Biophysical Journal Volume 77 July 1999 267–281

Hofmeister Effects of Anions on the Kinetics of Partial Reactions of the Na^+,K^+-ATPase

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ABSTRACT The effects of lyotropic anions, particularly perchlorate, on the kinetics of partial reactions of the Na^+,K^+-ATPase from pig kidney were investigated by two different kinetic techniques: stopped flow in combination with the fluorescent label RH421 and a stationary electrical relaxation technique. It was found that 130 mM $NaClO_4$ caused an increase in the K_d values of both the high- and low-affinity ATP-binding sites, from values of 7.0 (± 0.6) μM and 143 (± 17) μM in 130 mM NaCl solution to values of 42 (± 3) μM and 660 (± 100) μM in 130 mM $NaClO_4$ (pH 7.4, 24°C). The half-saturating concentration of the Na^+-binding sites on the E_1 conformation was found to decrease from 8–10 mM in NaCl to 2.5–3.5 mM in $NaClO_4$ solution. The rate of equilibration of the reaction, $E_1P(Na^+)_3 \leftrightarrow E_2P + 3Na^+$, decreased from 393 (± 51) s^{-1} in NaCl solution to 114 (± 15) s^{-1} in $NaClO_4$. This decrease is attributed predominantly to an inhibition of the $E_1P(Na^+)_3 \rightarrow E_2P(Na^+)_3$ transition. The effects can be explained in terms of electrostatic interactions due to perchlorate binding within the membrane and/or protein matrix of the Na^+,K^+-ATPase membrane fragments and alteration of the local electric field strength experienced by the protein. The kinetic results obtained support the conclusion that the conformational transition $E_1P(Na^+)_3 \rightarrow E_2P(Na^+)_3$ is a major charge translocating step of the pump cycle.

INTRODUCTION

It has been well established over many years that various inorganic anions are able to influence the effectiveness of various membrane-related physiological processes. For example, Kahn and Sandow (1955) found that nitrate and other anions cause an increase in the twitch tension of whole muscle. Similar observations were made by Hodgkin and Horowicz (1960) on single muscle fibers. Dani et al. (1983) reported that some anions, particularly thiocyanate and perchlorate, reversibly shift the voltage dependence of sodium channels of skeletal muscle, and Rychkov et al. (1998) recently found a similar effect on the voltage dependence of gating and the blocking potency of skeletal muscle ClC-1 chloride channels.

The order of anion effectiveness in influencing the various processes has generally been found to agree with the so-called Hofmeister, lyotropic, or chaotropic series (Collins and Washabaugh, 1985; Cacace et al., 1997), which was first described by Hofmeister (1888) based on the ability of neutral salts to precipitate egg globulin. Although slightly varying series have been reported, depending on the physiological process or protein under investigation, the effects of some common anions appear to increase in the approximate order $Cl^- < Br^- < NO_3^- < I^- < SCN^- < ClO_4^-$. Hodgkin and Horowicz (1960) noted that this order corresponds to the anions' "adsorbability" and suggested that the effects of the ions on membrane processes might result from their adsorption to the surface of the membrane, resulting in an alteration in the electric field strength.

The subject of the present paper is the effect of lyotropic ions on the kinetics of the electrogenic ion pump, the Na^+,K^+-ATPase. It was first shown by Post and Suzuki (1991) that lyotropic anions affect the equilibrium between two phosphorylated conformations of the enzyme, E_1P and E_2P. Their findings were subsequently given further support by measurements of Klodos (1991), Klodos and Plesner (1991), and Klodos et al. (1994), who showed that lyotropic anions decrease the rate of equilibration between the E_1P and E_2P conformations. No general agreement, however, has yet been reached as to the mechanism of how lyotropic anions modify the behavior of the enzyme. Post and Suzuki (1991) suggested that the anions might be acting by modifying the folding of the protein or by modification of the structure of the specific water molecule that attacks the active site of the phosphate group. An anion-induced change in the structure of the protein has also been suggested by Klodos et al. (1994). Post and Klodos (1996) attributed the effects to changes in the water structure. Some doubt in these hypotheses has, however, been raised by Nørby and Esmann (1997), who investigated the effects of various salts on the affinity of the Na^+,K^+-ATPase for ADP. Nørby and Esmann (1997) stressed the importance of electrostatic effects and showed that the effect of the salts NaCl and Na_2SO_4 on the ADP binding affinity could be treated as a pure ionic strength effect via the Debye-Hückel theory, i.e., due to screening of charge on the protein surface by the anions and cations in the adjacent aqueous medium. Nevertheless, Nørby and Esmann (1997) did note more pronounced effects of some salts, in particular $NaNO_3$, NaSCN, and $NaClO_4$, which could not be explained by the Debye-

Received for publication 2 February 1999 and in final form 27 April 1999.

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0006-3495/99/07/267/15 $2.00

Hückel theory alone. They suggested that the anions of these salts undergo a specific interaction with the protein and that the binding of NO_3^- to the protein is close to being competitive with ADP. At this point it is important to note, however, that the anions with which Nørby and Esmann (1997) obtained the greatest effects, i.e., NO_3^-, SCN^-, and ClO_4^-, belong to the lyotropic anions. It is reasonable to assume, therefore, that there should indeed be some specific lyotropic effect of these anions on the relative stability of the various enzyme conformations.

An explanation for the effects of lyotropic anions on the Na^+,K^+-ATPase that is an alternative to the ideas of changes in the protein or water structure is the electric field model, initially proposed by Hodgkin and Horowicz (1960) to explain lyotropic anion effects on muscle twitch tension. As stated earlier, this model incorporates the idea that lyotropic anions bind to the surface of the membrane, thus altering the electric field strength within the membrane. In this way they could stabilize or destabilize particular charged conformations of a membrane protein. Because the Na^+,K^+-ATPase is an electrogenic ion pump that translocates charge across the cell membrane, it is continually undergoing changes in the charge distribution of its conformation. It is reasonable to expect, therefore, that certain charge-translocating steps of the enzyme's reaction cycle might be affected by the local electric field strength. An electric field model has previously been employed by various authors (Stürmer et al., 1991; Klodos et al., 1995; Cornelius, 1995) to explain the effects of the hydrophobic ions tetraphenylborate (TPB^-) and tetraphenylphosphonium (TPP^+) on partial reactions of the Na^+,K^+-ATPase. Because it is well known (Liberman and Topaly, 1969; Flewelling and Hubbell, 1986) that these ions bind within phospholipid membranes and alter the intramembrane electric field strength, Stürmer et al. (1991), Klodos et al. (1995), and Cornelius (1995) all assumed that the electric field created by the ions within the lipid matrix was the cause for the effects on the partial reactions of the Na^+,K^+-ATPase. In principle, however, it cannot be excluded that such ions could also bind to hydrophobic domains within the protein itself or near the protein surface and there also induce changes in the electric field strength.

In a recent paper it was shown by Clarke and Lüpfert (1999) from measurements using the voltage-sensitive dyes RH421 and di-8-ANEPPS that lyotropic anions bind to phosphatidylcholine (PC) vesicles and significantly reduce the intramembrane electrical dipole potential. These findings, therefore, give added weight to the hypothesis of Hodgkin and Horowicz (1960). The order of effectiveness of the anions in reducing the dipole potential was found by Clarke and Lüpfert (1999) to be $ClO_4^- > SCN^- > I^- > NO_3^- > Br^- > Cl^- > F^- > SO_4^{2-}$. Therefore, to judge whether the electric field model is a feasible explanation for the effects of lyotropic anions on the Na^+,K^+-ATPase, it is first important to establish whether the effects of the anions on the enzyme follow the same order of effectiveness as that found for their effects on the dipole potential. If it can be shown that lyotropic anions do in fact act on the Na^+,K^+-ATPase by changing the local electric field strength, then this opens the way for the interesting possibility of using them to identify electrogenic reaction steps of the enzyme cycle, because it is to be expected that the major electrogenic steps would be most affected by changes in the local electric field strength. As in the case of TPB^- and TPP^+, however, it should be noted that the exact location of the electric field induced by lyotropic anions is unclear. Although the measurements of Clarke and Lüpfert (1999) show that they bind within the lipid matrix, it has been known since the work of Hofmeister (1888) that they also interact with water-soluble proteins. It is therefore to be expected that they could also interact with hydrophobic domains of the Na^+,K^+-ATPase and induce a change in local electric field strength directly within the protein matrix.

To resolve the kinetics of the partial reactions of the Na^+,K^+-ATPase, we have applied two different kinetic methods. The first is that of stopped-flow in combination with the fluorescence probe RH421. This method has previously proved very useful in resolving the kinetics of the enzyme in the absence of lyotropic anions (Pratap and Robinson, 1993; Kane et al., 1997; Clarke et al., 1998; Cornelius et al., 1998). The second method is a stationary electrical relaxation technique, whereby electrogenic steps of the Na^+,K^+-ATPase are perturbed by an alternating electric field of varying frequency. This method was first applied to the Na^+,K^+-ATPase by Sokolov et al. (1992). In principle it allows the determination of rate constants for fast electrogenic steps of the Na^+,K^+-ATPase enzyme cycle, which in the case of the stopped-flow method could be rate-limited by preceding nonelectrogenic steps. The two methods therefore yield useful complementary information. Comparison of the results obtained here with those previously reported enables the effects of lyotropic anions on individual rate constants and equilibrium constants to be identified.

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. RH421 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 were prepared and purified from the red outer medulla of pig kidney according to a modification (Fendler et al., 1985) of procedure C of Jørgensen (1974a,b), as described previously (Kane et al., 1997, 1998). The specific ATPase activity at 37°C and pH 7.5 was ~2400 μmol of P_i h^{-1} (mg of protein)$^{-1}$ in 30 mM histidine (Microselect, Fluka)/HCl containing 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, and 3 mM ATP (Boehringer Mannheim), and its protein concentration was typically 1.5 mg/ml. The enzymatic activity in the presence of 1 mM ouabain was less than 1%. The protein concentration was determined by the Lowry method (Lowry et al., 1951) and was subsequently divided by the correction factor of 1.35 determined from amino acid analysis (Peters et al., 1981). For the calculation of the molar protein concentration, a molecular mass for an $\alpha\beta$ unit of the Na^+,K^+-ATPase of 147,000 g mol^{-1} (Jørgensen and Andersen, 1988) was assumed.

