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Mechanism of the Rate-Determining Step of the Na^+,K^+-ATPase Pump Cycle†

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ABSTRACT: The kinetics of the $E_2 \rightarrow E_1$ conformational change of unphosphorylated Na^+,K^+-ATPase from rabbit kidney and shark rectal gland were investigated via the stopped-flow technique using the fluorescent label RH421 (pH 7.4, 24 °C). The enzyme was pre-equilibrated in a solution containing 25 mM histidine and 0.1 mM EDTA to stabilize initially the E_2 conformation. When rabbit kidney enzyme was mixed with NaCl alone, tris ATP alone or NaCl, and tris ATP simultaneously, a fluorescence decrease was observed. The reciprocal relaxation time, $1/\tau$, of the fluorescent transient was found to increase with increasing NaCl concentration and reached a saturating value in the presence of 1 mM tris ATP of $54 \pm 3\ s^{-1}$ in the case of rabbit kidney enzyme. The experimental behavior could be described by a binding of Na^+ to the enzyme in the E_2 state with a dissociation constant of 31 ± 7 mM, which induces a subsequent rate-limiting conformational change to the E_1 state. Similar behavior, but with a decreased saturating value of $1/\tau$, was found when NaCl was replaced by choline chloride. Analogous experiments performed with enzyme from shark rectal gland showed similar effects, but with a significantly lower amplitude of the fluorescence change and a higher saturating value of $1/\tau$ for both the NaCl and choline chloride titrations. The results suggest that Na^+ ions or salt in general play a regulatory role, similar to that of ATP, in enhancing the rate of the rate-limiting $E_2 \rightarrow E_1$ conformational transition by interaction with the E_2 state.

The Na^+,K^+-ATPase is known to play a fundamental role in numerous physiological processes, e.g., nerve, kidney, and heart function. Its activity in the cell must, therefore, be under tight metabolic control. A major site of regulation of the enzyme at the molecular level must be at its rate-determining steps, since only changes in the rates of these steps will result in any significant change in the overall turnover number of the enzyme.

The kinetics of the Na^+,K^+-ATPase are generally described in terms of the Albers−Post model (*1*, *2*), a simplified version of which is shown in Figure 1. This simple model considers two conformations of the enzyme, E_1 and E_2, which can be either in a phosphorylated or an unphosphorylated state. The model, furthermore, describes a consecutive mechanism of Na^+ and K^+ ion transport across the membrane, whereby Na^+ ions normally bind from the cytoplasm and K^+ ions from the extracellular fluid. The location of the rate-determining steps within this cycle and the determination of rate constants for the various steps have been the subject of an intense research effort by many groups over the past thirty years. Although several different reaction steps have been discussed as possible candidates for the rate-determining step of the enzyme, there now appears to be conclusive evidence and an overall consensus that under physiological conditions it is in fact the $E_2 \rightarrow E_1$ transition of unphosphorylated enzyme (*3*).

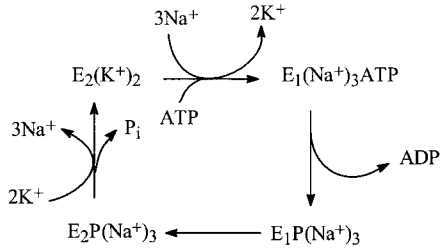

FIGURE 1: Albers-Post cycle.

It is known that the enzyme can be stabilized in the E_2 conformation by incubation with K^+ or Rb^+ ions (*4*, *5*) or by the appropriate choice of buffer solution composition (*6*−*13*). The kinetics of the conformational transition from E_2 to E_1 can be studied by rapidly mixing the enzyme with a sufficient excess of Na^+ ions over K^+, which leads to a conversion of the enzyme into the $E_1(Na^+)_3$ state. Such

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[1] Abbreviations: Na^+,K^+-ATPase, sodium and potassium ion-activated adenosine triphosphatase; ATP, adenosine 5′-triphosphate; E_1, E_2, and E_2P, intermediates of the Na^+,K^+-ATPase pump cycle; RH421, *N*-(4-Sulfobutyl)-4-(4-(p-(dipentylamino)phenyl)butadienyl)-pyridinium inner salt; EDTA, ethylenediaminetetraacetic acid; tris, tris-(hydroxymethyl)aminomethane; FITC, fluorescein 5′-isothiocyanate.

experiments were first carried out by Karlish and Yates (*5*), who used changes in the intrinsic tryptophan protein fluorescence to monitor the kinetics of the conformational change. They found that the rate of the transition was very dependent on the presence or absence of ATP, increasing from 0.29 s^{-1} at zero ATP to approximately 18 s^{-1} at 100 μM ATP. Unfortunately, they could not extend their measurements to higher ATP concentrations, either because of a limitation in the time resolution of their instrument or because ATP at high concentrations quenches tryptophan fluorescence. A further difficulty encountered in their measurements was the small amplitude of the fluorescence change observed. For this reason, many researchers have turned to using extrinsic fluorescent probes.

Consistent with the earlier findings of Karlish and Yates (*5*), using dog kidney enzyme covalently labeled with the fluorescent probe 5-iodoacetamidofluorescein (IAF), Steinberg and Karlish (*14*) found that the rate of the $E_2 \rightarrow E_1$ transition reached a saturating value at high ATP concentrations. At pH 7.0 and 20 °C, they determined a maximum rate of between 15.9 and 28.8 s^{-1}, depending on the buffer composition, and a half-saturating ATP concentration of 196 μM. Similar accelerations of the rate of the $E_2 \rightarrow E_1$ transition by ATP have also been found by Pratap et al. (*15*) using IAF-labeled dog kidney enzyme and by Kane et al. (*16*) and Clarke et al. (*17*) using the fluorescent probe RH421 on pig and rabbit kidney enzyme, respectively.

Another interesting kinetic approach which has been applied by several research groups is to monitor the release of K^+ or Rb^+ ions from the E_2 state of the enzyme which occurs as a consequence of its conformational change to E_1. The rate of this process has been determined using radioactive detection with the isotopes ^{42}K and ^{86}Rb. Consistent with the findings using fluorescence probes, it has been observed that ATP enhances the rate of release of both K^+ and Rb^+ (*18*, *19*). Forbush (*18*) found that ^{42}K was released with a saturating rate of 45 s^{-1} and that ATP acted thereby at a low-affinity site with a dissociation constant of approximately 300 μM.

In all of these experiments in which the effect of ATP on the rate of the $E_2 \rightarrow E_1$ transition was studied, Mg^{2+} ions were omitted or removed from solution to prevent phosphorylation of the enzyme. As well as its role in phosphorylating the enzyme and providing the energy to drive Na^+ transport, ATP must therefore also play a regulatory role in stimulating the $E_2 \rightarrow E_1$ transition and thus in increasing the overall pumping rate. If this is the case for ATP, the question can be asked whether Na^+ ions may also stimulate the $E_2 \rightarrow E_1$ transition. In the experiments described above, mixing with Na^+ ions initiates the conformational transition. This could, however, occur in two ways:

(a) binding of Na^+ ions to the E_1 state and perturbing an E_2-E_1 equilibrium in the favor of E_1, or

(b) binding of Na^+ ions to the E_2 state and stimulating a transition of the enzyme to the E_1 state.

The first mechanism could be described as a "lock-and-key" model of Na^+ binding because the Na^+ ions do not themselves directly induce any conformational change of the enzyme molecules to which they bind. The second mechanism can be termed an "induced-fit" model of Na^+ binding. The two mechanisms described here must be considered extreme cases, since in the lock-and-key model the conformational transition is only allowed when no Na^+ ions are bound and in the induced-fit model it is only allowed when Na^+ is bound. For the purposes of the introduction, however, we shall consider first these extreme models. It is worthwhile here also to point out that in both mechanisms, Na^+-induced stimulation of the $E_2 \rightarrow E_1$ transition does not necessarily mean that the Na^+ ions are binding to the ion transport sites. The possibility of regulatory Na^+ sites cannot be excluded.

