Mg^{2+} (30 s^{-1} with Mn^{2+} as opposed to 80 s^{-1} with Mg^{2+} [18]), and the magnitude of the fluorescent change was slightly larger than that with Mg^{2+} (−8% with Mn^{2+} as opposed to −6% with Mg^{2+} [18]).

In the presence of oligomycin, the initial increase in fluorescence disappeared at [Na^+] ≧ 15 mM. This greater sensitivity to Na^+ is consistent with oligomycin favoring E_1 conformations even in the absence of enzyme phosphorylation, as reported earlier [32]. Further, oligomycin reduced the rate constant for the fluorescent decrease at 155 mM Na^+ by only 60% (from 30 s^{-1} to 12 s^{-1}). By contrast, oligomycin in the presence of Mg^{2+} reduced k_{obs} by two orders of magnitude (from 80 s^{-1} to 1 s^{-1} [18]). Thus, the effect

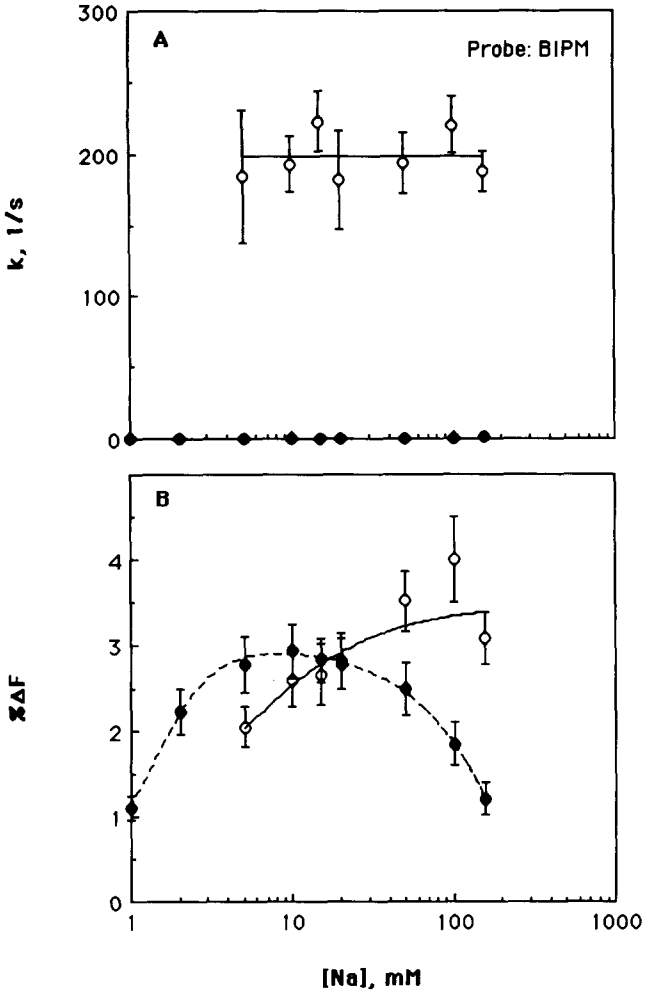

Fig. 8. Rate constant (A) and magnitude (B) of fluorescence change with BIPM-enzyme as a function of $[Na^+]$ in the absence (○) and presence (●) of oligomycin. In panel A, the solid line represents the average k_{obs} ($199 \pm 8\ s^{-1}$). In panel B, the solid line represents a fit to the Hill equation ($\%\Delta F_{max} = 3.5 \pm 0.4$, $K_{1/2} = 3.5 \pm 1.3$, and $n_H = 1.0 \pm 0.6$). The dashed line represents a fit to the Hill equation + a straight line ($\%\Delta F_{max} = 3.0 \pm 0.2$, $K_{1/2} = 1.3 \pm 0.1$, $n_H = 2.2 \pm 0.7$, and for the straight line, slope $= -0.0118 \pm 0.0018$).

of oligomycin in stabilizing the $E_1P(3Na^+)$ form of the enzyme seemed sensitive to the divalent cation used.

Fluorescence changes in enzyme labeled with BIPM

Taniguchi and co-workers [33] have reported that fluorescence changes in enzyme labeled with BIPM occur at several steps in the enzyme cycle, including conformational changes and Na^+ binding and occlusion. We have examined changes in the kinetics of fluorescence change in BIPM-enzyme as a function of Na^+ concentration, with and without oligomycin (Fig. 8).

In the absence of oligomycin, k_{obs} was 199 s^{-1} and largely independent of $[Na^+]$ over the range examined (Fig. 8A). This value for k_{obs} was much higher than that for IAF and RH421 signals, and also much higher than those reported by Taniguchi et al. [8]. The magnitude of the change also increased with a $K_{1/2}$ of about 3.5 mM and a maximum value of 3.5%. This value of $\%\Delta F_{max}$ is slightly higher than that observed by Taniguchi et al. [8].