Stopped-flow experiments were carried out using an SF-61 stopped-flow spectrofluorimeter from Hi-Tech Scientific (Salisbury, England). Details of the experimental set-up have been described elsewhere (Kane et al., 1998; Clarke et al., 1998). Each kinetic trace consisted of 1024 data points. To improve the signal-to-noise ratio, typically between 6 and 14 experimental traces were averaged before the reciprocal relaxation time was evaluated. The error bars shown in the figures correspond to the standard error of a fit of the averaged experimental trace of a set of measurements to a sum (either one or two) of exponential functions. Nonlinear least-squares fits of the reciprocal relaxation times to appropriate kinetic models were performed as previously described (Clarke et al., 1998). The errors quoted for the parameters determined (rate and equilibrium constants) correspond to the standard errors derived from the fits.

In one type of experiment, (a), the kinetics of phosphorylation, associated conformational changes and ion translocation reactions of the Na^+,K^+-ATPase were investigated in the stopped-flow apparatus by mixing Na^+,K^+-ATPase labeled with RH421 in one of the drive syringes with an equal volume of an ATP solution from the other drive syringe. The two solutions were prepared in the same buffer (composition given below), so that no change in the concentration of Na^+ or its associated anion occurred on mixing. Because RH421 concentrations above 1 μM are known to inhibit the steady-state hydrolytic activity (Frank et al., 1996) and the transient kinetics of Na^+-dependent partial reactions of the Na^+,K^+-ATPase (Kane et al., 1997), a noninhibitory RH421 concentration of 150 nM was used in the enzyme solution. The dead-time of the stopped-flow mixing cell was determined to be 1.7 ($\pm$ 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. The measurements were performed in a buffer containing 30 mM imidazole, 5 mM $MgCl_2$, and 1 mM EDTA at 24°C. The pH of the buffer was adjusted to 7.4 with HCl. For experiments at saturating Na^+ concentrations, in addition 130 mM of one of NaCl, NaBr, NaI, $NaNO_3$, NaSCN, or $NaClO_4$ was added to the buffer. In the case of experiments in which the $NaClO_4$ concentration was varied, choline perchlorate was added to the buffer medium to maintain a total concentration of $NaClO_4$ plus choline perchlorate of 130 mM. The total ionic strength was therefore kept constant at a value of 145 mM (excluding contributions from imidazole and EDTA). The pH of the ATP solutions were adjusted to pH 7.4 with *N*-methyl-D-glucamine.

In a second type of experiment, (b), the kinetics of K^+-stimulated dephosphorylation of the Na^+,K^+-ATPase were investigated in the stopped-flow apparatus by premixing Na^+,K^+-ATPase labeled with RH421 in one of the drive syringes with a small volume of 0.5 M Na_2ATP to a final ATP concentration of 2.0 mM. After phosphorylation of the enzyme had reached a steady state (this occurs within 2 s), the enzyme was mixed with an equal volume of a $KClO_4$ solution from the other drive syringe. Over the time necessary to perform the experiment, the decrease in the ATP concentration in the enzyme syringe can be considered to be negligible. If one assumes that the enzyme is hydrolyzing ATP at a rate of $\sim$5 s^{-1}, i.e., the rate determined for NaCl solution (Hobbs et al., 1980; Kane et al., 1998), for an enzyme concentration of 0.3 μM, it can be shown that after 2 s only $\sim$3 μM ATP has been consumed. To avoid any change in ionic strength on mixing, the $KClO_4$ solutions contained choline perchlorate of varying concentrations such that the total concentration of $KClO_4$ plus choline perchlorate was constant at 40 mM, and the same total concentration of choline perchlorate was included in the enzyme solution. The enzyme solution was prepared in a buffer containing, in addition to choline perchlorate, 30 mM imidazole, 130 mM $NaClO_4$, 5 mM $MgCl_2$, and 1 mM EDTA. The $KClO_4$ solutions were prepared in the same buffer.

In a third type of experiment, (c), the kinetics of K^+ dissociation, Na^+ binding, and the associated conformational change of unphosphorylated enzyme were investigated in the stopped-flow apparatus by mixing Na^+,K^+-ATPase labeled with RH421 and in the presence of 1 mM $KClO_4$ with an equal volume of 130 mM $NaClO_4$ and varying concentrations of Na_2ATP. Both solutions were in a buffer containing 30 mM imidazole and 1 mM EDTA, but in this case Mg^{2+} ions were omitted to prevent the phosphorylation reaction from occurring.

Each data set, in which the concentration of Na^+, K^+, or ATP was varied, was collected using a single Na^+,K^+-ATPase preparation. Solutions of choline perchlorate (130 mM or 40 mM), used for maintaining a constant ionic strength and a constant perchlorate concentration in the Na^+ and K^+ titrations of the enzyme, were prepared by slowly adding, with gentle stirring, an equal volume of either 260 mM or 80 mM choline hydroxide solution to a 260 mM or 80 mM solution of perchloric acid on ice in a fume cupboard.

The electrical relaxation measurements were performed on Na^+,K^+-ATPase-containing membrane fragments adsorbed to optically black lipid membranes (BLMs). The BLMs, with an area of 0.01–0.02 cm^2, were formed in a thermostatted Teflon cell as described elsewhere (Fendler et al., 1985). Each of the two compartments of the cell was filled with 1.5 ml of electrolyte containing 25 mM imidazole, 1 mM dithiothreitol, 3 mM $MgCl_2$, and 130 mM of either NaCl or $NaClO_4$ at 24°C. The pH of the solution was adjusted to 7.4 with HCl before the cell was filled. Fifteen microliters of the Na^+,K^+-ATPase-containing membrane fragments (1.5 mg/ml protein) were added to one compartment of the cell and stirred for 40 min, during which time the membrane fragments adsorbed to the BLM in a sandwich-like structure.

The external measuring circuit consists of a lock-in amplifier (model 7220; EG&G Instruments, Wokingham, England), which applies a sinusoidal potential difference across the compound membrane (BLM together with the adsorbed membrane fragments). The alternating current signal generated by this potential can then be monitored by the lock-in amplifier, which divides the signal into two components, one in phase with the reference sinusoidal potential difference (I_x) and one that is 90° out of phase (I_y). Upon the addition of Na^+,K^+-ATPase-containing membrane fragments, the system was equilibrated until the two components of the current signal on the lock-in amplifier were constant. Then 0.225 mM P^3-1-(2-nitrophenyl)ethyl ATP sodium salt (caged ATP) was added to the cell compartment containing the membrane fragments. The solutions in the cell were stirred for a further 10 min, and then an ATP concentration jump was generated by the photolysis of caged ATP with a light flash from an excimer laser (10 ns pulse, λ = 308 nm). The light pulses were focused on the lipid bilayer membrane, and their intensity was attenuated by neutral density filters. Approximately 15–20% of the caged ATP is released as ATP by each light flash. From the increase of the I_y component of the current signal (ΔI_y) after the release of ATP it is possible to calculate the change in capacitance (ΔC) of the adsorbed membrane fragments due to enzyme-related electrogenic processes according to $\Delta C = \Delta I_y/2U\pi\nu$ (ν and U are the frequency and amplitude of the applied alternating potential). The frequency dependence of ΔC yields the relaxation time of the electrogenic reaction. Further details of the method and its principles can be found elsewhere (Sokolov et al., 1992, 1994, 1997, 1998; Babes and Fendler, manuscript in preparation). Frequency spectra between 3 and 1015 Hz of the capacitance increment of the attached membrane fragments were obtained after flash photolysis of caged ATP. Flashes were performed at 3-min intervals to remove ATP from the compound membrane system by stirring.

The origins of the various reagents used were as follows: imidazole (99+%, Sigma, or $\geq$ 99.5%, Fluka), EDTA (99%, Sigma), HCl (1.0 M titrisol solution, Merck), ethanol (analytical grade, Merck), ATP magnesium salt·5.5H_2O ($\sim$97%, Sigma), ATP disodium salt·3H_2O (special quality, Boehringer Mannheim), caged ATP (Calbiochem), NaCl (Suprapur, Merck), $MgCl_2$·6H_2O (analytical grade, Merck), NaBr (Suprapur, Merck), NaI (Suprapur, Merck), $NaNO_3$ (Suprapur, Merck), NaSCN (>98.5%, Merck), $NaClO_4$ (analytical grade, Merck), $KClO_4$ (analytical grade, Merck), choline hydroxide (20% w/w solution, Aldrich), and perchloric acid (70% w/w solution, Fluka).

RESULTS

Effect of lyotropic anions on the reaction $E_1(Na^+)_3 \rightarrow E_2P$

Initially we wish to investigate the influence of lyotropic anions on the Na^+ branch of the Na^+,K^+-ATPase reaction

cycle. K^+ ions were therefore excluded from the buffer solutions. Because it has previously been found that perchlorate ions have particularly large effects on the membrane dipole potential (Clarke and Lüpfert, 1999), on the E_1P/E_2P distribution of the Na^+,K^+-ATPase (Post and Suzuki, 1991), and on the ADP affinity of the enzyme (Nørby and Esmann, 1997), we have decided to concentrate on the effects of this anion on the enzyme's partial reactions. To determine the order of effectiveness of anions in modifying the kinetics of the enzyme, however, experiments have also been performed in the presence of bromide, iodide, nitrate, and thiocyanate. In the discussion, the results obtained here will be compared with those previously found for the enzyme in the presence of chloride, so that the effects of perchlorate on the various reaction steps can be identified.

On mixing RH421-labeled Na^+,K^+-ATPase membrane fragments in the presence of 5 mM $MgCl_2$ and 130 mM of NaCl, $NaClO_4$, NaBr, $NaNO_3$, NaI, or NaSCN with Na_2ATP, as described in Materials and Methods (experiment (a)), an increase in fluorescence is observed (see Fig. 1). In agreement with previous measurements (Kane et al., 1997; Clarke et al., 1998), in which 130 mM NaCl was used, it was found that two exponential time functions were necessary to adequately fit the data. At 2 mM, the highest ATP concentration used (after mixing), for the experiments in the presence of 130 mM $NaClO_4$ the total increase in fluorescence was 28 (± 0.4)% over the value immediately after mixing, and the faster phase was responsible for the majority of the fluorescence change (80% of the total amplitude).

