Stopped-Flow Kinetic Investigations of Conformational Changes of Pig Kidney Na^+,K^+-ATPase†

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ABSTRACT: The kinetics of Na^+-dependent partial reactions of the Na^+,K^+-ATPase were investigated via the stopped-flow technique using the fluorescent labels RH421 and BIPM. After the enzyme is mixed with MgATP, both labels give almost identical kinetic responses. Under the chosen experimental conditions two exponential time functions are necessary to fit the data. The dominant fast phase, $1/\tau_1 \approx 180\ s^{-1}$ (saturating [ATP] and [Na^+], pH 7.4 and 24 °C), is attributed to phosphorylation of the enzyme and a subsequent conformational change ($E_1ATP(Na^+)_3 \rightarrow E_2P(Na^+)_3 + ADP$). The rate of the phosphorylation reaction measured by the acid quenched-flow technique was 190 s^{-1} at 100 μM ATP, suggesting that phosphorylation controls the kinetics of the RH421 signal and that the conformational change is very fast ($\geq 600\ s^{-1}$). The rate of the RH421 signal was optimal at pH 7.5. The Na^+ concentration dependence of $1/\tau_1$ showed half-saturation at a Na^+ concentration of 8−10 mM with positive cooperativity involved in the occupation of the Na^+ binding sites. The apparent dissociation constant of the high affinity ATP binding site determined from the ATP concentration dependence of $1/\tau_1$ was 7.0 (±0.6) μM, while the apparent K_d for the low affinity site and the rate constant for the E_2 to E_1 conformational change evaluated in the absence of Mg^{2+} were 143 (±17) μM and ≤ 28 s^{-1}. At RH421 concentrations in the micromolar range, a decrease in the value of $1/\tau_1$ is observed. On the basis of rapid quenched-flow measurements, this inhibition can be attributed to a reaction step subsequent to phosphorylation. This accounts for previously observed kinetic discrepancies between RH421 and BIPM.

The Na^+,K^+-ATPase, which is found in the plasma membrane of almost all animal cells, utilizes the free energy derived from the hydrolysis of ATP for the transport of Na^+ ions out of and K^+ ions into the cell (Glynn, 1993; Läuger, 1991; Cornelius, 1991; Skou, 1990). The concentration gradients of Na^+ and K^+ thus generated across the cell membrane have numerous important physiological functions, e.g. in the maintenance of the resting potential in nerve cells (Dudel, 1989) and as a secondary source of energy in the reabsorption of nutrients in the kidney (Deetjen, 1989).

The enzymatic mechanism of the Na^+,K^+-ATPase is often described by the so-called Albers−Post model (Läuger, 1991; Albers, 1967; Post et al., 1972), which considers two conformations of the enzyme, E_1 and E_2, which can be in either a phosphorylated or an unphosphorylated state. The model, furthermore, describes a consecutive mechanism of Na^+ ion and K^+ ion transport across the membrane. Although the assumption of only two enzyme conformations would seem, considering the size and complexity of the enzyme, to be an over-simplification, the Albers-Post model has so far been quite successful in explaining a great deal of kinetic data. Recently it was suggested, however, by Pratap and Robinson (1993) that two conformations were insufficient to explain transient kinetic data. This conclusion was based on stopped-flow kinetic measurements of ATP-induced fluorescence changes of Na^+,K^+-ATPase preparations labeled with three fluorescent probes: 5-iodoacetamidofluorescein (IAF) (Steinberg & Karlish, 1989), *N*-[*p*-(2-benzimidazoyl)phenyl]maleimide (BIPM) (Nagai et al., 1986), and RH421 (Forbush & Klodos, 1991; Bühler et al., 1991). Pratap and Robinson (1993) found that the probe BIPM yielded significantly higher values of the observed rate constants than RH421 (approximately 200 s^{-1} for BIPM in comparison to 100 s^{-1} for RH421 at 24 °C), i.e., apparently incompatible with a single conformational change from E_1 to E_2P. They, therefore, suggested that the two probes responded to two different steps in the Na^+-branch of the Na^+,K^+-ATPase pump cycle. Previously, Forbush and Klodos (1991) had also investigated the kinetics of the ATP-induced fluorescence change of RH421-labeled Na^+,K^+-ATPase membrane fragments. The value of the observed rate constants they calculated (60 s^{-1} at 20 °C) was, however, even lower than that of Pratap and Robinson under similar experimental conditions.

Another method of rapidly adding ATP to the system is to photochemically release it from a caged complex. This approach has been used by Klodos and Forbush (1988), who

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detected the fluorescence change of the probe RH160 and subsequently by Bühler et al. (1991) using RH421. Klodos and Forbush (1988) reported an observed rate constant at 20 °C of 42 s^{-1}. Bühler et al. (1991) found a half-life, also at 20 °C, of about 40 ms, which, assuming first-order kinetics, corresponds to an observed rate constant of 25 s^{-1}. It is, thus, apparent that widely varying rate constants, between 25 and 200 s^{-1} have been reported for Na^+-dependent partial reactions of the Na^+,K^+-ATPase under similar experimental conditions but using different kinetic methods and different probes.