Now let us consider the expected kinetic behavior for the two mechanisms. For simplicity, to demonstrate the predicted dependence of the observed rate constants for the two mechanisms on the Na^+ concentration, let us at this stage ignore ATP binding to the enzyme and consider the simplest case of a single Na^+ ion binding to the enzyme. Furthermore, we shall assume that the actual ion binding steps are much faster than the conformational change and can so be considered to be in equilibrium on the time-scale of the conformational change. The two mechanisms can be schematically represented as follows:

$$\text{Lock-and-key model} \qquad E_2 \underset{k_{-0}}{\overset{k_0}{\rightleftharpoons}} E_1 + Na^+ \overset{K_N'}{\rightleftharpoons} E_1Na^+ \quad \text{(a)}$$

$$\text{Induced-fit model} \qquad E_2 + Na^+ \overset{K_N}{\rightleftharpoons} E_2Na^+ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_1Na^+ \quad \text{(b)}$$

K_N and K_N' represent here equilibrium dissociation constants for Na^+ binding site on the E_2 and the E_1 enzyme conformation, respectively, k_0 and k_{-0} represent the forward and backward rate constants of the conformational transition when no Na^+ ions are bound, whereas k_1 and k_{-1} represent the forward and backward rate constants when the enzyme has bound Na^+. Applying relaxation kinetics theory (*20*), it can be shown that the reciprocal relaxation times (or observed rate constants) following a Na^+ concentration jump for the two mechanisms are given by

$$\text{Lock-and-key model} \qquad \frac{1}{\tau} = k_0 + k_{-0} \cdot \frac{K_N'}{K_N' + [Na^+]} \quad (1)$$

$$\text{Induced-fit model} \qquad \frac{1}{\tau} = k_1 \cdot \frac{[Na^+]}{K_N + [Na^+]} + k_{-1} \quad (2)$$

From these two equations, it can be seen that the two mechanisms predict very different dependencies of the rate of relaxation of the Na^+-induced $E_2 \rightarrow E_1$ transition on the Na^+ concentration. If the lock-and-key mechanism applies, then the rate of relaxation into equilibrium following mixing with Na^+ must decrease hyperbolically with increasing Na^+ concentration. If, on the other hand, the induced-fit mechanism applies, the rate of the relaxation may increase hyperbolically with increasing Na^+ concentration. In principle, therefore, the Na^+ concentration dependence of the rate should allow a distinction between the mechanisms. Such an approach has been followed by a number of research groups. Conflicting results have, however, been obtained.

In his studies of the $E_2 \rightarrow E_1$ transition with enzyme covalently labeled with fluorescein 5′-isothiocyanate (FITC), Karlish (*6*) observed no effect of Na^+ in the range 100−750 mM on the rate of the transition. The observed rate constants he measured were 0.73, 0.81, and 0.67 s^{-1} at 100, 250, and 750 mM NaCl. The lack of any Na^+ effect could, however,

possibly be explained by saturation of the Na^+ binding sites within this concentration range. It should be noted that the low values of the observed rate constants measured by Karlish (*6*) are due to the blockage of the ATP binding site by the FITC label (*21*−*23*), so their measurements were limited to unphysiological concentrations of zero ATP.

Using the probe eosin, Skou and Esmann (*7*) observed a decrease in the half-time of the $E_1 \rightarrow E_2$ transition with increasing Na^+ concentration from 5.2 s at 50 mM Na^+ to 1.25 s at 300 mM Na^+. If one assumes first-order kinetics, these half-times correspond to observed rate constants of 0.13 s^{-1} (50 mM Na^+) and 0.6 s^{-1} (300 mM Na^+). This result would suggest an induced-fit mechanism (b), i.e., that Na^+ ions bind directly to the $E_2(K^+)_2$ state and stimulate its conversion to $E_1(Na^+)_3$. Interestingly, Skou and Esmann (*7*) also found that choline cations were also capable of stimulating the transition. They attributed this to a Na^+-like effect of choline rather than an ionic strength effect. From their data, however, it is not possible to conclude anything concerning the relative effectiveness of Na^+ and $choline^+$ in stimulating the transition. Similar to the case of the experiments of Karlish (*6*), however, the observed rate constants found by Skou and Esmann (*7*) are very low, due to a blockage of the ATP binding site by eosin (*24*−*27*).

From rapid filtration studies of ^{42}K and ^{86}Rb release from unphosphorylated enzyme, Forbush (*18*) also observed an increase in the observed rate constant with increasing cation concentration. Using *N*-methylglucamine to maintain a constant ionic strength, he observed that within the precision of his experiments the apparent affinities of the various cations were not substantially different from one another (20−40 mM). The magnitude of increase in the rate constant at saturating cation concentrations was found, however, to increase in the order $Li^+ < choline^+ = tris^+ =$ *N*-methylglucamine $< Cs^+ < Rb^+ < Na^+ < K^+$. Forbush (*18*) thus concluded that other cation sites can be occupied at the same time as the K^+ occlusion sites, i.e., direct binding of cations to $E_2(K^+)_2$ stimulates the transition to E_1, consistent with the induced-fit mechanism (b) above. Hasenauer et al. (*19*) similarly found that Na^+ increased the rate of spontaneous release of Rb^+ from the enzyme, which they noted as "clearly indicating the simultaneous bindings of Na^+ and Rb^+ to the enzyme." Hasenauer et al. (*19*) have hypothesized that Na^+ binding at a regulatory site causes a widening of an ion access channel, thus allowing the more rapid dissociation of Rb^+. On the basis of these results, therefore, in addition to the alternate binding of Na^+ and K^+ to transport sites described by the Albers-Post model, one must consider simultaneous cation binding, i.e., K^+ or Rb^+ to transport sites and at the same time Na^+ to regulatory sites.

Results consistent with those described above have also been reported by Esmann (*26*), who observed both a Na^+-induced increase in the rate of Rb^+ release as well as an increase in the rate of the $E_2 \rightarrow E_1$ transition as measured using the fluorescence probe eosin. Esmann (*26*) also noted that these effects must be due to Na^+ binding to sites other than the Rb^+ occlusion sites. In the absence of both K^+ and Rb^+, an acceleration of a conformational change of unphosphorylated enzyme by various cations, including Na^+, $choline^+$, and $guanidinium^+$ was, furthermore, detected by Doludda et al. (*12*) via stopped-flow measurements using the covalently labeled fluorescence probe FITC. The apparent dissociation constants of the cations were found to be in the range 10−20 mM.

Very different results have, however, been reported by Faller and co-workers (*28*−*32*). Using FITC-modified enzyme (*28*−*30*), enzyme noncovalently labeled with eosin (*31*), and fluorescence resonance energy transfer between IAF-labeled enzyme and trinitrophenyl-ATP bound to the active site (*32*), they observed that the reciprocal relaxation time (or observed rate constant) decreased with increasing Na^+ concentration. Their results are, therefore, more consistent with the lock-and-key mechanism a above, i.e., a binding of Na^+ to the E_1 conformation which shifts an E_2-E_1 equilibrium to the side of E_1. They explained (*31*) the apparent discrepancy of their results with those of the studies of other workers by an ionic strength effect on the rate constants of the conformational change and the dissociation constants for K^+ and Na^+, which they suggested was responsible for the Na^+-induced increase in the rate observed elsewhere. They therefore stressed the importance of maintaining a constant ionic strength when performing such measurements. Their explanation of a nonspecific ionic strength effect would not appear to apply, however, to the results of Forbush (*18*), who did in fact control the ionic strength using *N*-methylglucamine and still observed an increase in the rate of ^{42}K and ^{86}Rb release with increasing Na^+ concentration. Forbush (*18*) also observed that various cations had a different effectiveness in inducing ^{42}K or ^{86}Rb release, which would seem to imply binding to specific sites rather than a nonspecific ionic strength effect.

Because of the disagreement in the literature concerning the mechanism of the $E_2 \rightarrow E_1$ transition, we decided to reinvestigate the Na^+ concentration dependence of its rate of relaxation using the voltage-sensitive fluorescent probe RH421. This probe has advantages over other commonly used probes. First, it inserts itself noncovalently into Na^+,K^+-ATPase-containing membrane fragments so that no covalent modification of the enzyme is necessary (*33*, *34*). Second, it does not interfere with the ATP binding site of the enzyme, as is the case with FITC and eosin, so that measurements can be made in the presence of ATP, i.e., closer to physiological conditions. The detection of the $E_2 \rightarrow E_1$ transition using RH421 was first reported by Kane et al. (*16*) using enzyme from pig kidney, and it was subsequently also observed using enzyme from rabbit kidney (*17*). In both of these studies, the effect of ATP on the rate of the transition was investigated. In agreement with previous investigations using other methods, a hyperbolic increase in the rate with increasing ATP concentration was found. The effect of Na^+ concentration on the transition observed using RH421 has, however, not yet been investigated.

MATERIALS AND METHODS

N-(4-Sulfobutyl)-4-(4-(p-(dipentylamino)phenyl)butadienyl)-pyridinium inner salt (RH421) was obtained from Molecular Probes (Eugene, OR) and was used without further purification. It was added to Na^+,K^+-ATPase-containing membrane fragments from an ethanolic stock solution. The dye is spontaneously incorporated into the membrane fragments.

Na^+,K^+-ATPase-containing membrane fragments from the red outer medulla of rabbit kidney were prepared and purified according to procedure C of Jørgensen (*35*, *36*). The specific

ATPase activity was measured by the pyruvate kinase/lactate dehydrogenase assay (*37*), and the protein concentration was determined by the Lowry method (*38*) using bovine serum albumin as a standard. The specific activity of the Na^+,K^+-ATPase preparations used were in the range 1800−2300 μmol Pi/h per mg protein at 37 °C. The protein concentration was in the range 2.6−2.7 mg/mL.