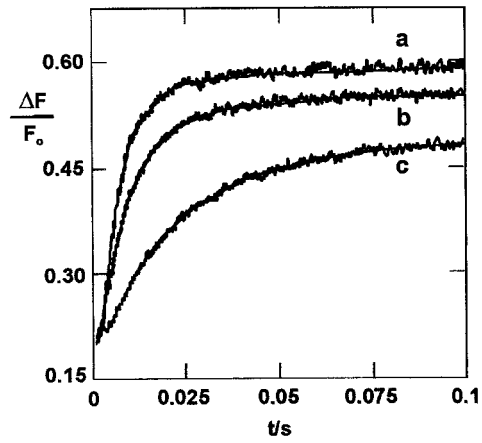

FIGURE 1 Stopped-flow fluorescence transients of Na^+,K^+-ATPase membrane fragments from pig kidney noncovalently labeled with RH421 (75 nM, after mixing). Na^+,K^+-ATPase (9 μg/ml or 0.061 μM, after mixing) was rapidly mixed with an equal volume of Na_2ATP (0.5 mM, after mixing). Each solution was in a buffer containing 30 mM imidazole, 5 mM $MgCl_2$, 1 mM EDTA, and 130 mM of either NaCl (*curve a*), $NaNO_3$ (*curve b*), or $NaClO_4$ (*curve c*). The fluorescence of membrane-bound RH421 was measured using an excitation wavelength of 577 nm at emission wavelengths ≥ 665 nm (RG665 glass cutoff filter). The solid lines represent fits to a sum of two exponential time functions. (*a*) 130 mM NaCl. The calculated reciprocal relaxation times were 191 (± 7) s^{-1} (90% of the total amplitude) and 39 (± 8) s^{-1} (10%). (*b*) 130 mM $NaNO_3$. The calculated reciprocal relaxation times were 145 (± 6) s^{-1} (85%) and 33 (± 5) s^{-1} (15%). (*c*) 130 mM $NaClO_4$. The calculated reciprocal relaxation times were 54 (± 2) s^{-1} (78%) and 8 (± 2) s^{-1} (22%).

In the case of the experiments previously performed in the presence of NaCl (Kane et al., 1997; Clarke et al., 1998), the fast phase could be attributed to phosphorylation of the enzyme, a subsequent conformational change, and the release, depending on the Na^+ concentration in the solution, of some or all of its Na^+ ions ($E_1(Na^+)_3$ + ATP → $E_2P(Na^+)_3 \leftrightarrow E_2P + 3Na^+$). That RH421 detects the formation of enzyme in the E_2P conformation rather than phosphorylation alone is supported by a number of experimental observations. It has been shown, for example, that incubation with oligomycin (Pratap and Robinson, 1993) and treatment with chymotrypsin (Stürmer et al., 1991; Klodos, 1994), which both inhibit the E_1P-to-E_2P conformational transition, cause inhibition of the ATP-induced RH421 fluorescence change. Furthermore, it has been shown by Cornelius (1999) from a comparison of quenched-flow and stopped-flow measurements and by an analysis of the effect of ADP on the reciprocal relaxation time of the RH421 fluorescence signal, that the probe must be responding to a reaction occurring after the formation of the E_1P conformation.

Because even in the absence of K^+ ions it is known that the E_2P conformation of the enzyme can undergo a slow spontaneous dephosphorylation (Hobbs et al., 1980; Kane et al., 1998), the slow phase was attributed (Clarke et al., 1998) to the relaxation of the dephosphorylation/rephosphorylation (via ATP) equilibrium according to the reaction sequence $E_2P \rightarrow E_2 \rightarrow E_1 + 3Na^+ \rightarrow E_1(Na^+)_3$ + ATP → $E_2P(Na^+)_3 \leftrightarrow E_2P + 3Na^+$. The reciprocal relaxation time for the establishment of the dephosphorylation/rephosphorylation equilibrium would be expected to be equal to the sum of the rate constants for the rate-determining steps of the forward and backward reactions, i.e., the rate of spontaneous dephosphorylation plus the rate of the E_2 to $E_1(Na^+)_3$ conformational change (which limits the rate of rephosphorylation in the above sequence). A different explanation of the slow phase has been offered by Cornelius (1999), who suggests that the slow phase could be associated with the conversion of enzyme from the E_2P conformation into a further phosphoenzyme conformation ($E_2'P$) via a dead-end pathway, which does not occur in the presence of K^+ ions. In the rest of this section, however, we limit ourselves to the presentation of results for the fast phase.

The reciprocal relaxation time for the fast phase of the ATP-induced RH421 fluorescence change, $1/\tau_1$, was found to depend on the sodium salt used. The values of $1/\tau_1$ determined at a concentration of 1 mM Na_2ATP (after mixing) were 191 (± 7) s^{-1} (130 mM NaCl), 154 (± 6) s^{-1} (130 mM NaBr), 145 (± 6) s^{-1} (130 mM $NaNO_3$), 75 (± 4) s^{-1} (130 mM NaI), 69 (± 2) s^{-1} (130 mM NaSCN), and 54 (± 2) s^{-1} (130 mM $NaClO_4$). The amplitude of the fluorescence change was also found to decrease significantly when 130 mM NaCl was replaced with 130 mM $NaClO_4$ (see Fig. 1). Assuming that perchlorate has no

significant effect on the sensitivity of RH421 toward enzyme activity, the decrease in amplitude could simply be explained by a decrease in the rate of formation of enzyme in the E_2P state relative to the rate of its decay back into the $E_1(Na^+)_3$ state via dephosphorylation and the $E_2 \rightarrow E_1(Na^+)_3$ conformational change, so that in the steady state the amount of enzyme that accumulates in the high-fluorescent E_2P state is reduced in the presence of perchlorate.

The order of effectiveness of the anions in slowing the kinetics of the enzyme relative to measurements in 130 mM NaCl is thus $ClO_4^- > SCN^- > I^- > NO_3^- > Br^-$. This order is in complete agreement with the order of effectiveness of anion binding to lipid membranes and of reducing the internal membrane dipole potential (Clarke and Lüpfert, 1999).

ATP concentration dependence of the rate of the reaction $E_1(Na^+)_3 \rightarrow E_2P$

The reciprocal relaxation time for the fast phase of the ATP-induced RH421 fluorescence change, $1/\tau_1$, was found to depend on the concentration of Na_2ATP (see Fig. 2). At a $NaClO_4$ concentration of 130 mM, $1/\tau_1$ increased with increasing Na_2ATP concentration until it leveled out at a maximum value in the range of 44–48 s^{-1}. The fact that the reciprocal relaxation time reaches a maximum value indicates that the process being observed is not simply the binding of ATP to the enzyme, because this would be expected to show a linear dependence of the reciprocal relaxation time on the ATP concentration. Therefore, it can be assumed that the observed process is a reaction of the enzyme occurring subsequent to ATP binding. Because of the close analogy between the experimental behavior presented here and that previously found in NaCl solution (Kane et al., 1997; Clarke et al., 1998), the same reaction scheme as presented in the earlier papers is proposed (see Fig. 3). According to this scheme, it can be shown (Kane et al., 1997) that, at saturating Na^+ concentrations, the ATP dependence of the reciprocal relaxation time for the fast phase is described by the following equation:

$$\frac{1}{\tau_1} = k_3 \cdot \frac{K_A[ATP]}{1 + K_A[ATP]} \quad (1)$$

The total relative fluorescence change (fast and slow phases), $\Delta F/F_o$, increased with increasing ATP concentration, from a value of 0.15 at the lowest ATP concentration used (0.5 μM), until it reached a saturating value of ~0.25–0.28 at ATP concentrations above ~20 μM.

Fitting the reciprocal relaxation time data according to the model shown in Fig. 3 to Eq. 1 yields the following parameters: $k_3 = 48\ (\pm 1)\ s^{-1}$ and $K_A = 2.4\ (\pm 0.2) \cdot 10^4\ M^{-1}$, where k_3 represents the rate constant for the rate-determining step subsequent to ATP and Na^+ binding, and K_A represents the apparent binding constant of ATP to its binding site on the enzyme. The reciprocal of K_A, i.e., 42 ($\pm$ 3) μM, corresponds to the apparent dissociation constant of the ATP binding site.

Na^+ ion concentration dependence of the rate of the reaction $E_1(Na^+)_3 \rightarrow E_2P$

The reciprocal relaxation time for the fast phase of the ATP-induced RH421 fluorescence change, $1/\tau_1$, was also found to be dependent on the Na^+ ion concentration. $1/\tau_1$ increased with increasing Na^+ from a value close to zero in the absence of Na^+ to a saturating value of ~45 s^{-1} at 130 mM Na^+ (see Fig. 4). This behavior is consistent with the

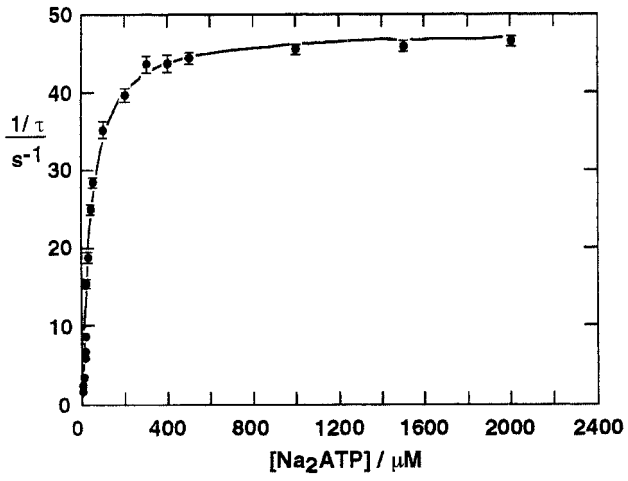

FIGURE 2 Dependence of the reciprocal relaxation time, $1/\tau$, of the fast phase of the RH421 fluorescence change on the concentration of Na_2ATP (after mixing) for stopped-flow experiments in which Na^+,K^+-ATPase was rapidly mixed with Na_2ATP in a nominally K^+-free medium. [Na^+,K^+-ATPase] = 9 μg/ml (≡ 0.061 μM), [$NaClO_4$] = 130 mM, [RH421] = 75 nM, [imidazole] = 30 mM, [$MgCl_2$] = 5 mM, [EDTA] = 1 mM, λ_{ex} = 577 nm, $\lambda_{em} \geq$ 665 nm; pH 7.4, T = 24°C. The solid line represents a nonlinear least-squares fit of the data to Eq. 1.