In the presence of oligomycin, k_{obs} decreased by a factor of 200. The magnitude of the fluorescence change increased to a maximum value of 3.0%, as in the absence of oligomycin; however, this maximum was obtained at lower values of $[Na^+]$ than in the absence of oligomycin (10 mM vs. 155 mM). As Na^+ was increased further, $\%\Delta F$ declined, so that at 155 mM Na^+ the magnitude was about the same as at 1 mM Na^+. Such an increase and decrease in $\%\Delta F$ was also observed in RH421 fluorescence response with oligomycin (Fig. 8B).

Discussion

Early experiments on the Na^+/K^+-ATPase identified two distinct conformations 'E_1' and 'E_2', distinguished in terms of numerous criteria [1–11]. In previous work [18], fluorescence changes of IAF-enzyme were followed as a function of $[Na^+]$. Assuming the IAF fluorescence change reflected the conformational change from E_1P to E_2P, those results indicated that the release of at least one Na^+ preceded the conformational change, and that the rate-limiting step over the sequence examined was the release of the second and/or the third Na^+. But how valid is the assumption that the IAF fluorescent signal represents *the* conformational change? The related question is: do the properties of all indicators of conformational transitions change at the same step in the enzyme cycle, or is there a series of steps over which these change? From this question arises a fundamental question: are there just two conformations for the enzyme, or is there a spectrum of conformations that the enzyme goes through between the traditional E_1 and E_2 conformations? And: at what step in the enzyme cycle is charge transferred across the membrane?

The experiments presented here address these questions through comparing, under various conditions, the signals generated by two fluorescent indicators of conformational change and one indicator of charge transfer. From the data presented here, we conclude:

(i) The rate-constant (k_{obs}) of the RH421 fluorescence change increased with $[Na^+]$, similar to that of IAF-fluorescence change [18]. The $K_{1/2}$ for Na^+ for both signals was approximately the same. Thus, assuming RH421 reports the transfer of charge across the membrane [26,27], the rate-limiting step for charge transfer was the same as for the IAF fluorescence change – the release of the second and/or the third Na^+. Nevertheless, the maximum k_{obs} for RH421 fluorescence change was consistently higher (by about 25%)

than the k_{obs} for the IAF fluorescence change (100 s^{-1} for RH421 vs. 80 s^{-1} for IAF [18]), indicating that RH421 reports an earlier step in the enzyme cycle than IAF. A caveat to this conclusion is the possibility that RH421 increases the rate of release of the second and/or the third Na^+.

(ii) With oligomycin-treated enzyme, k_{obs} for the RH421 fluorescence change decreased by two orders of magnitude and was Na^+-independent over the concentration range studied; $\%\Delta F_{max}$ did not change significantly, similar to the sensitivity of IAF fluorescence change to oligomycin. Further, $\%\Delta F$ for RH421 (and BIPM; see below) increased to a maximum at 20 mM [Na^+] and then decreased, so $\%\Delta F$ at 155 mM Na^+ was approximately the same as that for 1 mM Na^+. These results indicate that treatment with oligomycin slows down some (earlier) Na^+-independent step so that this step then becomes rate-limiting. Moreover, the k_{obs} is consistent with the rate-constant for the dissociation of oligomycin from the enzyme [34]. Our observations are consistent with oligomycin inhibiting Na^+ deocclusion, a step that is expected to be independent of [Na^+], in accord with previous studies.

(iii) Oligomycin is thought to increase the affinity of the enzyme for Na^+ [32,33]. Such an increased affinity would shift the equilibrium for the release of Na^+ toward the sodium-bound forms ($EP \cdot 3Na^+_{out}$, $EP \cdot 2Na^+_{out}$, and $EP \cdot Na^+_{out}$). Under these conditions, the reaction sequence $EP(3Na^+) \leftrightarrow EP \cdot 3Na^+_{out} \leftrightarrow EP \cdot 2Na^+_{out} \leftrightarrow EP \cdot Na^+_{out} \leftrightarrow EP$ could be reversed at lower [Na^+] than in the absence of oligomycin. Thus, the decrease in fluorescence seen at higher [Na^+] in the presence of oligomycin could be attributed to diminished Na^+ release from the enzyme.

(iv) When the total Mg^{2+} concentration was varied from 0.1 to 5 mM, the rate-constant of the RH421 fluorescence change (k_{obs}) increased from 10 s^{-1} to 90 s^{-1} with a $K_{1/2}$ of 0.22 mM. Analysis of these data in terms of [MgATP] and $[Mg]_{free}$ yielded $K_{1/2}$ values of 200 μM and 13.2 μM, respectively. It is not obvious which of these is functionally pertinent.