Na^+,K^+-ATPase-containing membrane fragments from shark rectal glands were purified as described by Skou and Esmann (*39*). The specific ATPase activity at 37 °C and pH 7.4 was measured according to Ottolenghi (*40*) to be ∼1800 μmol ATP hydrolyzed h^{-1} (mg of protein)$^{-1}$, and the protein concentration was 2.9 mg/mL. The protein concentration was determined according to Lowry (*38*) using bovine serum albumin as a standard.

Stopped-flow experiments on rabbit kidney Na^+,K^+-ATPase were carried out using an SF-61 stopped-flow spectrofluorimeter from Hi-Tech Scientific (Salisbury, England). Details of the experimental setup have been described elsewhere (*16*, *17*). Each kinetic trace consisted of 1024 data points. To improve the signal-to-noise ratio, we averaged typically between 6 and 20 experimental traces before the reciprocal relaxation time was evaluated. The error bars shown in the figures correspond to the standard error of a fit of an exponential function to the averaged experimental traces. Nonlinear least-squares fits of the reciprocal relaxation times to appropriate kinetic models were performed using the commercially available program ENZFITTER. To take into account the greater absolute errors of the higher values of the reciprocal relaxation times, we weighted the individual points according to the reciprocal of their value. The errors quoted for the parameters determined (rate and equilibrium constants) correspond to the standard errors derived from the fits.

The kinetics of the conformational change of unphosphorylated enzyme were investigated in the stopped-flow apparatus by mixing 20 μg/mL of Na^+,K^+-ATPase labeled with 150 nM RH421 with an equal volume of 2 mM trisATP and varying concentrations of NaCl in the range 0−600 mM. Both the enzyme suspension and the trisATP/NaCl mixtures were prepared in a solution containing 25 mM histidine and 0.1 mM EDTA. The pH of this solution was adjusted to pH 7.4 with HCl. Because the solution is weakly buffered in this pH range, the pH of the trisATP/NaCl mixtures was readjusted to 7.4 after the addition of the acidic trisATP by adding a small amount of tris. Mg^{2+} ions were excluded from the solutions to prevent phosphorylation of the enzyme occurring.

The solutions in the drive syringes were equilibrated to a temperature of 24 °C before each experiment. Because RH421 concentrations above 1 μM are known to inhibit the steady-state hydrolytic activity (*41*) and the transient kinetics of Na^+-dependent partial reactions of the Na^+,K^+-ATPase (*16*), a noninhibitory RH421 concentration of 150 nM was used in the enzyme solution. The dead-time of the mixing cell was determined to be 1.7 ± 0.2 ms. The electrical time constant of the fluorescence detection system was set at a value of not less than 10 times faster than the relaxation time of the fastest enzyme-related transient. Interference of photochemical reactions of RH421 with the kinetics of Na^+,K^+-ATPase-related fluorescence transients was avoided by inserting neutral density filters in the light beam to reduce the excitation light intensity.

Analogous stopped-flow experiments were also performed on Na^+,K^+-ATPase from shark rectal gland. These were carried out using an SX.17MV rapid mixing stopped-flow spectrofluorometer (Applied Photophysics, U.K.). The flow volume was 100−300 μL. The excitation wavelength was 546 nm (using a combined xenon/mercury lamp), and the fluorescence was measured at emission wavelengths ≥630 nm using a cutoff filter. The dead time of the stopped-flow apparatus was ∼1.5 ms.

Each data set, in which the concentration of Na^+ was varied, was collected using a single Na^+,K^+-ATPase preparation. All solutions were prepared using deionized water. The nominally K^+-free buffers were analyzed by total-reflection X-ray fluorescence spectroscopy, atomic absorption spectroscopy, or ion chromatography and found to contain not more than 25 μM K^+ ions.

A 7.5 mM stock solution of sodium orthovanadate was prepared for control inhibition experiments by titrating an aqueous solution of the compound to pH 7.4 with HCl and then boiling the resultant yellow-colored solution until it became colorless, to remove polymerized vanadate species (*42*).

The origins of the various reagents used were as follows: tris((hydroxymethyl)amino)methane (99.9%, Sigma), L-histidine (≥ 99.5%, Fluka), choline chloride (99+%, 3× crystallized, Sigma), EDTA (99%, Sigma), HCl (1.0 M titrisol solution, Merck), ethanol (analytical grade, Merck), ATP tris salt·1.5H_2O (98%, Sigma), ATP magnesium salt·3H_2O (97%, Sigma), NaCl (Suprapur, Merck), ouabain 7.5H_2O (≥95%, Sigma), sodium orthovanadate (≥90%, Sigma), and $MgCl_2$·6H_2O (analytical grade, Merck).

RESULTS

Na^+-Induced Stopped-Flow Fluorescence Traces. To investigate the kinetics of the $E_2 \rightarrow E_1$ conformational transition of unphosphorylated Na^+,K^+-ATPase, we have carried out experiments in which rabbit kidney enzyme is initially equilibrated in a solution containing 25 mM histidine and 0.1 mM EDTA and is subsequently rapidly mixed with tris ATP and NaCl. The composition of the initial equilibrating solution was chosen so as to minimize the presence of any cations in solution, which via a Na^+-like effect can convert the enzyme into an E_1 conformation (*3*, *6*−*13*). The conformational equilibrium of the enzyme was thus initially poised toward the E_2 conformation. It was furthermore noncovalently labeled with the fluorescence probe RH421 to allow for fluorescence detection of the kinetics. On mixing with tris ATP alone, NaCl alone, or tris ATP and NaCl together in the absence of $MgCl_2$, a decrease in fluorescence is observed (see Figure 2, curve a, and Figure 3), which indicates that the fluorescence intensity of dye associated with enzyme in the E_2 conformation is higher than that of dye associated with enzyme which has bound ATP, Na^+ or both ATP and Na^+. In the case of experiments in which the enzyme was mixed with ATP or with ATP and NaCl simultaneously, the kinetics of the fluorescence decrease could be closely approximated with a single-exponential time function. When the enzyme was mixed with NaCl alone, a

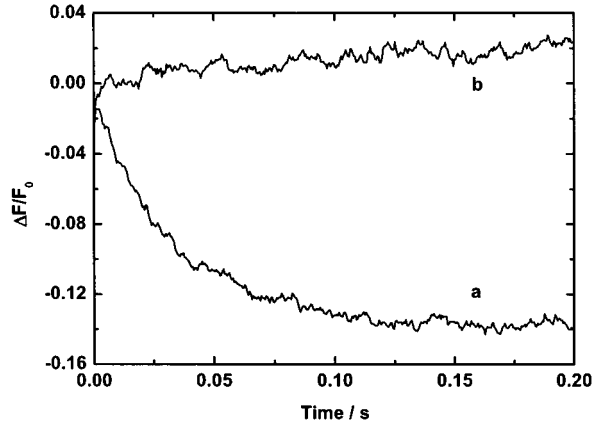

FIGURE 2: Stopped-flow fluorescence transients of rabbit kidney Na^+,K^+-ATPase. Curve a: 40 μg/mL (before mixing) of Na^+,K^+-ATPase labeled with 150 nM RH421 was mixed simultaneously with the same volume of a solution containing 100 mM NaCl and 2 mM tris ATP (pH 7.4, 24 °C). Both the enzyme suspension and the NaCl/tris ATP solution were prepared in a solution containing 25 mM histidine and 0.1 mM EDTA. The fluorescence of membrane-bound RH421 was measured at an excitation wavelength of 577 nm at emission wavelengths of ≥665 nm (RG665 glass cutoff filter). The calculated reciprocal relaxation time was 32 ± 1 s^{-1}. Curve b: The same as curve a, except that the enzyme was equilibrated with 1.5 mM sodium orthovanadate prior to mixing with NaCl and tris ATP.