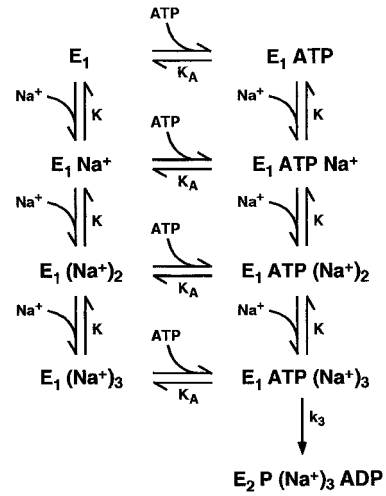

FIGURE 3 Reaction scheme describing the Na^+ and ATP binding steps of the Na^+,K^+-ATPase and its subsequent phosphorylation and conformational change.

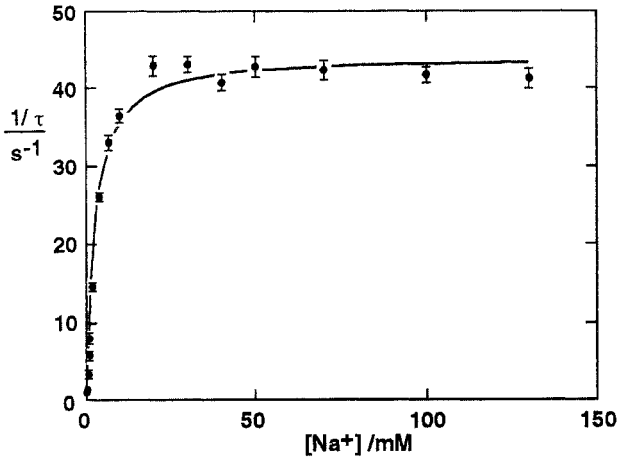

FIGURE 4 Dependence of the reciprocal relaxation time, $1/\tau$, of the fast phase of the RH421 fluorescence change on the concentration of Na^+ ions for stopped-flow experiments in the presence of 130 mM ClO_4^- in which Na^+,K^+-ATPase was rapidly mixed with MgATP in a nominally K^+-free buffer medium. [Na^+,K^+-ATPase] = 12 μg/ml ($\equiv$ 0.080 μM), [MgATP] = 0.5 mM, [RH421] = 125 nM, [imidazole] = 30 mM, [$MgCl_2$] = 5 mM, [EDTA] = 1 mM; pH 7.4, T = 24°C. The total ionic strength and the perchlorate concentration were maintained at constant values at $NaClO_4$ concentrations below 130 mM by replacing $NaClO_4$ in the solution with choline perchlorate, so that the total concentration of choline plus Na^+ ions was always 130 mM. The excitation and emission wavelengths were as in Fig. 2. The solid line represents a nonlinear least-squares fit of the data to Eq. 2.

idea that phosphorylation of the Na^+,K^+-ATPase only occurs at a significant rate when all of the Na^+ ion binding sites of the enzyme are occupied (as described in Fig. 3).

The total relative fluorescence change (fast and slow phases), $\Delta F/F_o$, increased with increasing Na^+ ion concentration, from a value of 0.08 at the lowest Na^+ ion concentration used (0.1 mM), until it reached a saturating value of $\sim$0.37 at Na^+ concentrations above $\sim$20 mM.

The total ionic strength in these measurements was maintained at 160 mM, and the total perchlorate concentration was maintained at 130 mM by the addition of choline perchlorate. This avoided any jump in either the ionic strength or the perchlorate concentration on mixing.

The reciprocal relaxation time data shown in Fig. 4 were fitted according to the model shown in Fig. 3, assuming that there are three identical Na^+ binding sites and there is no interaction between them. The fit to this model is shown as the solid line in Fig. 4. Using the derivation method described in Kane et al. (1997), it can be shown that the dependence of $1/\tau_1$ on the Na^+ concentration should be given by

$$\frac{1}{\tau_1} = k_3 \cdot \frac{K_A[\mathrm{ATP}]}{1 + K_A[\mathrm{ATP}]} \cdot \frac{K^3[\mathrm{Na}^+]^3}{(1 + K[\mathrm{Na}^+])^3} \qquad (2)$$

K represents here the apparent microscopic (or intrinsic) association constant of the Na^+ binding sites. It is mathematically defined by the following expression:

$$K = \frac{E_1Na^+}{3E_1 \cdot Na^+} = \frac{E_1ATP\ Na^+}{3E_1ATP \cdot Na^+} = \frac{E_1(Na^+)_2}{E_1Na^+ \cdot Na^+} = \frac{E_1ATP(Na^+)_2}{E_1ATP\ Na^+ \cdot Na^+} = \frac{3E_1(Na^+)_3}{E_1(Na^+)_2 \cdot Na^+} = \frac{3E_1ATP(Na^+)_3}{E_1ATP(Na^+)_2 \cdot Na^+} \qquad (3)$$

The factor 3 in Eq. 3 is a statistical coefficient that takes into account the number of free and occupied binding sites per enzyme molecule (Cantor and Schimmel, 1980). The values of the parameters obtained from the fit to Eq. 2 were k_3 = 46 ($\pm$ 1) s^{-1} and K = 1.32 ($\pm$ 0.08) $\cdot$ 10^3 M^{-1}. The reciprocal of K corresponds to an apparent microscopic dissociation constant of 0.76 ($\pm$ 0.04) mM. The value of K_A was taken from the previous ATP titration.

The experimental data were also fitted to kinetic models involving positive cooperativity in the Na^+ binding, because in 130 mM chloride solution it has been found that this yields a better description of the experimental behavior (Kane et al., 1997; Clarke et al., 1998). In this case, however, it was found that the positive cooperativity models did not show any significant improvement on an identical site model.

Effect of perchlorate on the reaction $E_1P(Na^+)_3 \rightarrow E_2P + 3Na^+$

The stopped-flow measurements described above allow the effects of perchlorate on the reaction sequence $E_1(Na^+)_3$ + ATP $\rightarrow$ $E_1P(Na^+)_3 \leftrightarrow E_2P(Na^+)_3 \leftrightarrow E_2P + 3Na^+$ to be identified. However, because this is a sequence containing several reaction steps, the stopped-flow measurements alone do not allow one to decide which individual step is being influenced by the presence of perchlorate ions. For this reason experiments have been performed using the stationary electrical relaxation technique described in Materials and Methods. This technique allows an analysis of the effect of perchlorate on the reactions $E_1P(Na^+)_3 \leftrightarrow E_2P(Na^+)_3 \leftrightarrow E_2P + 3Na^+$, independently of the preceding phosphorylation step (Sokolov et al., 1992, 1994).

Before the release of ATP from caged ATP via a light flash, in the presence of 130 mM NaCl or $NaClO_4$, the enzyme is stabilized in the $E_1(Na^+)_3$ conformation. Upon photolysis of caged ATP, the Na^+,K^+-ATPase binds and hydrolyzes ATP and undergoes a conformational change to the $E_2P(Na^+)_3$ state. In the absence of K^+ ions, the dephosphorylation reaction occurs at a rate of $\sim$5 s^{-1} (Hobbs et al., 1980; Kane et al., 1998), which is significantly lower than the rate of rephosphorylation by ATP via the $E_2 \rightarrow E_1(Na^+)_3$ transition of $\leq$ $\sim$40 s^{-1} (Clarke et al., 1998). Therefore, as long as excess ATP is present, a steady state will be reached in which the enzyme accumulates in a phosphorylated state. It can thus be assumed that the in-

creased components of the alternating current signal detected by the stationary electrical relaxation measurements characterize either the equilibrium between phosphorylated forms of the enzyme ($E_1P(Na^+)_3 \leftrightarrow E_2P(Na^+)_3$) or an equilibrium involving the release of either the first, second, or third Na^+ ion from the E_2P form of the enzyme ($E_2P(Na^+)_3 \leftrightarrow E_2P(Na^+)_2 + Na^+ \leftrightarrow E_2PNa^+ + Na^+ \leftrightarrow E_2P + Na^+$).

From the alternating current signal the capacitance increase due to phosphorylation of the enzyme was determined at different frequencies. The spectra can be fitted with a Lorentzian function, $\Delta C = A + B/(1 + \nu^2/f_o^2)$, where ΔC is the change in capacitance after the release of ATP, f_o is the corner frequency (inflection point of the ΔC versus ν curve), and A and B are constants. The constant component A, which persists at high frequencies, is probably due to a fast charge moving reaction that is not resolved in the frequency range used in the experiment (Lu et al., 1995). f_o is related to the reciprocal relaxation time of the charge-moving reaction by $1/\tau = 2\pi f_o$. The frequency dependence of the capacitative signal of the enzyme has been measured at pH 7.4 in buffer containing 25 mM imidazole, 3 mM $MgCl_2$, and 130 mM of either NaCl or $NaClO_4$. As shown in Fig. 5, the reciprocal relaxation time in 130 mM NaCl is 393 ($\pm$ 51) s^{-1}, whereas in 130 mM $NaClO_4$ the value is shifted to 114 ($\pm$ 15) s^{-1}.

K^+ ion concentration dependence of the rate of the reaction EP $\rightarrow$ E

The kinetics of the dephosphorylation reaction of the Na^+,K^+-ATPase were investigated as a function of the K^+ concentration, using experimental procedure (b) described under Materials and Methods. After RH421-labeled enzyme is mixed with ATP, it undergoes phosphorylation and a subsequent conformational change. In the presence of 130 mM $NaClO_4$, it has been shown above that the overall reaction ($E_1(Na^+)_3 + ATP \rightarrow E_2P + 3Na^+$) proceeds with a rate constant of $\sim$50 s^{-1}. The reaction is accompanied by an increase in the fluorescence of the probe. Because it is known that K^+ ions significantly accelerate the rate of dephosphorylation (Hobbs et al., 1980; Kane et al., 1998), in their absence a steady state is reached in which there is an accumulation of enzyme in a phosphorylated form ($E_1P \leftrightarrow E_2P$). Subsequent mixing of the phosphorylated enzyme with $KClO_4$ accelerates the rate of dephosphorylation, so that there is an increase in the proportion of enzyme in a dephosphorylated state and a decay in the concentration of phosphorylated enzyme until a new steady state is reached. This can be detected as a decrease in the fluorescence of RH421. The time course of the RH421 decrease, therefore, allows the determination of rate constants for the dephosphorylation reaction and K^+ dissociation constants.