More recently, detailed investigations (Frank et al., 1996) of the interaction of the fluorescent probe RH421 with Na^+,K^+-ATPase membrane fragments have indicated that RH421 concentrations in the micromolar range can inhibit the hydrolytic activity of the enzyme. It may, therefore, be expected that the value of the observed rate constant found in stopped-flow experiments using RH421 could depend on the concentration of probe used. This could possibly account for some of the discrepancies in the values of the rate constants discussed above. It should be noted, however, that in the kinetic studies referred to so far, several different enzyme sources have been used. The possibility that some of the differences in rate constants may be related to species differences (Forbush & Klodos, 1991), therefore, also deserves attention.

In the light of the observed inhibition by RH421, we decided to perform stopped-flow measurements using non-inhibiting RH421 concentrations to determine if the pre-steady state kinetics detected by the probe differed from the behavior measured previously at higher probe concentrations. In the case that the kinetics are sensitive to the concentration of RH421, then we would try to determine if these measurements justify modification of the Albers−Post scheme with additional conformational states as proposed by Pratap and Robinson (1993). In addition, time-resolved studies of charge translocation in black lipid membranes and ATP-dependent phosphoenzyme formation were carried out in the presence and absence of RH421 for comparison with the kinetic behavior determined by stopped-flow mixing. The results obtained from this comparison provide clues to the identification of the probable step(s) in the reaction mechanism being monitored and/or inhibited by RH421 and allow a lower limit to be placed on the rate constant for a protein conformational change subsequent to phosphorylation. On the basis of our experimental observations, it will be shown that the observed kinetic behavior can be explained in terms of the Albers−Post model without recourse to additional conformational states. Moreover, rate limitation of the overall cycle is likely to be predominantly due to the E_2 to E_1 conformational transition of unphosphorylated enzyme.

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. In contrast to RH421, *N*-(*p*-(2-benzimidazolyl)phenyl)-maleimide (BIPM) is linked covalently to the Na^+,K^+-ATPase at Cys 964 (Nagai et al., 1986; Taniguchi et al., 1994).

Na^+,K^+-ATPase-containing membrane fragments were prepared and purified from the red outer medulla of pig kidney by a procedure that involved treatment with NaI followed by deoxycholate (Taniguchi et al., 1982) or according to a modification (Fendler et al., 1985) of procedure C of Jørgensen (1974a,b). In the case of the Jørgensen-type preparation, selected density gradient fractions were diluted five times in 25 mM imidazole (Microselect, Fluka)/HCl (Suprapur, Merck) solution containing 0.2 mM dithiothreitol (Aldrich) at pH 7.5, leading to an enzyme concentration of about 0.1 mg/mL. The fractions were then centrifuged for 4 h at 72000*g* and 4 °C. The pellets were then suspended at a concentration of about 3 mg/ml in 25 mM imidazole (Microselect for luminescence spectroscopy, Fluka, sublimed)/HCl containing 0.2 mM dithiothreitol, incubated for 20 min at room temperature, and centrifuged for 20 min at 200000*g* and 4 °C. The pellets were then resuspended in the same volume of the imidazole buffer used for the 200000*g* centrifugation and were kept on ice. The specific ATPase activity of the Jørgensen-type preparation at 37 °C was approximately 1800 μmol of P_i/h per mg of protein 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 2 mg/mL. The enzymatic activity in the presence of 1 mM ouabain was less than 1%. For both preparations the protein concentration was determined by the Lowry method (1951). In the case of enzyme prepared using NaI and deoxycholate treatment, the protein concentration was typically 8 mg/ml.

Optically black lipid membranes (BLM) with an area of 0.01−0.02 cm^2 were formed in a thermostated 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 130 mM NaCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, and 25 mM imidazole at pH 6.2. The temperature was kept at 24 °C. The membrane-forming solution contained 1.5% (wt/vol) diphytanoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) and 0.025% (wt/vol) octadecylamine (Riedel de Haen, Hannover, Germany) dissolved in *n*-decane. P^3-1-(2-Nitrophenyl)ethyl ATP, tripropylammonium salt (caged ATP) was prepared as described previously (Fendler et al., 1985).