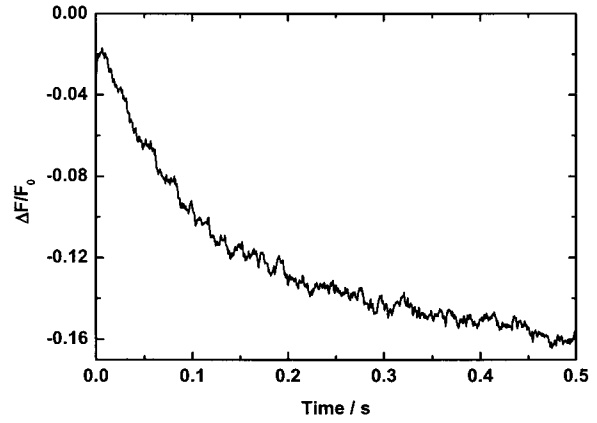

FIGURE 3: Stopped-flow fluorescence transient of rabbit kidney Na^+,K^+-ATPase on mixing with NaCl alone. 40 μg/mL (before mixing) of Na^+,K^+-ATPase labeled with 150 nM RH421 was mixed simultaneously with the same volume of a solution containing 600 mM NaCl (pH 7.4, 24 °C). Both the enzyme and the NaCl solution were prepared in a solution containing 25 mM histidine and 0.1 mM EDTA. The wavelengths for excitation and emission were as specified in Figure 2. A fit of a biexponential time function to the data yielded reciprocal relaxation times of 20 ± 2 s^{-1} (38% of the total amplitude) and 5.2 ± 0.5 s^{-1} (62% of the total amplitude).

double-exponential time function was necessary to describe the data, with both phases causing a fluorescence decrease (see Figure 3). This is in agreement with a previous report (*17*), in which it was furthermore found that the double exponential kinetic behavior disappeared on increasing the ATP concentration above a concentration of approximately 50 μM. The double exponential behavior could perhaps be attributed to the presence of more than one conformational state prior to mixing or to the presence of more than one class of Na^+ sites, e.g., allosteric sites and transport sites. Further investigations of the kinetics in the absence of ATP are planned. Here we shall concentrate, however, on measurements at saturating ATP concentrations, since this is of more relevance to the functioning of the enzyme under physiological conditions.

Before proceeding to investigate the Na^+-concentration dependence of the observed fluorescence change, we performed two control experiments to establish that the effect observed is actually arising from a reaction specific to the Na^+,K^+-ATPase. First of all, 1.5 mM of sodium orthovanadate was added to the drive syringe containing the Na^+,K^+-ATPase membrane fragments. This resulted in the complete disappearance of the fluorescence decrease (see Figure 2, curve b). In fact, a slight increasing trend in the fluorescence was then observed. Such an increase in fluorescence is also observed for RH421 bound to lipid vesicles in the absence of protein. This effect can, therefore, be attributed to a photochemical reaction of membrane-bound dye (*16*). Since it is believed that vanadate inhibits the enzyme by binding at the phosphate discharge site and stabilizing the enzyme in the E_2 state (*43*), this suggests that the fluorescence decrease is due to a reaction starting from the E_2 conformation.

A second control experiment was performed by preequilibrating the enzyme with 1.5 mM ouabain, a specific inhibitor of the Na^+,K^+-ATPase. In this case, the fluorescence change was not entirely abolished, but the reciprocal relaxation time of the change decreased dramatically, from a value of 51 ± 1 s^{-1} after mixing with 100 mM NaCl and 1 mM tris ATP (concentrations after mixing) in the absence of ouabain to 2.9 ± 0.3 s^{-1} in its presence. There was also a small reduction in the amplitude of the change ($-\Delta F/F_o$), from ~0.13 in the absence of ouabain to ~0.11 in its presence. Ouabain is thought to inhibit the enzyme reversibly by binding to the E_2P or, in this case, the E_2 conformation (*43*). As in the case of vanadate, the inhibition of the signal is consistent with it arising from a reaction beginning in the E_2 conformation. The absence of complete inhibition may be due to the lack of Mg^{2+} ions in the buffer, which are known to significantly enhance ouabain binding (*44*).

To investigate the Na^+ specificity of the fluorescence change, experiments were performed in which the enzyme was mixed with choline chloride rather than NaCl. Tris ATP was omitted in this case, since tris ATP alone causes a fluorescence decrease. It was found that mixing with 600 mM choline chloride also produced a fluorescence decrease. In contrast to the measurements carried out using NaCl, however, the fluorescence change induced by choline chloride did not appear to show biexponential behavior. A monoexponential time function was found to adequately fit the data. The amplitude of the change ($-\Delta F/F_o$) was in this case ~0.07, and the reciprocal relaxation time was 11 ± 1 s^{-1}, which is on the same order of magnitude as the values observed (20 and 5 s^{-1} for the fast and slow phases, respectively) on mixing with NaCl alone. The fact that a signal was observed under these conditions is consistent with the frequently reported Na^+-like effect of a variety of cations, including choline (*3*, *6*−*13*). The choline-induced fluorescence change was completely abolished if the enzyme was pre-equilibrated with 1.5 mM sodium orthovanadate.

Effect of Na^+ Concentration. The reciprocal relaxation time for the ATP and Na^+-induced RH421 fluorescence change, $1/\tau$, was found to be dependent on the Na^+ ion concentration. $1/\tau$ increased with increasing Na^+ from a value of approximately 10 s^{-1} on mixing with tris ATP alone to a saturating

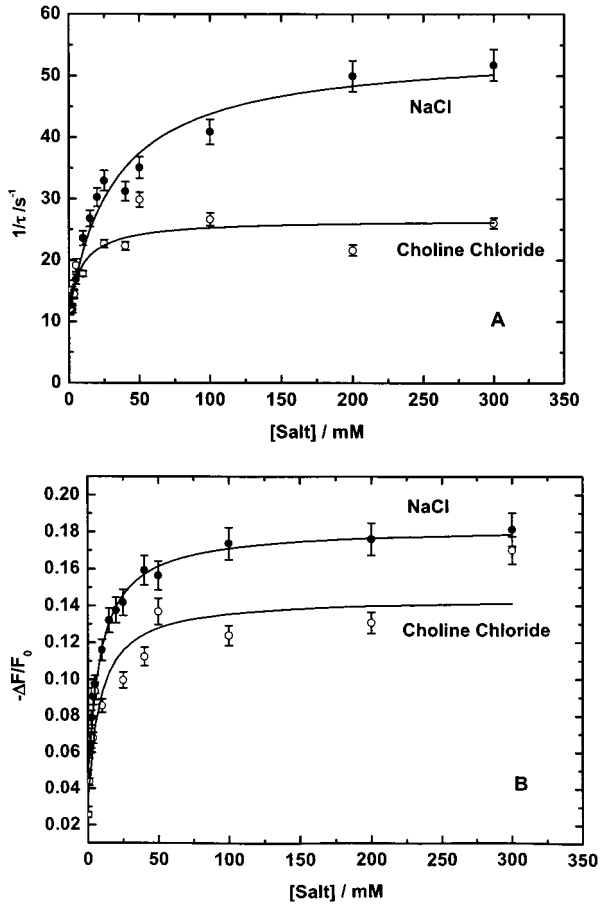

FIGURE 4: Effect of varying salt concentration, i.e., NaCl (filled circles) and choline chloride (open circles), on the reciprocal relaxation time ($1/\tau$) (panel A) and the relative fluorescence change ($-\Delta F/F_o$) (panel B) of RH421 fluorescence transients of rabbit kidney Na^+,K^+-ATPase induced by mixing simultaneously with salt plus 2 mM of tris ATP. The salt concentrations given are those after mixing. All other experimental conditions were the same as those given in Figure 2. The solid lines represent nonlinear least-squares fits of eq 4 (reciprocal relaxation times) and eq 5 (relative fluorescence changes) to the data.

value of approximately 50 s^{-1} at Na^+ concentrations $\geq$ 200 mM (see Figure 4A). The total relative fluorescence change, $-\Delta F/F_o$, also increased with increasing Na^+ concentration, from a value of ~0.05 in the absence of NaCl to a saturating value of ~0.18 (see Figure 4B). There was no obvious sigmoidicity apparent in the dependence of either $1/\tau$ or $\Delta F/F_o$ on the NaCl concentration. In both cases, the increase appeared to be hyperbolic, suggesting the lack of any cooperativity.

The fact that the reciprocal relaxation time reaches a maximum value indicates that the process being observed is not simply the binding of Na^+ to the enzyme, since this would be expected to show a linear dependence of the reciprocal relaxation time on the Na^+ concentration. Saturation of $1/\tau$ implies that Na^+ binding is coupled to a slower conformational change. The data cannot, however, be explained by the simple lock-and-key mechanism described in the Introduction, which involves binding of Na^+ to the E_1 conformation alone, since this mechanism predicts a decrease in $1/\tau$ with increasing Na^+ concentration. The simplest explanation is, therefore, that the observed process is a conformational change of the enzyme occurring after Na^+ binding, i.e., an induced-fit mechanism. Since it has previously been found that the RH421 fluorescence change also shows an increase in $1/\tau$ to a similar saturating value on increasing the ATP concentration (*16*, *17*), it would seem that the conformational change also occurs after ATP binding. The results are thus consistent with both Na^+ and ATP binding to enzyme in the E_2 state and stimulating either independently or together the transition to the E_1 state. As shown by the experiments of Forbush (*18*), in which the enzyme was initially in the $E_2(K^+)_2$ state, the conformational change also results in the release of K^+ ions from the enzyme. In the experiments reported here, the buffer solutions were nominally K^+-free, i.e., the background K^+ concentration was $\leq$25 μM. Whether this low K^+ ion concentration is sufficient to stabilize the enzyme initially in the $E_2(K^+)_2$ state is not entirely clear. Grell et al. (*13*) and Matsui and Homareda (*45*) have reported apparent dissociation constants of the enzyme for K^+ ions in the absence of Na^+ of 8 and 6 μM, respectively. On the basis of these values, it is possible that a significant proportion of the enzyme in our experiments may have had bound K^+ ions prior to mixing with NaCl. It is, however, very unlikely that the stimulation of the E_2-to-E_1 conformational transition is due to a competition between Na^+ and K^+ ions for the same sites, since, as discussed in the Introduction, the results of Forbush (*18*) and Hasenauer et al. (*19*) indicate that Na^+ and K^+ or its analogue Rb^+ bind simultaneously to the enzyme. It appears more likely, as suggested by Hasenauer et al. (*19*), that Na^+ is binding at a regulatory site on the enyzme.