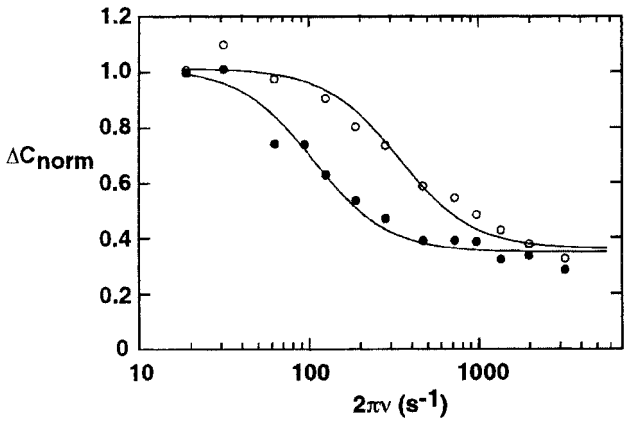

FIGURE 5 Dependence of the capacitance change, ΔC_{norm}, of Na^+,K^+-ATPase membrane fragments adsorbed to a black lipid membrane after the photolytic release of ATP (35–45 μM) from a caged complex on the frequency, ν, of the applied sinusoidal potential difference (amplitude 10 mV). The capacitance changes have been normalized to the values obtained at a frequency of 3 Hz. The Na^+,K^+-ATPase concentration was 15 μg/ml ($\equiv$ 0.10 μM). The aqueous buffer contained 25 mM imidazole, 1 mM dithiothreitol, 3 mM $MgCl_2$, and 130 mM of either NaCl (○) or $NaClO_4$ (●); pH 7.4, T = 24°C. The solid lines represent nonlinear least-squares fits of the data to the equation $\Delta C = A + B/(1 + \nu^2/f_o^2)$. The calculated reciprocal relaxation times were 393 ($\pm$ 51) s^{-1} in the presence of 130 mM NaCl and 114 ($\pm$ 15) s^{-1} in the presence of 130 mM $NaClO_4$.

Measurements over a range of K^+ concentrations showed an increase in the reciprocal relaxation time of the fluorescence decrease with increasing K^+ concentration, from a value of $\sim$30 s^{-1} at infinitely low K^+ concentrations to a maximum value in the range 95–110 s^{-1} at high K^+ concentrations (see Fig. 6). The relative fluorescence change, $-\Delta F/F_o$, increased with increasing K^+ ion concentration, from a value of 0.02 at the lowest K^+ ion concentration used (0.25 mM), until it reached a saturating value of $\sim$0.10 in the K^+ concentration range 1.5–5 mM. At higher K^+ concentrations there appeared to be a decrease in $-\Delta F/F_o$ to $\sim$0.05 at 15 mM K^+, and the observed relaxations appeared to become biexponential, i.e., a fast initial drop in fluorescence was observed, followed by a slower phase. In the case of the measurements above 5 mM K^+, the reciprocal relaxation times shown in Fig. 6 represent monoexponential fits to the portion of the curve following the initial fluorescence drop. Because of the small amplitudes of the fluorescence changes observed at high K^+ concentrations, an accurate analysis of both phases would probably require measurements to be performed at lower temperatures, where the fluorescence quantum yield of the dye is increased and the relaxation rates of both processes are well within the time resolution of the stopped-flow technique.

The fact that the reciprocal relaxation times shown in Fig. 6 reach a maximum value indicates that the process being observed is not simply the initial binding of K^+ to the enzyme, because this would be expected to show a linear dependence of the reciprocal relaxation time on the K^+ concentration. The simplest explanation is, therefore, that the observed process is a reaction of the enzyme induced by K^+ binding. Possible candidates are a conformational change or the dephosphorylation of the enzyme. As previously discussed for measurements in the presence of KCl (Kane et al., 1998), the intercept value of 29 s^{-1} at infinitely low K^+ concentrations is attributed to the relaxation of the

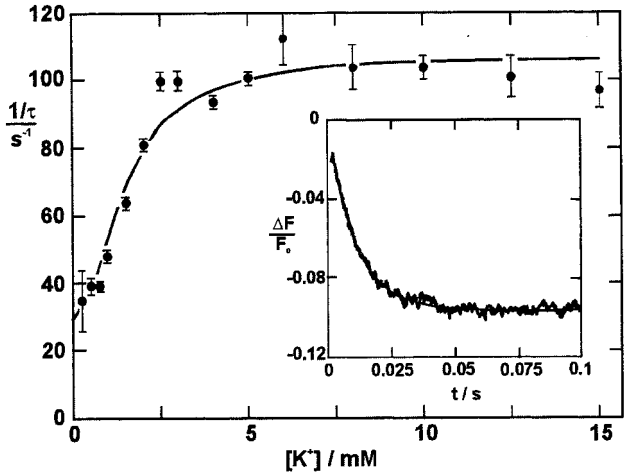

FIGURE 6 Dependence of the reciprocal relaxation time, $1/\tau$, of the RH421 fluorescence change on the concentration of K^+ (after mixing) for sequential mixing stopped-flow experiments in which Na^+,K^+-ATPase was premixed with Na_2ATP (2.0 mM after mixing) for 10 s to allow for phosphorylation and was subsequently rapidly mixed with $KClO_4$. [Na^+,K^+-ATPase] = 22 μg/ml (0.15 μM), [$NaClO_4$] = 130 mM, [RH421] = 150 nM, [imidazole] = 30 mM, [$MgCl_2$] = 5 mM, [EDTA] = 1 mM; pH 7.4, T = 24°C. The excitation and emission wavelengths were as in Fig. 2. The solid line represents a nonlinear least-squares fit of the data to Eq. 4. The inset shows an experimental stopped-flow relaxation obtained at a concentration of 4 mM $KClO_4$ after mixing.

dephosphorylation/rephosphorylation equilibrium of enzyme in the absence of bound K^+ ions.

In light of the present and previous findings, we propose the reaction scheme shown in Fig. 7 as a description of the experimentally observed behavior. E refers to a pool of E_1 and E_2 conformations, and EP refers to a pool of E_1P and E_2P conformations. K_1 and K_2 represent the association constants for the binding of the first and second K^+ ions to the EP or the E state of the enzyme, k_4 represents the rate constant for dephosphorylation of the enzyme with both K^+ binding sites occupied, and k'_4 represents the rate constant for dephosphorylation when the K^+ binding sites are vacant

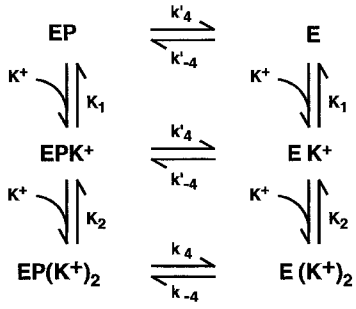

FIGURE 7 Reaction scheme describing the K^+ binding equilibria to EP and E conformations of the Na^+,K^+-ATPase as well as the coupled dephosphorylation and rephosphorylation reaction steps of enzyme with all of its K^+ binding sites occupied (k_4 and k_{-4}) and enzyme with either vacant or partially occupied sites (k'_4 and k'_{-4}). E refers to a pool of E_1 and E_2 conformations of the enzyme. EP refers to a pool of E_1P and E_2P conformations of the enzyme.

or only partially occupied. k_{-4} and k'_{-4} represent the corresponding rate constants for the back-reaction, i.e., rephosphorylation of the enzyme either directly by inorganic phosphate or indirectly via conversion to the $E_1(Na^+)_3$ conformation and phosphorylation by ATP. The reaction scheme chosen includes the basic assumption that the K^+ binding steps are so fast that they are always in equilibrium on the time scale of the dephosphorylation reaction. It should be noted that the model shown in Fig. 7 also assumes that the change in the dephosphorylation rate constant comes about entirely by the binding of the second K^+ ion to the enzyme. A more general model in which the rate constant for enzyme with a single bound K^+ ion could assume an intermediate value between that of enzyme with completely vacant and fully occupied K^+ binding sites cannot be excluded, however. Similarly, the model shown in Fig. 7 assumes that the binding constants of K^+ to the E and EP states are identical, whereas this may not necessarily be the case. The accuracy of the experimental data unfortunately does not allow a reliable determination of an intermediate rate constant or a discrimination between the strength of K^+ binding to the E and EP states, and, therefore, we have chosen to use the simplified model shown in Fig. 7. Fitting to the more general model is likely to change the calculated values of K_1 and K_2, but it would not significantly alter the calculated value of k_4 or k'_4.

For such a model the K^+ concentration dependence of the reciprocal relaxation time is described by the following equation (Kane et al., 1998):

$$\frac{1}{\tau} = \frac{(k_4 + k_{-4})K_1K_2[K^+]^2 + (k'_4 + k'_{-4})(1 + K_1[K^+])}{1 + K_1[K^+] + K_1K_2[K^+]^2} \qquad (4)$$

The fit to this model, which incorporates positive cooperativity in the K^+ ion binding, is shown in Fig. 6. Unfortunately, because of the relatively large number of free parameters involved in the model, it was found that widely varying values of K_1 and K_2 were able to give equally good fits to the experimental data, so that only limiting values can be given. In contrast, the values of ($k_4 + k_{-4}$) and ($k'_4 + k'_{-4}$) are reasonably well defined by the saturating value of $1/\tau$ at high K^+ concentrations and by the intercept. The values of the parameters calculated from the fit to Eq. 4 were $k_4 + k_{-4} = 108\ (\pm 3)\ s^{-1}$, $k'_4 + k'_{-4} = 29\ (\pm 4)\ s^{-1}$, $K_1 \leq 9.5 \cdot 10^1\ M^{-1}$, and $K_2 \geq 4.9 \cdot 10^3\ M^{-1}$. The values of K_1 and K_2 correspond to apparent dissociation constants of $\geq$11 mM and $\leq$0.21 mM, respectively. These values must be considered as apparent dissociation constants because of the presence of 130 mM $NaClO_4$ in the reaction mixture and because the possibility of competition between K^+ and Na^+ ions for the same binding sites is not included in the model shown in Fig. 7. The presence of 130 mM $NaClO_4$ is necessary to ensure maximum phosphorylation of the enzyme.

An attempt was also made to fit the data to a model in which the K^+ binding sites are identical and there is no interaction between them. It was found, however, that sig-

nificant positive and negative deviations of the experimental points from the fitted curve were apparent, and therefore this model was abandoned.

ATP concentration dependence of the rate of the reaction $E_2(K^+)_2 \rightarrow E_1(Na^+)_3$

To investigate the kinetics of K^+ dissociation, Na^+ binding, and the associated conformational change of unphosphorylated Na^+,K^+-ATPase, we have carried out experiments in which the enzyme in the presence of 1 mM of a potassium salt is rapidly mixed with 130 mM of a sodium salt. This induces the transition $E_2(K^+)_2 \rightarrow E_1(Na^+)_3$. The experiments were carried out as described in Materials and Methods (experiment (c)). Perchlorate salts of both sodium and potassium were used. To investigate the effect of ATP on this reaction, various concentrations of Na_2ATP were added to the sodium perchlorate solution. Such experiments have previously been performed using the RH421 probe, but with NaCl instead of $NaClO_4$ (Kane et al., 1997).