(v) When Mg^{2+} was replaced with Ca^{2+}, IAF fluorescence changes were small as well as slow. On the other hand, $\%\Delta F$ for RH421-enzyme was 80% of that with Mg^{2+}, although k_{obs} was reduced by a factor of 100. These observations support our conclusion [(i) above] that RH421 reports a step in the enzyme cycle prior to that reported by IAF. Oligomycin abolished the RH421 fluorescence change with Ca^{2+}, indicating that Ca^{2+} affects the enzyme cycle at two steps: (a) the step reported by IAF, which is largely blocked; and (b) the step that is also blocked by oligomycin. Since oligomycin and Ca^{2+} each reduced the rate-constant by a factor of 100, then if these reductions occurred at different steps, the rate-constant with oligomycin plus Ca^{2+} would be the same as the rate-constant with either oligomycin or Ca^{2+}; however, if the reductions were at the same step, then the factor by which the rate-constant was reduced in the presence of oligomycin plus Ca^{2+} would be a product of the factors by which the rate-constant were reduced with either alone (i.e., a factor of 100×100): these signals would be too slow to be observed in the time-scale that these experiments were performed, and the fluorescence would appear to be unchanged. This was observed.

(vi) When Mn^{2+} was substituted for Mg^{2+}, the enzyme was initially in the E_2 conformation, as indicated by the steady-state level of IAF fluorescence. This low level of fluorescence was maintained with [Na^+] < 50 mM. When the Mn^{2+}-enzyme was then rapidly mixed with ATP, the IAF fluorescence increased, indicating a transition to the E_1 conformations. The rate constant for this conformational change was comparable to that observed by Steinberg and Karlish for the $E_2(K) \rightarrow E_1 \cdot ATP$ conformational change, indicating that this fluorescence change reflected an $E_2(Mn^{2+}) \rightarrow E_1 \cdot ATP$ transition.

The ATP-induced changes in the presence of Mn^{2+} and either 1 or 155 mM Na^+ (Table I) were measured with the ionic strength kept constant with choline chloride. ATP-induced changes have also been measured earlier [18] with enzyme plus Mg^{2+} in low ionic-strength buffer (NaCl $\leqq$ 5 mM, 25 mM imidazole-HCl (pH 7)) in the absence of K^+; under these conditions, the enzyme underwent a rapid transition from the E_2 to the E_1 conformation, as indicated by a rapid increase in fluorescence ($k_{obs} > 100$ s^{-1}). The latter rate constant was much higher than that for the fluorescence increases seen with Mn^{2+} at low Na^+ (this work) and in the presence of K^+ [20]. These observations imply either (a) the fluorescence change indicates a step that occurs after K^+ deocclusion, as proposed by Skou and Esmann [9], or (b) the pathway for conformational transition from $E_2 \rightarrow E_1 \cdot ATP$ is different in the presence or absence of K^+ (or Mn^{2+}).

(vii) The rate-constant of the fluorescence change with BIPM-labelled enzyme was about two-fold greater than that with IAF-enzyme or with RH421, and independent of [Na^+] over the range examined (1–155 mM). Thus BIPM reported a step prior to those reported by IAF or RH421 (i.e., the release of the second and/or third Na^+). Under the conditions of our experiments ([Na^+] = 1–155 mM), the release of the first Na^+ was practically irreversible, so this step could be the one reported by BIPM. This possibility is strengthened by the observation that DCCD, which blocks Na^+ occlusion [36], binds close to the BIPM binding site (Glu-953 for DCCD, vs. Cys-964 for BIPM [37]).

Note that k_{obs} at 155 mM Na^+ was much greater than the rate-constant observed by Taniguchi et al. under similar conditions (160 mM Na^+; [8]): approxi-

mately 200 s^{-1} vs. 30–35 s^{-1}. Those low rate constants are inconsistent with the rate-constants observed with IAF- and RH421-enzyme. Possible explanations for this discrepancy are that Taniguchi et al. [8] did most of their experiments at high ionic strength (0.6–2 M), or that they used a much lower concentration of ATP (5 μM). The latter possibility is particularly appealing because Steinberg and Karlish [20] have reported that k_{obs} for IAF-enzyme increases with [ATP] with a $K_{1/2}$ of approx. 50 μM. In fact, Taniguchi et al. [38] found that at 2 M Na^+, they could not saturate the rate-constant for BIPM fluorescence change with 300 μM ATP, although $\%\Delta F$ and the amount of EP formed were saturated at a much lower [ATP].