A generalized reaction scheme allowing all sequences of Na^+ and ATP binding as well as the conformational change occurring either before or after Na^+ and ATP binding is shown in Figure 5. The derivation of the dependence of the reciprocal relaxation time, $1/\tau$, on the Na^+ and ATP concentrations for this mechanism is described in the Appendix (see eq A8). This equation can, however, be significantly simplified if one considers the results of previous investigations as well as those presented here. Stopped-flow measurements on rabbit kidney enzyme (*17*) at varying ATP concentrations previously suggested that the sum of the forward and backward rate constants for the conformational transition at saturating Na^+ concentrations but in the absence of ATP, $k_1 + k_{-1}$, is 0.8 $\pm$ 0.2 s^{-1} at 24 °C and pH 7.4. Because of the biexponential nature of the fluorescence transients observed on mixing with NaCl alone, however, a completely reliable estimation of values for k_1 and k_{-1} is difficult and still necessitates further investigation. Here reciprocal relaxation times of 5 and 20 s^{-1} were determined on mixing enzyme with 600 mM NaCl. On the basis of the present data available, therefore, one can only conclude that k_1 and k_{-1} have values not greater than 20 s^{-1}.

More precise values are available for the rate constants when both Na^+ and ATP are absent. In this case, the rate constants of the conformational change have been determined by experiments in which the conformational equilibrium was perturbed by the addition of inorganic phosphate (*46*−*48*). Measurements on rabbit kidney (*46*) yielded values of 0.023 $\pm$ 0.001 s^{-1} for k_0 and 0.059 $\pm$ 0.003 s^{-1} for k_{-0} at 21 °C and pH 7.1. Similar measurements carried out on enzyme from shark rectal gland yielded values of 0.95 s^{-1} (*47*; 20 °C and pH 7.5) and 0.4 s^{-1} (*48*; 24 °C and pH 7.4) for k_0 and values of 0.18 s^{-1} (*47*; 20 °C and pH 7.5) and 0.3 s^{-1} (*48*; 24 °C and pH 7.4) for k_{-0}. Values of K_A and K_A' are

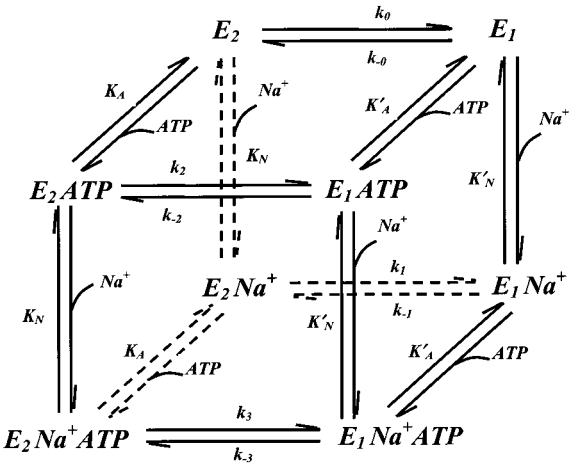

FIGURE 5: Generalized reaction scheme for the conformational change, Na^+ binding and ATP binding of unphosphorylated Na^+,K^+-ATPase. The scheme allows all possible sequences of the $E_2 \rightarrow E_1$ conformational change and substrate (Na^+ and ATP) binding. The scheme assumes that the substrate binding steps are in equilibrium on the time scale of the conformational change and are characterized by equilibrium dissociation constants of K_N and K_A for the binding of Na^+ and ATP, respectively, to the E_2 state and dissociation constants of K_N' and K_A' for the binding of the same substrates to the E_1 state. The rate constants k_0, k_1, k_2, and k_3 refer to the first-order rate constants for the conformational change $E_2 \rightarrow E_1$ when no substrate molecules are bound (k_0), one Na^+ is bound (k_1), one ATP is bound (k_2), and both one Na^+ and one ATP are bound (k_3). The rate constants k_{-0}, k_{-1}, k_{-2}, and k_{-3} are defined in the same way, but they refer to the reverse reaction, i.e., $E_1 \rightarrow E_2$. Note that the sites to which the Na^+ ions bind in this scheme are not necessarily transport sites.

also known from previous kinetic studies (*17*). For rabbit kidney enzyme, K_A and K_A' were determined at pH 7.4 and 24 °C to be 71 ± 7 and 8.0 ± 0.7 μM, respectively. Applying these values at the high ATP concentration used here of 1 mM after mixing, $[ATP]/K_A = 14.1$ and $[ATP]/K_A' = 125$, i.e., both much greater than 1. Equation A8 can, therefore, be simplified by approximating the terms $(1 + [ATP]/K_A)$ and $(1 + [ATP]/K_A')$ by $[ATP]/K_A$ and $[ATP]/K_A'$, respectively. Furthermore, using the values of k_0 and k_1 given above, the terms $k_0/([ATP]/K_A)$ and $k_1/([ATP]/K_A)$, with values of <0.07 and $<1.4\ s^{-1}$, are both much smaller than the smallest reciprocal relaxation time measured here ($\sim 10\ s^{-1}$). Similarly, the terms $k_{-0}/([ATP]/K_A')$ and $k_{-1}/([ATP]/K_A')$, with values of <0.002 and $<0.16\ s^{-1}$, are also much less than $10\ s^{-1}$. Under saturating ATP concentrations, the terms in equation A8 containing k_0, k_{-0}, k_1, and k_{-1} can, therefore, be neglected, and the equation describing the dependence of $1/\tau$ on the Na^+ concentration reduces to

$$\frac{1}{\tau} = \frac{k_2 + (k_3[Na^+]/K_N)}{1 + ([Na^+]/K_N)} + \frac{k_{-2} + (k_{-3}[Na^+]/K_N')}{1 + ([Na^+]/K_N')} \quad (3)$$

The meanings of the various parameters are defined in Figure 5 and its caption. In principle, this equation could explain an increase or a decrease in $1/\tau$, depending on whether the presence of bound Na^+ accelerates or retards the conformational transition. Experimentally, it has been observed (see Figure 4A), however, that $1/\tau$ increases with increasing Na^+ concentration, which means that bound Na^+ must accelerate the conformational transition.

Equation 3 describes in principle a two-phase hyperbolic increase in $1/\tau$. The first term describes the saturation of Na^+ binding to E_2 and saturates at the value of k_3. The second term describes the saturation of Na^+ binding to E_1 and saturates at the value of k_{-3}. The experimental data show, however, no evidence for a biphasic increase in $1/\tau$. This could be due to two factors. First, the rate constants for the backward reactions ($E_1 \cdot ATP \rightarrow E_2 \cdot ATP$ and $E_1 \cdot Na^+ \cdot ATP \rightarrow E_2 \cdot Na^+ \cdot ATP$), k_{-2} and k_{-3}, could be significantly smaller than the rate constant for the forward reaction ($E_2 \cdot ATP \rightarrow E_1 \cdot ATP$ and $E_2 \cdot Na^+ \cdot ATP \rightarrow E_1 \cdot Na^+ \cdot ATP$), k_2 and k_3, when Na^+ and ATP are bound. This is, however, certainly not the case in the absence of Na^+ and ATP (*46*−*48*). Second, if Na^+ bound with comparable strengths to both enzyme conformations, i.e., $K_N \approx K_N'$, then only one phase might be resolvable. This might seem unlikely, since it is generally believed that Na^+ binds much more strongly to the E_1 conformation than to the E_2 conformation. However, it is important to note that the Na^+ ions which are responsible for the stimulation of the $E_2 \rightarrow E_1$ transition are not necessarily the same Na^+ ions which bind to the transport sites. The enzyme may have allosteric Na^+ sites which are completely independent of the transport sites and whose binding constant is not significantly affected by the conformational state of the enzyme. To fit the data we have, therefore, approximated eq 3 by assuming that K_N and K_N' are equal. In this case, eq 3 reduces to

$$\frac{1}{\tau} \approx \frac{k_2 + k_{-2} + (k_3 + k_{-3})[Na^+]/K_N}{1 + ([Na^+]/K_N)} \quad (4)$$

Fitting of eq 4 to the data for rabbit kidney enzyme shown in Figure 4A yields the following parameters:

$$k_2 + k_{-2} \approx 11 \pm 1\ s^{-1}$$

$$k_3 + k_{-3} \approx 54 \pm 3\ s^{-1}$$

$$K_N \approx 31 \pm 7\ mM$$

Note that in the measurements in which enzyme was mixed with tris ATP in the absence of NaCl, the solution contained 2.5 mM tris (2.5 mol tris/mol ATP). Tris itself has been shown (*3*) to induce a transition to an E_1-like state, but only at significantly higher concentrations. The fluorescent transient observed at zero NaCl concentrations can, thus, be attributed to an ATP-induced conformational change.