It was found that on mixing with $NaClO_4$, a decrease in fluorescence occurred. The kinetics of the fluorescence decrease could be closely approximated with a single exponential time function. The value of the reciprocal relaxation time for the observed fluorescence transient increases with increasing Na_2ATP concentration, reaching a saturating value of $\sim$45 s^{-1} (see Fig. 8). The total relative fluorescence change, $-\Delta F/F_o$, also increased with increasing Na_2ATP concentration, from a value of $\sim$0.04 at the lowest Na_2ATP concentration used (5 μM) to a maximum value of $\sim$0.10 at a Na_2ATP concentration of $\sim$500 μM. From the ATP concentration dependence of the reciprocal relaxation time, it is possible to estimate the binding constant for the low-affinity ATP binding site. If one assumes that the ATP binding step is in equilibrium on the time scale of the conformational change, then it can be shown that the reciprocal relaxation time, $1/\tau$, is related to the concentration of ATP by

$$\frac{1}{\tau} = \left[\left(\frac{1}{\tau}\right)_{max} - \left(\frac{1}{\tau}\right)_{min}\right]\left(\frac{K'_A[ATP]}{1 + K'_A[ATP]}\right) + \left(\frac{1}{\tau}\right)_{min} \quad (5)$$

where K'_A is the apparent binding constant of ATP for the low-affinity binding site of the enzyme, $(1/\tau)_{min}$ is the reciprocal relaxation time for the formation of enzyme in the $E_1(Na^+)_3$ conformation from the $E_2(K^+)_2$ conformation in the absence of ATP, and $(1/\tau)_{max}$ is the reciprocal relaxation time at a saturating concentration of ATP. This equation is based on a model in which there are two pathways from $E_2(K^+)_2$ to $E_1(Na^+)_3$: one in the absence of bound ATP and one that is ATP stimulated. Fitting the data shown in Fig. 8 to Eq. 5 yields a value for K'_A of 1.5 ($\pm$ 0.2) $\cdot$ 10^3 M^{-1}. This corresponds to an apparent dissociation constant of 660 ($\pm$ 100) μM. The values of $(1/\tau)_{min}$ and $(1/\tau)_{max}$ determined from the fit were 4.0 ($\pm$ 0.7) s^{-1} and 53 ($\pm$ 2) s^{-1}, respectively.

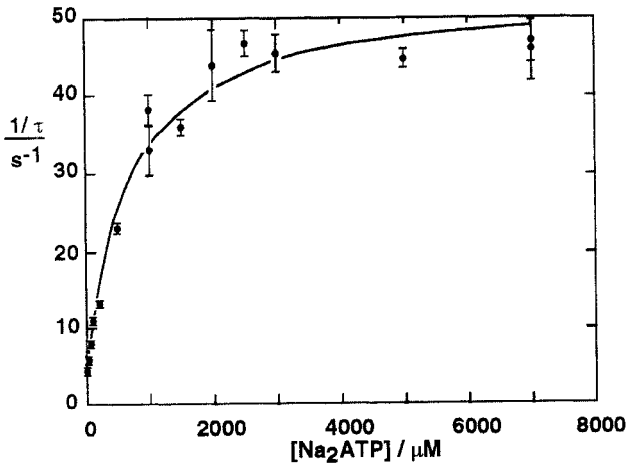

FIGURE 8 Dependence of the reciprocal relaxation time, $1/\tau$, of the RH421 fluorescence change on the concentration of Na_2ATP (after mixing) for stopped-flow experiments in which Na^+,K^+-ATPase in a buffer containing 1 mM $KClO_4$ was rapidly mixed with a buffer containing 130 mM $NaClO_4$. [Na^+,K^+-ATPase] = 12 μg/ml ($\equiv$ 0.08 μM), [RH421] = 125 nM, [imidazole] = 30 mM, [EDTA] = 1 mM; pH 7.4, T = 24°C. The excitation and emission wavelengths were as in Fig. 2. The solid line represents a nonlinear least-squares fit of the data to Eq. 5.

DISCUSSION

The effects of lyotropic anions, particularly perchlorate, on the kinetics of the partial reactions of pig kidney Na^+,K^+-ATPase have been investigated by two different kinetic methods: the stopped-flow technique in combination with the fluorescent probe RH421 and a stationary electrical relaxation technique. The results obtained are summarized in Table 1. In addition to the values determined in the present paper, data from previous papers in which measurements were performed in chloride solution have been included in the table, so that the effects of perchlorate ions relative to those of chloride can be seen.

From the results shown in Table 1 it is apparent that, in addition to effects on the various rate constants, which will be discussed shortly, perchlorate ions have a significant influence on the binding affinity of ATP for the enzyme. The affinities of ATP for both the high-affinity and low-affinity sites decrease significantly, i.e., K_d for the high-affinity site increases from 7.0 ($\pm$ 0.6) μM to 42 ($\pm$ 3) μM, and that for the low-affinity site increases from 143 ($\pm$ 17) μM to 660 ($\pm$ 100) μM. From equilibrium titrations, Nørby and Esmann (1997) also found that the affinity of the enzyme for ADP decreased significantly in the presence of 100 mM $NaClO_4$. It should be noted that the terms "high-affinity" and "low-affinity" ATP binding sites do not necessarily imply that the enzyme possesses two distinct ATP binding sites. It is also possible that the affinity of a single ATP-binding site could change as a consequence of an enzyme conformational change.

Table 1 also shows that the apparent affinity of Na^+ for the E_1 conformation is increased significantly when perchlorate is substituted for chloride, i.e., the concentration at

TABLE 1 Comparison of the effects of chloride and perchlorate (130 mM) on reaction steps of pig kidney Na^+,K^+-ATPase*

Reaction step	Chloride solution	Perchlorate solution[§]
$E_1(Na^+)_3 + ATP \rightarrow E_2P + 3Na^+$		
k_3	180 (±10) s^{-1} [#]	48 (±2) s^{-1}
$1/K_A$	7.0 (±0.6) μM[#]	42 (±3) μM
$K_{1/2}(Na^+)$[¶]	8–10 mM[#]	2.5–3.5 mM
$E_1P(Na^+)_3 \rightarrow E_2P + 3Na^+$		
$k_+ + k_-$	393 (±51) s^{-1} [§]	114 (±15) s^{-1}
$EP \rightarrow E + P_i$		
$k'_4 + k'_{-4}$	60 (±6) s^{-1} [‖]	29 (±4) s^{-1}
$EP + 2K^+ \rightarrow E(K^+)_2 + P_i$		
$k_4 + k_{-4}$	366 (±35) s^{-1} [‖]	108 (±3) s^{-1}
$K_{1/2}(K^+)$[¶]	2.4–2.6 mM[‖]	1.4–1.6 mM
$E_2(K^+)_2 + 3Na^+ \rightarrow E_1(Na^+)_3 + 2K^+$		
$k_+ + k_-$	3.6 (±0.4) s^{-1} [#]	4.0 (±0.7) s^{-1}
$E_2(K^+)_2 + 3Na^+ + ATP \rightarrow E_1(Na^+)_3ATP + 2K^+$		
$k_+ + k_-$	28 (±1) s^{-1}*	53 (±2) s^{-1}
$1/K'_A$	143 (±17) μM[#]	660 (±100) μM

*In the case of the majority of reactions investigated, a significant backward reaction is likely to be present, so that only the sum of the forward and backward rate constants could be determined accurately. The values of the errors given are standard deviations derived from fits of the data to appropriate mathematical models.

[#]Kane et al. (1997).

[§]This paper.

[‖]Kane et al. (1998).

[¶]$K_{1/2}$ is defined as the concentration of Na^+ or K^+ at which the difference between the value of $1/\tau$ and its value at an infinitely low concentration of Na^+ or K^+ has reached half its final saturating value.

which the reciprocal relaxation time has reached half its saturating value decreases from 8–10 mM to 2.5–3.5 mM. Whether in fact this represents a true change in the Na^+ affinity is, however, doubtful. Because, as discussed earlier, the dye RH421 detects formation of enzyme in the E_2P conformation, the apparent increase in the Na^+ affinity in perchlorate solution could be explained by the reduction in the rate of equilibration of the step $E_1P(Na^+)_3 \leftrightarrow E_2P(Na^+)_3$ (discussed later), assuming that the rate of phosphorylation is unaffected by perchlorate. Direct measurements of the kinetics of formation of enzyme in the $E_1P(Na^+)_3$ state would be necessary to test this hypothesis.

The fact that perchlorate causes a decrease in the affinity of the enzyme for ATP could be explained by electrostatic considerations. It has been shown elsewhere that perchlorate ions bind to the surface of phospholipid membranes, causing a decrease in the intrinsic positive intramembrane dipole potential (Clarke and Lüpfert, 1999) and the induction of a negative surface potential (McLaughlin et al., 1975; Tatulian, 1983, 1987). Furthermore, it is known that lyotropic anions can interact with the surface of water-soluble proteins, thus inducing a change in local electric field strength within the protein matrix (Cacace et al., 1997). According to the Boltzmann relation, the change in the dissociation constant of a charged species for its binding site due to its interaction with a local electric field is given by

$$K_d = K_{di} \cdot \exp\left(\frac{ze_0\psi}{kT}\right) \quad (6)$$

where K_{di} is the intrinsic dissociation constant of charged substrate (defined as that found in chloride solution), K_d is the dissociation constant in perchlorate solution, z is the valence of the charged substrate (-3 to -4 for ATP), e_0 is the elementary charge, k is the Boltzmann constant, T is the absolute temperature, and ψ is the change in the electrical potential sensed by the charged substrates at their binding sites due to the binding of perchlorate to the membrane and/or protein. Based on Eq. 6, it can be shown that a value of ψ of at most -16 mV would be necessary to produce the affinity change in ATP shown in Table 1. In the presence of 100 mM $NaClO_4$ measurements using the probe di-8-ANEPPS (Clarke and Lüpfert, 1999; Clarke, 1997) indicate that the positive intrinsic intramembrane dipole potential of dimyristoylphosphatidylcholine vesicles is reduced by a value of ~125 mV. This change in potential, however, could be partially shielded from the ATP binding site by charged amino acid residues of the protein, so that the effective change in potential experienced by ATP is significantly less. McLaughlin et al. (1975) reported a zeta potential of phosphatidylethanolamine vesicles of -13 mV at 250 mM $NaClO_4$. Approximately the same value was found by Tatulian (1983) for dimyristoylphosphatidylcholine vesicles at 100 mM $KClO_4$. Because the changes in the dipole potential and in the surface potential are both approximately large enough to explain the changes in ATP affinity induced by perchlorate, it is difficult to decide which might actually be the cause. In fact, it may be more likely that the change in affinity arises from a local electric field induced by perchlorate binding within hydrophobic domains on the cytoplasmic face of the protein, because the phosphorylation site of the Na^+,K^+-ATPase is generally believed to be located within a cytoplasmic loop of the amino acid chain (Jørgensen and Andersen, 1988).