Because oligomycin reduced k_{obs} but not $\%\Delta F_{max}$, the BIPM fluorescence change represents a step that is either irreversible or is separated from the oligomycin-sensitive step by an irreversible step. Again, this conclusion is consistent with BIPM reporting the release of the first Na^+.

(viii) The rate constant for fluorescence change of BIPM-enzyme incubated with oligomycin was comparable to that of enzyme labelled with IAF and RH421 with oligomycin. Thus, the oligomycin-inhibited step occurs at or before the step reported by BIPM. Since oligomycin inhibits the deocclusion of Na^+, the step reported by BIPM is either Na^+ deocclusion or the release of the first Na^+.

(ix) The magnitude of the fluorescence change with BIPM increased with Na^+ to a maximum of about 3.5% and a $K_{1/2}$ of 3.5 mM. In the presence of oligomycin, this curve shifted to lower Na^+ concentrations – the $K_{1/2}$ was now 1.4 mM; however, $\%\Delta F_{max}$ remained the same. This implies that the step reported by BIPM is separated from the rate limiting step in the presence of oligomycin (Na^+ deocclusion) by an irreversible step. Therefore, these results support the conclusion that BIPM reports the release of the first Na^+ from the enzyme.

The decrease in $\%\Delta F$ at higher [Na^+] (seen also with RH421-enzyme treated with oligomycin) reflects the reversal of the enzyme cycle from E(high BIPM fluorescence) to E(low BIPM fluorescence), consistent with oligomycin preventing the deocclusion of Na^+. Such a shift should be observed even in the absence of oligomycin, but at much higher [Na^+], and has been observed with IAF-enzyme [20].

(x) From the observations presented here, we have constructed a scheme (Fig. 9) for the transition of the enzyme cycle with emphasis on the portion involved in Na^+ (and charge) transport, indicating the steps that we believe are reported by IAF, BIPM and RH421, and the steps inhibited by oligomycin and Ca^{2+}. This scheme does not refer to the E_1 and E_2 conformations; instead, it considers each step in the enzyme sequence to be a distinct conformation with Na^+ bound to the

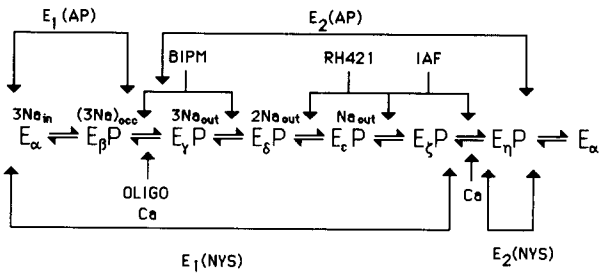

Fig. 9. Suggested model for the action of the Na^+/K^+-ATPase based on the results with IAF, RH421 and BIPM. The Greek letters represent different conformations; the steps affected by oligomycin and Ca^{2+} are shown. The proposed steps indicated by IAF, RH421, and BIPM are indicated. E_1(AP) and E_2(AP) represent the species in this scheme that correspond to the E_1 and E_2 conformations proposed by Albers and Post; E_1(NYS) and E_2(NYS) represent the assigned conformations in the Nørby-Yoda-Skou scheme. The subscripts 'in' and 'out' indicate that the bound Na^+ is in equilibrium with intracellular or extracellular Na^+, respectively; 'occ' represents occluded Na^+.

enzyme as shown. Subscripts indicate the side of the membrane from which the cation binding sites are accessible; occlusion is further indicated by enclosing Na^+ within parentheses.

We have indicated also which of the species in the scheme would be identified as 'E_1' and 'E_2' according to the Albers-Post (AP) and the Nørby-Yoda-Skou (NYS) schemes [18]; for the latter scheme, following Skou's proposal [17], we have included two EP forms with no Na^+ bound ($E_\zeta P$ and $E_\eta P$, corresponding to EP with three and two cation binding sites, respectively), although a change from three to two cation sites (and thus conversion of 'E_1P' to 'E_2P') could also be placed between $E_\gamma P$ and $E_\delta P$.

(xi) We believe that the analysis of transient kinetic data must de-emphasize the traditional interpretation of Na^+/K^+-ATPase kinetics in terms of two conformations. We believe that such interpretations are prone to misleading and contradictory conclusions. A similar point was raised by Glynn and Karlish [39], who pointed out that the nomenclature in terms of E_1 and E_2 is insufficient for explaining many experimental results. Nørby [40] suggested that a hierarchy of conformational states be considered, with transitions between subconformations within each hierarchical level. Jencks [41] has raised similar arguments for the Ca^{2+}-ATPase from the sarcoplasmic reticulum.

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