Now let us consider the dependence of the relative change in fluorescence, $\Delta F/F_o$, on the Na^+ concentration. If one applies a simple noncooperative model for one-site Na^+ binding, $\Delta F/F_o$ should be related to the Na^+ concentration by

$$\frac{\Delta F}{F_o} = \left(\frac{\Delta F}{F_o}\right)_{min} + \left[\left(\frac{\Delta F}{F_o}\right)_{max} - \left(\frac{\Delta F}{F_o}\right)_{min}\right] \cdot \frac{[Na^+]}{K_N^{app} + [Na^+]} \quad (5)$$

where $(\Delta F/F_o)_{min}$ and $(\Delta F/F_o)_{max}$ are the relative fluorescence changes on mixing with ATP alone in the absence of Na^+

and on mixing with saturating concentrations of Na^+, respectively. K_N^{app} is an apparent dissociation constant of the enzyme for Na^+. Its value is not necessarily the same as K_N in eq 4 because K_N^{app} includes additional Na^+ binding which may occur after the conformational change to E_1 and may possibly perturb the conformational equilibrium. Fitting of eq 5 to the data for rabbit kidney enzyme shown in Figure 4B yields the following values of the parameters:

$$(\Delta F/F_o)_{min} = -0.048 \pm 0.002$$

$$(\Delta F/F_o)_{max} = -0.182 \pm 0.002$$

$$K_N^{app} = 9.1 \pm 0.6 \text{ mM}$$

The lack of any obvious sigmoidicity apparent in the dependence of either $1/\tau$ or $\Delta F/F_o$ on the NaCl concentration (see Figure 4A,B) is in marked contrast to the behavior observed in studies of the kinetics of phosphorylation of the enzyme by ATP (*16*, *17*). There the dependence of $1/\tau$ on the NaCl concentration was clearly sigmoid, indicating that more than one Na^+ site must be occupied before phosphorylation can occur. In fact, the data were fitted by a positive cooperative model involving the binding of three Na^+ ions. This is in agreement with the Albers−Post model, in which the binding of three Na^+ ions is considered to be necessary to allow ATP hydrolysis to proceed. In the case of the $E_2 \rightarrow E_1$ conformational change investigated here, however, the data could be adequately described by a model in which only a single Na^+ ion is necessary to stimulate the reaction.

The fact that the value of K_N^{app} is only a factor of less than 4 smaller than K_N would imply either that the perturbation in the equilibrium binding of Na^+ due to the conformational transition is not very great, i.e., k_3 and k_{-3} have similar values, or that the dissociation constants of these enzyme sites for Na^+ have similar values in the E_2 and E_1 states.

Effect of Choline Concentration. The reciprocal relaxation time for the ATP and choline$^+$-induced RH421 fluorescence change, $1/\tau$, was found to be dependent on the choline$^+$ ion concentration. $1/\tau$ increased from a value of approximately 10 s^{-1} on mixing with tris ATP alone to a saturating value of approximately 25 s^{-1} at choline$^+$ concentrations ≥ 100 mM (see Figure 4A). The total relative fluorescence change, $-\Delta F/F_o$, also increased with increasing choline$^+$ concentration, from a value of ∼0.03 in the absence of choline chloride to a saturating value of ∼0.14 (see Figure 4B). Both the reciprocal relaxation time and the relative fluorescence change observed at saturating choline chloride concentrations are significantly lower than those observed at saturating sodium chloride concentrations. It seems, therefore, that Na^+ is significantly more effective in stimulating the rate of the $E_2 \rightarrow E_1$ transition than choline$^+$. This is in agreement with the rapid filtration studies of Forbush (*18*).

As in the case of the sodium chloride titration data, the reciprocal relaxation times and the relative fluorescence changes obtained for the rabbit kidney enzyme at varying choline chloride concentrations were fitted to eqs 4 and 5, respectively, replacing [Na^+] by [choline$^+$] in each case. The values of the parameters derived were as follows:

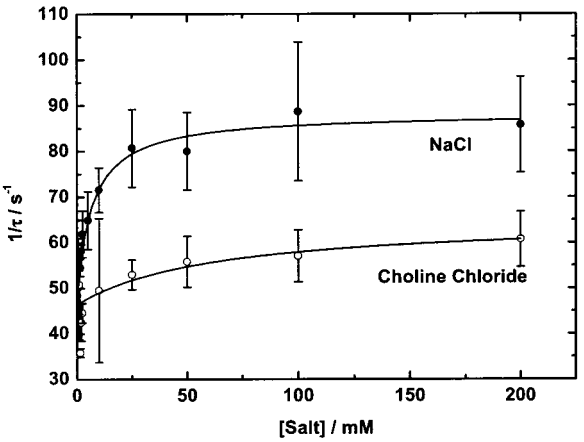

FIGURE 6: Effect of varying salt concentration, i.e., NaCl (filled circles) and choline chloride (open circles), on the reciprocal relaxation time ($1/\tau$) of RH421 fluorescence transients of shark rectal gland Na^+,K^+-ATPase induced by mixing simultaneously with salt plus 2 mM of tris ATP. The salt concentrations given are those after mixing. All other experimental conditions were the same as those given in Figure 2. The solid lines represent nonlinear least-squares fits of eq 4 to the data.

$$k_2 + k_{-2} \approx 13 \pm 2 \text{ s}^{-1}$$

$$k_3 + k_{-3} \approx 27 \pm 2 \text{ s}^{-1}$$

$$K_d \approx 11 \pm 9 \text{ mM}$$

$$(\Delta F/F_o)_{min} = -0.034 \pm 0.012$$

$$(\Delta F/F_o)_{max} = -0.144 \pm 0.010$$

$$K_d^{app} = 9 \pm 5 \text{ mM}$$

As in the case of Na^+, the similarity of the values of K_d and K_d^{app} implies either that the values of k_3 and k_{-3} have similar magnitudes or that the strengths of binding of choline$^+$ to the E_2 and E_1 states are similar.

Na^+ and Choline-Induced Stopped-Flow Results with Shark Enzyme. For comparison, experiments were also performed using enzyme derived from shark rectal gland. The approach used was analogous to that applied to the rabbit kidney Na^+,K^+-ATPase. The enzyme was pre-equilibrated in a solution containing 25 mM histidine and 0.1 mM EDTA and noncovalently labeled with RH421. It was subsequently mixed with a solution containing, in addition to 25 mM histidine and 0.1 mM EDTA, 2 mM tris ATP and different concentrations of either NaCl or choline chloride (between 0 and 400 mM). Upon mixing, a fluorescence decrease was observed in all cases. The transients could be fitted with monoexponential time functions. The relative fluorescence changes ($-\Delta F/F_o$) were always between 0.025 and 0.05. Because of the small amplitudes of the fluorescence changes, the signal-to-noise ratio is relatively high. The reciprocal relaxation times, $1/\tau$, are, therefore, only approximate. The reciprocal relaxation time was found to increase with increasing Na^+ concentration, from a value of 40−50 s^{-1} on mixing with 2 mM tris ATP alone up to a value of approximately 90 s^{-1} on mixing with 2 mM tris ATP plus 400 mM NaCl (see Figure 6). In the case of the experiments

where the enzyme was mixed with choline chloride solution, a slight increase in the reciprocal relaxation was also apparent but it was far less pronounced than with NaCl. On mixing with 400 mM choline chloride solution, the reciprocal relaxation time was found to have a value of approximately 60−70 s^{-1}.

Fitting of the experimental data shown in Figure 6 with eq 4 or its equivalent for choline yields the following parameters:
From the NaCl titration

$$k_2 + k_2 \approx 48 \pm 2\ s^{-1}$$

$$k_3 + k_3 \approx 88 \pm 2\ s^{-1}$$

$$K_N \approx 7 \pm 2\ mM$$

From the choline chloride titration

$$k_2 + k_2 \approx 46 \pm 2\ s^{-1}$$

$$k_3 + k_3 \approx 65 \pm 12\ s^{-1}$$

$$K_d \approx 62 \pm 104\ mM$$

The behavior observed with shark rectal gland enzyme is qualitatively similar to that found with the rabbit enzyme. In both cases, the reciprocal relaxation time increases with increasing Na^+ concentration. The amplitudes of the fluorescence changes of 0.025−0.05 are, however, clearly smaller than those observed for rabbit kidney enzyme (0.045−0.18), and the reciprocal relaxation times for shark enzyme (40−∼90 s^{-1} in the case of the NaCl mixing experiments) are higher than those of the rabbit enzyme (10−54 s^{-1}). The higher reciprocal relaxation times of the shark enzyme are consistent with its higher steady-state turnover number, which was recently calculated (*3*) on the basis of data of Cornelius (*49*) to be 70 s^{-1} at 23 °C and pH 7.0, in comparison to a value of 43 s^{-1} for rabbit kidney enzyme under similar experimental conditions (24 °C and pH 7.4).