Table 1 also shows that the affinity of K^+ ions for the EP state of the enzyme appears to increase somewhat with the substitution of chloride for perchlorate. The effect is significantly less, however, than that found for the binding of Na^+ ions to the E_1 conformation. As in the case of the Na^+ affinity, it is possible that this is only an apparent change in the K^+ affinity due to a secondary effect resulting from the decrease in the rate of equilibration of the $E_1P(Na^+)_3 \leftrightarrow E_2P(Na^+)_3$ step and a breakdown of the single EP pool assumption (see later). The true K^+ affinity for the E_2P conformation may, in fact, be unaffected by perchlorate.

Further evidence supporting an electrostatic origin of both the shifts in ATP affinities as well as the changes in rate constants is given by measurements in the presence of various anions. There it was found that the order of effectiveness in which the anions slowed the kinetics of the reaction $E_1(Na^+)_3 + ATP \rightarrow E_2P + 3Na^+$ was $ClO_4^- >$

$SCN^- > I^- > NO_3^- > Br^- > Cl^-$. This is in full agreement with the order of effectiveness of anion binding to dimyristoylphosphatidylcholine vesicles and of reducing the internal membrane dipole potential (Clarke and Lüpfert, 1999), as well as the order of their binding strength to egg PC vesicles (Tatulian, 1987).

Now let us consider the changes in the magnitudes of the various rate constants induced by perchlorate. As shown in Table 1, the rate constant of the reaction $E_1(Na^+)_3 + ATP \rightarrow E_2P + 3Na^+$ was found from stopped-flow measurements to decrease from 180 s^{-1} in chloride solution to 50 s^{-1} in perchlorate solution. To attribute this change in rate constant more specifically to an individual reaction step, measurements were performed with the stationary electrical relaxation technique, which yielded reciprocal relaxation times for the reaction $E_1P(Na^+)_3 \leftrightarrow E_2P + 3Na^+$ of 393 s^{-1} in chloride solution and 114 s^{-1} in perchlorate solution. Therefore, it seems likely that the major effect of perchlorate might be on this reaction rather than on the preceding phosphorylation step, $E_1(Na^+)_3 + ATP \rightarrow E_1P(Na^+)_3$. In chloride solution the phosphorylation reaction has been found via acid quenched-flow measurements to occur at a rate of ~200 s^{-1} (Kane et al., 1997). If one uses this value for phosphorylation and 393 s^{-1} for the reaction $E_1P(Na^+)_3 \rightarrow E_2P + 3Na^+$, it is possible, based on the differential rate equations for the complete sequence of reactions, $E_1(Na^+)_3 + ATP \rightarrow E_1P(Na^+)_3 \rightarrow E_2P + 3Na^+$, to numerically simulate the stopped-flow measurements (i.e., the build-up in the concentration of E_2P, assuming that RH421 is detecting this species alone). From the simulated curves a theoretically expected reciprocal relaxation time can be obtained. This yields a value in the range 153–166 s^{-1}, which is only slightly less than the experimental value of 180 s^{-1}. Now if we assume in the first instance that the phosphorylation reaction is unaffected by perchlorate, the same type of simulation can be carried out for the stopped-flow measurements in the presence of perchlorate. In this case the simulations yield reciprocal relaxation times in the range 84–100 s^{-1}. This appears to be significantly higher than the experimental value of 50 s^{-1}. Therefore, in addition to its effect on the reaction $E_1P(Na^+)_3 \rightarrow E_2P + 3Na^+$, it cannot be excluded at this stage that perchlorate may also cause some reduction in the rate of the phosphorylation reaction. Direct measurements of the phosphorylation reaction in the presence and absence of perchlorate would be necessary to test this possibility.

The reduction in the rate of equilibration of the step $E_1P(Na^+)_3 \leftrightarrow E_2P + 3Na^+$ found here from electrical relaxation measurements is consistent with the findings of Post and Suzuki (1991), Klodos (1991), and Klodos and Plesner (1991) showing that lyotropic anions affect the position of the equilibrium between the E_1P and E_2P forms of the enzyme, as well as with quenched-flow measurements of Klodos et al. (1994) indicating that lyotropic anions decrease the rate of equilibration between the E_1P and E_2P forms. This reaction step, however, is composed of several individual substeps: $E_1P(Na^+)_3 \leftrightarrow E_2P(Na^+)_3 \leftrightarrow E_2P(Na^+)_2 + Na^+ \leftrightarrow E_2PNa^+ + Na^+ \leftrightarrow E_2P + Na^+$, i.e., there is the initial conformational change followed by equilibria describing the sequential release of the three Na^+ ions. It is interesting now to discuss whether the effect of perchlorate is actually acting on the initial conformational equilibrium or on a subsequent Na^+ dissociation equilibrium, because the question of the electrogenicity of the Na^+-transporting steps of the Na^+,K^+-ATPase has been an interesting question of discussion for a number of years (Fendler et al., 1993; Wuddel and Apell, 1995).

According to electrical measurements of Wagg et al. (1997), the equilibration of the Na^+ release steps occurs with reciprocal relaxation times of $\geq$1000 s^{-1}. They can therefore be considered to be in equilibrium on the time scale of the relaxation of the conformational change. Under these conditions it can be shown that, for the simplest case of three identical Na^+ binding sites on E_2P, the reciprocal relaxation time for the relaxation of the step $E_1P(Na^+)_3 \leftrightarrow E_2P(Na^+)_3$ is given by

$$\frac{1}{\tau} = k_+ + k_- \cdot \frac{K_N^3[Na^+]^3}{(1 + K_N[Na^+])^3} \qquad (7)$$

where k_+ and k_- are the forward and backward rate constants for the conformational change, respectively, and K_N is the microscopic association constant of the extracellular Na^+ binding sites on the E_2P conformation. Because the electrical relaxation measurements were carried out at 130 mM NaCl or $NaClO_4$, which is far above the half-saturating Na^+ concentrations of the cytoplasmic Na^+ binding sites (see Table 1), dissociation of Na^+ ions from the E_1P conformation can be neglected in Eq. 7, and the Na^+ binding sites of the E_1P state are assumed to be always fully occupied. Equation 7 shows that even if k_+ and k_- were unaffected by perchlorate, a change in the rate of relaxation of the overall reaction $E_1P(Na^+)_3 \leftrightarrow E_2P + 3Na^+$ could occur if perchlorate caused a change in the Na^+ affinity of the E_2P conformation. At first glance it may seem, therefore, that the observed decrease in the reciprocal relaxation time in the presence of perchlorate (see Fig. 5) could be explained by a decrease in K_N, i.e., a decrease in the Na^+ affinity. However, the maximum decrease in $1/\tau$ that could be observed according to Eq. 7 would be the value of k_-. An analysis of quenched-flow data by Suzuki and Post (1997) indicates a concentration ratio of $E_1P(Na^+)_3$ to E_2P in 160 mM NaCl solution of at least 5. Assuming this value for k_+/k_-^* ($k_-^* = k_- K_N^3[Na^+]^3(1 + K_N[Na^+]^3)$ and using the experimentally determined $1/\tau$ value of 393 s^{-1}, individual values for k_+ of 328 s^{-1} and for k_-^* of 66 s^{-1} can be estimated. Therefore, the maximum possible drop in $1/\tau$ that could be produced by an effect of perchlorate on either the Na^+ binding equilibrium to the E_2P state or on the back-reaction itself, $E_2P(Na^+)_3 \rightarrow E_1P(Na^+)_3$, is 66 s^{-1}. Experimentally it was found, however, that $1/\tau$ decreased from 393 s^{-1} in chloride solution to 114 s^{-1} in perchlorate solution, i.e., a drop of 279 s^{-1}. On these grounds an effect of perchlorate on K_N or on k_- can be excluded as major causes of the perchlorate

effect on $1/\tau$. Furthermore, a decrease in K_N due to perchlorate would be the opposite of that expected on electrostatic grounds, because adsorption of negatively charged perchlorate should cause an attraction of Na^+ cations to the membrane surface. A decrease in k_- relative to k_+ would be inconsistent with the results of Post and Suzuki (1991), Klodos (1991), Klodos and Plesner (1991), and Klodos et al. (1994), which clearly show that perchlorate induces a shift in the conformational equilibrium in favor of the $E_1P(Na^+)_3$ state. Therefore, it must be concluded that perchlorate causes a significant reduction in the value of k_+, i.e., the forward reaction $E_1P(Na^+)_3 \rightarrow E_2P(Na^+)_3$ is significantly slowed by perchlorate. This effect can be explained electrostatically. If the Na^+ ions bound to the E_1P conformation of the enzyme are located at a site deeper within the membrane than the perchlorate ions, the local electric field created by the perchlorate ions on the intracellular side of the membrane would be expected to stabilize the enzyme in the $E_1P(Na^+)_3$ state and hence increase the activation energy barrier for transport of the Na^+ ions across the membrane. This is consistent with the concept that the reaction $E_1P(Na^+)_3 \rightarrow E_2P(Na^+)_3$ is an important electrogenic (charge-transporting) step of the Na^+,K^+-ATPase pump cycle.

This conclusion concerning the electrogenicity of the conformational transition would appear to be in contradiction to the results of charge-pulse and ATP concentration jump experiments of Wuddel and Apell (1995), who attributed the major charge-translocating process to the release of the first Na^+ ion from the $E_2P(Na^+)_3$ state. These findings were based on simulations of a kinetic model that included a rate constant of 25 s^{-1} for the $E_1P(Na^+)_3 \leftrightarrow E_2P(Na^+)_3$ conformational transition. This is in disagreement with rate constants for this transition found here (393 s^{-1}) and by other authors (Fendler et al., 1985; Clarke et al., 1998). The reason, however, for the apparent contradiction between the low electrogenicity reported for the conformational transition by Wuddel and Apell (1995) and the large effect of perchlorate on the same reaction found here remains unclear.