The fluorescence changes observed for the shark enzyme can be explained in the same fashion as that of the rabbit enzyme, i.e., the enzyme is at least partially in the E_2 conformation when equilibrated in histidine/EDTA solution and undergoes a transition to the E_1Na^+ATP form on mixing with NaCl and tris ATP. The lower amplitudes of the shark enzyme might possibly be explained by the enzyme initially not totally being in the E_2 conformation. Even in the absence of Na^+ ions perhaps some of the enzyme is already in an E_1-like conformation. The differences in behavior observed here between rabbit kidney enzyme and shark rectal gland enzyme may be related to the different Na^+,K^+-ATPase isoforms present. Whereas rabbit kidney consists of the α_1 isoform, shark rectal gland enzyme is an α_3-like isoform (*50*, *51*). It is possible that the E_1 or an E_1-like conformation is more stable in enzyme of the α_3-isoform even at low cation concentrations. Another possible explanation for the lower amplitudes observed with the shark enzyme could be that the fluorescent probe RH421 is less sensitive to the conformational change in this preparation, perhaps due to a different lipid composition (*52*, *53*).

DISCUSSION

The kinetics of the rate-determining $E_2 \rightarrow E_1$ conformational transition of rabbit kidney Na^+,K^+-ATPase have been investigated via the stopped-flow technique by mixing enzyme fluorescently labeled with RH421 simultaneously with NaCl and ATP in the absence of Mg^{2+} ions (to prevent phosphorylation and halt turnover). A decrease in fluorescence was observed, whose time course could be fitted by a single-exponential time function. At a constant ATP concentration of 1 mM ATP after mixing, the reciprocal relaxation time was found to increase hyperbolically with increasing NaCl concentration to a saturating value of 56 ± 2 s^{-1}. Previous studies on enzyme from the same source (*17*) showed that the reciprocal relaxation time also increases hyperbolically with increasing ATP concentration. These results suggest that the conformational change can occur with both Na^+ and ATP bound to the enzyme and that the conformational change is stimulated by binding of Na^+ and ATP to the E_2 form of the enzyme. The results are thus consistent with an induced-fit model of Na^+ and ATP binding, as described in the Introduction, whereby a relaxation of the protein conformation occurs after substrate binding. Both Na^+ and ATP would seem, therefore, in addition to their roles as transported ions and in protein phosphorylation, respectively, to act in a regulatory fashion by stimulating the rate of conversion of the protein into the E_1 conformation and thus enhancing the overall turnover number of the enzyme.

The degree to which the stimulation of the conformational change by Na^+ ions does in fact cause an acceleration of the enzyme turnover under physiological conditions depends on where they bind, i.e., intra- or extracellularly. The results shown in Figure 4A indicate that significant stimulations of the $E_2 \rightarrow E_1$ transition and the overall turnover can only be expected at Na^+ concentrations up to approximately 200 mM, after which saturation of the Na^+ binding sites occurs. The degree to which fluctuations in the Na^+ concentration could modify the turnover number via an effect on the $E_2 \rightarrow E_1$ transition, therefore, decreases as the Na^+ concentration increases. According to electron microprobe results of Thurau (*54*), the typical intracellular Na^+ concentration of kidney tubule cells is approximately 16 mM, whereas that of the extracellular fluid is approximately 160 mM. If the Na^+ ions which stimulate the $E_2 \rightarrow E_1$ transition bind from the intracellular side, small variations in the intracellular Na^+ concentration would, thus, be expected to have a significant effect on the rate of the $E_2 \rightarrow E_1$ transition and hence on the turnover number. On the other hand, if the Na^+ ions bind from the extracellular side, only small effects on the turnover number would be expected because the extracellular Na^+ concentration is already approaching the saturating level (see Figure 4A). From a regulatory point of view, because Na^+ ions are pumped out of the cell, stimulation of the turnover by intracellular Na^+ would make more physiological sense for the enzyme, since this would increase its pumping rate and remove Na^+ ions from the cytoplasm if the intracellular Na^+ concentration increased above the desired level.

Since the experiments described here were performed on open membrane fragments with both sides of the enzyme

accessible to Na^+ ions, it is not possible from these experiments alone to decide whether the Na^+ ions are binding to the enzyme from the cytoplasmic or the extracellular face. Thus, it is also not possible to make any further conclusions about the degree to which Na^+ stimulation of the $E_2 \rightarrow E_1$ transition would modify the turnover number in an intact cell. Some light may be shed on these questions, however, by considering results obtained from other studies. On the basis of steady-state activity studies of the effect of sodium ion concentration on vanadate inhibition, Sachs (*55*) concluded that intracellular Na^+ does not bind to the enzyme prior to the release of K^+. On the basis of his results, it would seem, therefore, unlikely that Na^+ binding to the cytoplasmic face of the enzyme is responsible for acceleration of the $E_2 \rightarrow E_1$ transition. Particularly interesting results have been observed using sided preparations of enzyme reconstituted into lipid vesicles. Using reconstituted pig kidney enzyme, Karlish and Stein (*56*) found that the sigmoidicity of cytoplasmic Na^+ activation of the enzyme's steady-state activity decreased with an increase in the extracellular Na^+ concentration. On the basis of their results, they concluded that "allosteric" Na^+ sites on the extracellular surface of the enzyme were occupied simultaneously with Na^+ transport sites on the cytoplasmic surface. Similar to the Na^+ effect on the $E_2 \rightarrow E_1$ transition studied here, Karlish and Stein found that the effect they observed was not specific to Na^+ but could also be induced to a lesser extent by Li^+ and tris ions. They estimated an apparent affinity of the allosteric Na^+ sites of roughly 50 mM. This value is of the same order of magnitude as that found here for the dissociation constant of Na^+ ions interacting with the E_2 form of rabbit kidney enzyme, i.e., 31 ± 7 mM. The reasonable agreement in the values may, however, be fortuitous because agreement between values derived from transient kinetic measurements and steady-state is not necessarily expected, if in the steady state Na^+ binding is followed by further reactions which perturb the Na^+ binding equilibrium. Further support for allosteric extracellular Na^+ binding can be found in measurements of Cornelius and Skou (*57*) using reconstituted shark rectal gland enzyme. They found that extracellular Na^+ enhanced the ATP hydrolytic activity of the enzyme with a high affinity of about 2 mM. This is also in accord with the higher Na^+ dissociation constant found here for the shark rectal gland enzyme of 7 ± 2 mM. Very interesting and relevant are also the results of van der Hijden and de Pont (*58*) on reconstituted rabbit kidney enzyme. They found that the steady-state phosphorylation level of the enzyme was enhanced by extracellular Na^+ ions and amine buffers (e.g., tris). In their interpretation of this effect, they considered that the most likely explanation was that extracellular ligands induce a transition to the E_1 conformation of the enzyme, which binds ATP and can be subsequently phosphorylated. The stopped-flow results reported here would certainly support such an interpretation. On the basis of all of these results, it appears very likely, therefore, that the Na^+-induced stimulation of the $E_2 \rightarrow E_1$ transition found here is in fact due to Na^+ binding to extracellular sites.

Now let us compare the reciprocal relaxation time measured at saturating NaCl and ATP of 54 ± 3 s^{-1} to recent estimates of the rate of the $E_2 \rightarrow E_1$ transition under phosphorylating conditions (i.e. saturating concentrations of Na^+, ATP, and Mg^{2+}, pH 7.4, 24 °C) of 90 s^{-1} for rabbit kidney enzyme and 65 s^{-1} for pig kidney enzyme (*3*). The slightly higher values measured under phosphorylating conditions may be due to a further slight stimulation of the rate of the conformational transition by Mg^{2+} ions, which were omitted in the present studies so that the transition could be uncoupled from subsequent phosphorylation.