It is interesting to note that, using a similar electrical relaxation technique, Sokolov et al. (1998) observed a similar decrease in $1/\tau$ for the reaction $E_1P(Na^+)_3 \leftrightarrow E_2P + 3Na^+$ upon increasing the chloride concentration from 150 mM to 1 M. The chloride concentration needed to produce the decrease was 10 times higher than that of perchlorate used here. Post and Suzuki (1991) found that similarly high concentrations of chloride were needed to shift the relative sensitivity of phosphoenzyme to ADP and K^+ ions. Because the results of Clarke and Lüpfert (1999) show no evidence for binding of chloride to lipid vesicles, even in the molar range, it would seem most likely that the effect seen by Sokolov et al. (1998), as they suggested themselves, is due to an effect of ionic strength on the conformational stability of the enzyme. It is possible that, in analogy to the direct effect of perchlorate binding to the membrane, high ionic strength may screen positively charged amino acid residues or lipid headgroups on the intracellular face of the membrane, thus stabilizing the enzyme in the $E_1P(Na^+)_3$ state.

A significant effect of perchlorate ions has also been found on the rates of both spontaneous and K^+-stimulated dephosphorylation (see Table 1). In both cases it was found that the reciprocal relaxation time decreased significantly. This is consistent with quenched-flow measurements carried out by Post and Suzuki (1991), Klodos (1991), and Klodos and Plesner (1991) at either 2°C or 0°C, which showed a decrease in the sensitivity of the enzyme toward dephosphorylation by K^+ ions in the presence of perchlorate and an increase in the sensitivity toward ADP. Post and Suzuki (1991), Klodos (1991), and Klodos and Plesner (1991) have suggested that the reduction in the rate of K^+-stimulated dephosphorylation is a consequence of the stabilization of enzyme in the $E_1P(Na^+)_3$ state. It is interesting to note that the phosphoenzyme decays observed by Klodos (1991) and Klodos and Plesner (1991) generally appear to be double exponential, whereas the stopped-flow fluorescence measurements reported here (at least at K^+ concentrations $\leq$ 5 mM) and elsewhere (Kane et al., 1998) could be adequately fitted by a single exponential function. This difference is likely to be due to the different temperatures used for the measurements, i.e., 24°C for the stopped-flow measurements and 0°C for the quenched-flow measurements of Klodos (1991) and Klodos and Plesner (1991). Computer simulations indicate that if the relaxation of the E_1P/E_2P transition is as fast or faster than the dephosphorylation/rephosphorylation equilibrium, the E_1P and E_2P conformations can be considered as a single EP pool, and a single exponential phosphoenzyme decay can be expected. Presumably this is no longer the case at 0°C, because of the very high activation energy of the $E_1P \rightarrow E_2P$ transition (Friedrich and Nagel, 1997).

With respect to the dephosphorylation measurements, it is important to mention the double-exponential behavior that was apparent at high K^+ concentrations (above 5 mM). Under these conditions it would seem that the assumption of a single EP pool may break down. The fast fluorescence drop could then presumably be attributed to dephosphorylation of enzyme in the $E_2P(K^+)_2$ state and the slower phase to a subsequent relaxation of the $E_1P(Na^+)_3 \leftrightarrow E_2P(Na^+)_3$ equilibrium, coupled to the rapid dephosphorylation of $E_2P(K^+)_2$. This interpretation is further supported by the good agreement between $1/\tau$ values determined from dephosphorylation measurements at saturating K^+ concentrations (366 ($\pm$ 35) s^{-1} and 108 ($\pm$ 3) s^{-1} in chloride and perchlorate solution) and the corresponding $1/\tau$ values determined for the reaction $E_1P(Na^+)_3 \rightarrow E_2P + 3Na^+$ from the electrical relaxation measurements (393 ($\pm$ 51) s^{-1} in chloride and 114 ($\pm$ 15) s^{-1} in perchlorate), which strongly suggests that in both cases the same rate-limiting reaction is being measured. Therefore, the saturating values of 366 ($\pm$ 35) s^{-1} (chloride) and 108 ($\pm$ 3) s^{-1} (perchlorate) determined from the dephosphorylation measurements most likely represent not the rate of relaxation of the dephosphor-

ylation/rephosphorylation equilibrium $E_2P(K^+)_2 \leftrightarrow E_2(K^+)_2$ (as was suggested previously; Kane et al., 1998), but the relaxation of the conformational equilibrium $E_1P(Na^+)_3 \leftrightarrow E_2P(Na^+)_3$. The dephosphorylation of enzyme in the $E_2P(K^+)_2$ state could then be considered to be much faster, in agreement with the ^{31}P NMR measurements of Dahms and Miara (1983), who determined the rate of P_i dissociation from the enzyme to be $\geq 80{,}000\ s^{-1}$.

In contrast to the inhibitory effect of perchlorate on the relaxation of the E_1P/E_2P equilibrium, it has been found that the relaxation of the ATP-stimulated $E_2(K^+)_2/E_1(Na^+)_3$ equilibrium is accelerated by perchlorate (see Table 1). This effect could easily be explained by an electrostatic stabilization of the more highly positively charged $E_1(Na^+)_3$ species relative to the $E_2(K^+)_2$ species by the negatively charged perchlorate anion, which would therefore provide an increased driving force for the forward reaction.

Finally, if one accepts the electrostatic interpretation of the Hofmeister effect of lyotropic anions on the partial reactions of the Na^+,K^+-ATPase presented here, the results obtained can be compared with the effects of the hydrophobic ions tetraphenylborate (TPB^-) and tetraphenylphosphonium (TPP^+) on the enzyme. As mentioned in the Introduction, these ions are well known to bind within phospholipid membranes and alter the intramembrane electric field strength.

Both Stürmer et al. (1991) and Klodos et al. (1995) found that TPP^+ reduced the affinity of the E_2P conformation of the enzyme for K^+ ions. Unfortunately the effect of TPB^- on K^+ affinity could not be investigated because of the low solubility of TPB^- in K^+-containing solution. According to electrostatic considerations, however, the TPP^+-induced decrease in K^+ affinity is consistent with the perchlorate-induced increase in apparent K^+ affinity of the E_2P conformation found here. Stürmer et al. (1991) and Klodos et al. (1995) found that the effect of TPP^+ was independent of the ionic strength, thus suggesting that TPP^+ affects the K^+ binding affinity by changing the local electric field strength within the membrane and/or protein matrix rather than by inducing a surface potential.

From measurements of the steady-state rate of Na^+/Na^+ exchange using Na^+,K^+-ATPase reconstituted into lipid vesicles, Cornelius (1995) found that TPP^+ decreased the affinity of the enzyme for cytoplasmic Na^+ ions, whereas TPB^- increased the affinity. This is also in good agreement with the perchlorate-induced increase in apparent affinity of the E_1 form of the enzyme for Na^+ ions reported here. Both the results found here and those of Cornelius (1995) would appear to be in contradiction to the findings of Klodos et al. (1995), who were of the opinion that lipophilic ions do not influence the interaction of Na^+ and K^+ with intracellular sites. Klodos et al. (1995) based their conclusion on the absence of any effect of TPP^+ or TPB^- on the kinetics of dephosphoenzyme conversion ($E_2(K^+)_2 \leftrightarrow E_1(Na^+)_3$) in the absence of ATP. As shown in Table 1, however, although the kinetics of this process appear to be unaffected by perchlorate in the absence of ATP, a significant increase in the rate of equilibration is observed at saturating ATP concentrations. It is therefore possible that ATP causes a modification of the enzyme conformation such that its sensitivity to the local electric field strength is increased.

Neither Klodos et al. (1995) nor Cornelius (1995) found any effect of TPP^+ or TPB^- on the rate of phosphorylation by ATP. It therefore appears very likely that phosphorylation itself is not a major charge-translocating step of the enzyme cycle. Here it was not possible to exclude the possibility, however, that perchlorate may have some effect on the phosphorylation reaction. Klodos et al. (1995) did find a significant effect of TPP^+ and TPB^- on the conformational equilibrium between the two phosphoenzyme forms of the enzyme. They observed that TPP^+ shifted the equilibrium in favor of the E_2P form (K^+-sensitive) of the enzyme, whereas TPB^- favored the E_1P form (ADP-sensitive). The effect of TPB^- on the conformational equilibrium, therefore, parallels that observed here and elsewhere (Post and Suzuki, 1991; Klodos, 1991; Klodos and Plesner, 1991; Klodos et al., 1994) for perchlorate.

To summarize, it has been found that the lyotropic anion perchlorate has significant effects on reaction steps of the Na^+,K^+-ATPase in which charged species (Na^+, K^+, and ATP) are either bound or translocated. The effects can be explained in terms of electrostatic interactions due to perchlorate binding within the membrane and/or protein matrix and alteration of the local electric field strength experienced by the ion-binding domains of the protein, which is similar to the hypothesis initially proposed by Hodgkin and Horowicz (1960). A relatively simple model is therefore adequate for interpreting the Hofmeister effect of lyotropic anions on ion-transporting membrane proteins. The effects of lyotropic anions are comparable to those of hydrophobic ions, such as TPB^- and TPP^+, and can therefore be used in a similar fashion as tools for the identification of electrogenic reaction steps. The kinetic results obtained here in the presence of perchlorate support the conclusion that the conformational transition $E_1P(Na^+)_3 \rightarrow E_2P(Na^+)_3$ is a major charge translocating step of the pump cycle.

The authors thank Annelie Schacht for excellent technical assistance; Helga Volk for the preparation of the diagrams; Dr. David Kane for valuable discussions and for his help with preliminary stopped-flow measurements; and Dr. Irena Klodos, Prof. Hans-Jürgen Apell, Dr. Jeffrey Froehlich, and Dr. Peter Pohl for valuable discussions, correspondence, and suggestions. For his interest and support of this work, the authors thank Prof. Dr. Ernst Bamberg.

CG was the recipient of a short-term Max Planck Society fellowship and of partial financial support from the Ministry of Research and Technology, Romania. AB is grateful for financial support from the German Academic Exchange Service. RJC and CL acknowledge with gratitude financial support from the Max Planck Society and from the Deutsche Forschungsgemeinschaft.

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