Interestingly, it has been found that the conformational transition is also observed on mixing with choline chloride. This is in accord with kinetic studies of Skou and Esmann (*7*) and Esmann (*26*) using the probe eosin in the absence of ATP, with similar studies of Doludda et al. (*12*) using enzyme covalently labeled with FITC and with ^{42}K deocclusion experiments of Forbush (*18*). Acceleration of the $E_2 \rightarrow E_1$ transition and stabilization of the E_1 conformation by choline chloride and buffer cations has generally been interpreted as a Na^+-like action (*3*, *6*−*13*). Any increase in the cation concentration must, however, be accompanied by an increase in the anion concentration in order to maintain charge neutrality in the solution. Therefore, since other salts can replace sodium chloride in stimulating the transition, one might wonder whether the effect is actually due to cation binding to a site on the enzyme or whether instead it may be an ionic strength effect, as has been suggested by Smirnova and Faller (*31*). A change in ionic strength could, for example, easily lead to a change in enzyme conformation without direct ion binding via a charge screening effect according to Debye−Hückel theory. If the positive and negative charges of a salt bridge, e.g., between a carboxylate residue and a protonated amino residue, are shielded by intervening ions, the resultant weakening of the interaction could lead to a conformational change. Evidence for a salt bridge between transmembrane segments of the yeast plasma membrane H^+-ATPase, a P-type ATPase related to the Na^+,K^+-ATPase, was in fact recently reported by Gupta et al. (*59*) on the basis of steady-state kinetic measurements on site-directed mutants. According to their measurements the putative salt bridge of the H^+−ATPase is not directly involved in H^+-pumping, but it is essential for the correct folding of the protein and its insertion into the plasma membrane.

Grell et al. (*11*), on the other hand, favor a model whereby it is the cation alone, e.g., Na^+ or choline$^+$, which induces the conformational change. They have suggested a mechanism in which the cations coordinate electrostatically to two negatively charged carboxylate amino acid side-chains of the protein. As a consequence of the electrostatic interaction the two side chains could be drawn closer together, thus triggering the rearrangement from E_2 to E_1.

At this stage, it is not possible to clearly distinguish between these two possible mechanisms, i.e., an electrostatic cation coordination or an ionic strength charge screening. To do this, both a truly inert cation and a truly inert anion would have to be found. If such ions cannot be found, this would tend to lend support to the ionic strength screening mechanism. Nevertheless, the fact that NaCl induces a higher maximum rate of the transition than choline chloride (see Figure 4A) suggests at least some cation selectivity is present, i.e., Na^+ ions provide a greater acceleration than choline ions.

Irrespective of the exact molecular mechanism of the conformational change, it is, however, clear from the results presented here that the rate-determining $E_2 \rightarrow E_1$ transition is stimulated by the interaction of Na^+ or Na^+ and Cl^- with

the enzyme in the E_2 state, thus inducing a protein conformational relaxation into the E_1 state (i.e. an induced-fit mechanism). The results are not explicable by an alternative model, whereby Na^+ or Na^+ and Cl^- interact preferentially with the E_1 conformation alone and shift the conformational equilibrium in favor of E_1. Similar to the regulatory role played by ATP, it thus appears that salt or Na^+ ions also help to regulate the enzyme by stimulating K^+ release and its conversion to the E_1 conformation, in which further Na^+ ions can be bound and the enzyme can undergo phosphorylation. Since the $E_2 \rightarrow E_1$ transition is the rate-determining step, the Na^+ or salt effect on its rate will increase the overall turnover number of the enzyme.

Whether or not the Na^+ ion, which stimulates the conformational transition, is binding to one of the Na^+ transport sites is still, however, an open question. On the basis of experiments of Forbush (*18*) and Hasenauer et al. (*19*), the Na^+ ions stimulating the $E_2 \rightarrow E_1$ transition bind prior to the release of K^+ or Rb^+, so they cannot be binding to at least two of the transport sites, which are already occupied. Since in the ATP-dependent Na^+-Na^+ exchange mode of the enzyme, three Na^+ ions are transported from the cytoplasm to the extracellular fluid and only two Na^+ ions are transported in the opposite direction, Cornelius and Skou (*57*) considered that it was reasonable to assume that it is one of the three Na^+ transport sites exposed to the extracellular fluid which causes the allosteric effect. Although this is an attractive explanation, further experimental support would be necessary.

APPENDIX

Reciprocal Relaxation Time for NaCl Mixing Experiments. To describe the regulatory Na^+ and ATP concentration dependence of the reciprocal relaxation time, $1/\tau$, we have used the general reaction scheme shown in Figure 5. The reaction scheme incorporates the following essential points: (1) both Na^+ and ATP can bind to enzyme in both the E_1 and E_2 conformations; (2) the Na^+ and ATP binding steps are always in equilibrium on the time scale of the conformational change; (3) the dissociation constants of the enzyme for Na^+ and ATP in each conformation are assumed to be independent of whether ATP and Na^+ are bound, respectively; (4) all possible sequences of substrate binding and conformational change are allowed.

To derive an equation for $1/\tau$ containing the equilibrium and rate constants for the various steps as well as the total Na^+ and ATP concentrations, we have applied the theory of relaxation kinetics, as developed by Eigen (*60*) and applied by Kirschner and co-workers (*61−63*) to the kinetics of the allosteric enzyme glyceraldehyde-3-phosphate dehydrogenase. Normally this theory is applied to systems close to equilibrium, e.g., as in a temperature jump experiment. Under pseudo-first-order conditions, however, i.e., in our case excess of both Na^+ and ATP over enzyme, the theory is also applicable to stopped-flow data.

The total concentrations of enzyme in the E_2 and E_1 states are given by

$$\sum E_2 = E_2 + E_2Na^+ + E_2ATP + E_2Na^+ATP \quad \text{(A1)}$$

$$\sum E_1 = E_1 + E_1Na^+ + E_1ATP + E_1Na^+ATP \quad \text{(A2)}$$

The reaction scheme shown in Figure 5 can now be simplified to an overall reaction between the E_2 and E_1 states:

$$\sum E_2 \rightleftharpoons \sum E_1$$

The rate of change of the deviation of the total concentration in the E_2 states from its final equilibrium value is given by

$$-\frac{d\Delta\sum E_2}{dt} = k_0\Delta E_2 - k_{-0}\Delta E_1 + k_1\Delta E_2Na^+ - k_{-1}\Delta E_1Na^+ + k_2\Delta E_2ATP - k_{-2}\Delta E_1ATP + k_3\Delta E_2Na^+ATP - k_{-3}\Delta E_1Na^+ATP \quad \text{(A3)}$$

To integrate this differential equation and derive an expression for the reciprocal relaxation time, we must first derive equations relating ΔE_2, ΔE_1, ΔE_2Na^+, ΔE_1Na^+, ΔE_2ATP, ΔE_1ATP, ΔE_2Na^+ATP, and ΔE_1Na^+ATP to $\Delta\Sigma E_2$. For this, we make use of the assumption that the Na^+ and ATP binding steps are all independently in equilibrium on the time scale of the relaxation of the conformational change. The dissociation constants for the E_2 and E_1 states for Na^+ and ATP are defined as follows:

$$K_N = \frac{E_2{\cdot}Na^+}{E_2Na^+} = \frac{E_2ATP{\cdot}Na^+}{E_2Na^+ATP} \quad \text{(A4)}$$

$$K_N' = \frac{E_1{\cdot}Na^+}{E_1Na^+} = \frac{E_1ATP{\cdot}Na^+}{E_1Na^+ATP} \quad \text{(A5)}$$

$$K_A = \frac{E_2ATP}{E_2ATP} = \frac{E_2Na^+{\cdot}ATP}{E_2Na^+ATP} \quad \text{(A6)}$$

$$K_A' = \frac{E_1{\cdot}ATP}{E_1ATP} = \frac{E_1Na^+{\cdot}ATP}{E_2Na^+ATP} \quad \text{(A7)}$$

At excess concentrations of Na^+ and ATP over enzyme, it can be assumed that the free concentrations of Na^+ and ATP are constant for the duration of the stopped-flow experiment. Under these conditions, one can derive from eqs A4-A7 and the laws of conservation of mass (eqs A1 and A2) expressions for the deviations of the various enzyme species from their equilibrium values in terms of $\Delta\Sigma E_2$. Substituting these expressions into eq A3 and integrating, it can be shown that the reciprocal relaxation time is given by

$$\frac{1}{\tau} = \frac{k_0 + k_1[Na^+]/K_N + k_2[ATP]/K_A + k_3[Na^+][ATP]/(K_NK_A)}{(1 + [Na^+]/K_N)(1 + [ATP]/K_A)} + \frac{k_{-0} + k_{-1}[Na^+]/K_N' + k_{-2}[ATP]/K_A' + k_{-3}[Na^+][ATP]/(K_N'K_A')}{(1 + [Na^+]/K_N')(1 + [ATP]/K_A')} \quad \text{(A8)}$$

This equation describes in principle the dependence of $1/\tau$ on $[Na^+]$ and [ATP] for any sequence of substrate binding and conformational change. The equation can, however, be significantly simplified if one considers particular conditions, e.g., saturating $[Na^+]$ or saturating [ATP]. An equation for any more restricted mechanism, e.g., no binding of Na^+ to

E_2 or no conformational change with bound Na^+, can also be derived from it by setting the appropriate equilibrium dissociation constants to infinity or rate constants to zero.

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