The membrane was connected to an external measuring circuit via polyacrylamide gel salt bridges and Ag/AgCl electrodes. The signal was amplified, filtered and recorded with a digital oscilloscope. For further details see Fendler et al. (1985, 1987, 1993). Pig kidney Na^+,K^+-ATPase membrane fragments and caged ATP were added under stirring to one compartment of the cuvette. To photolyze the caged ATP, light pulses of an excimer laser with a duration of 10 ns and a wavelength of 308 nm were attenuated by neutral density filters and focused onto the lipid bilayer membrane. Under the conditions of the experiments approximately 20% of the caged ATP is released as ATP by each light flash. After performing experiments in the absence of RH421, the dye was added to the cuvette from an ethanolic stock solution and experiments were continued until no further change in the observed relaxation time was apparent. Control experiments in which small volumes of ethanol were added to the membrane showed no significant change of the measured rate constant.

Stopped-flow experiments were carried out using an SF-61 stopped-flow spectrofluorimeter from Hi-Tech Scientific Ltd. (Salisbury, U.K.). The solution in the observation chamber was excited using a 100 W short arc mercury lamp (Osram, Germany) and the fluorescence was detected at right angles to the incident light beam using an R928 multialkali side-on photomultiplier. The exciting light was passed through a grating monochromator with a blaze wavelength of 500 nm. In the case of experiments using RH421, the mercury line at 577 nm was used for excitation and the fluorescence was collected at wavelengths $\geq$665 nm by using an RG665 glass cutoff filter (Schott, Mainz, Germany) in front of the photomultiplier. For experiments using BIPM-labeled enzyme, the mercury line at 313 nm was used for excitation and the fluorescence was collected at wavelengths $\geq$360 nm by using a WG360 glass cutoff filter (Schott, Mainz, Germany) in front of the photomultiplier. The kinetic data were collected via a high-speed 12-bit analog-to-digital data acquisition board and were analyzed using software developed by Hi-Tech Scientific Ltd. (Salisbury, U.K.). Each individual kinetic trace consisted of either 512 or 1024 (in the case of the data presented in Figures 6 and 8) data points. In order to improve the signal-to-noise ratio, typically between 6 and 14 experimental traces were averaged before the reciprocal relaxation time was evaluated. The errors bars shown on 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. The relaxation time is defined here as the time necessary for the difference in fluorescence intensity from its final steady state value to decay to 1/e of its value at any point in time. This is based on the standard definition for all relaxation kinetic methods. It should be noted that none of the kinetic methods employed here is strictly a relaxation method, but, because all experiments were carried out under pseudo-first-order conditions, exponential decay behavior is to be expected and for simplicity the term relaxation time is, therefore, used throughout. Nonlinear least squares fits of the reciprocal relaxation times to appropriate kinetic models were performed using the commercially available program ENZFITTER. In order to take into account the greater absolute errors of the higher values of the reciprocal relaxation times, the individual points were weighted 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. Computer simulations of experimental stopped-flow transients were carried out using the commercially available program Mathematica 2.2.

The kinetics of the Na^+,K^+-ATPase conformational changes and ion translocation reactions were investigated in the stopped-flow apparatus by mixing Na^+,K^+-ATPase labeled with either RH421 or BIPM in one of the drive syringes with an equal volume of an ATP solution from the other drive syringe. Both solutions were made up in the same buffer (composition given below), so that no change in the Na^+ concentration occurred on mixing. The solutions in the drive syringes were equilibrated to a temperature of 24 °C prior to each experiment. The drive syringes were driven by compressed air. 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 to a value of not greater than one-tenth of the relaxation time of the fastest enzyme-related transient, i.e., from 0.33 ms for measurements at saturating ATP and Na^+ concentrations down to 10 ms at the lowest ATP and Na^+ concentrations. It was found that RH421 bound to Na^+,K^+-ATPase membrane fragments undergoes a slow photochemical reaction which is characterized by an increase in fluorescence and has an apparent rate constant $\leq$1.6 s^{-1} depending on the intensity of the exciting light. Interference of the photochemical reaction with the kinetics of the Na^+,K^+-ATPase-related fluorescence transients was, however, avoided by inserting neutral density filters in the light beam in front of the monochromator. The kinetics of K^+ deocclusion and conformational changes of dephosphorylated enzyme were investigated in the stopped-flow apparatus by mixing Na^+,K^+-ATPase labeled with RH421 and in the presence of 1 mM KCl with an equal volume of 130 mM NaCl containing varying concentrations of Na_2ATP. In this case Mg^{2+} ions were omitted from the buffer to prevent the phosphorylation reaction from occurring.

All stopped-flow experiments with the Na^+,K^+-ATPase in which the enzyme was mixed with ATP, except those in which the pH, the Na^+ concentration or the K^+ concentration were varied, were performed in a buffer containing 30 mM imidazole, 130 mM NaCl, 5 mM $MgCl_2$, and 1 mM EDTA. In the case of experiments in which the Na^+ concentration was varied, choline chloride was added to the buffer medium to maintain a total concentration of NaCl plus choline chloride of 130 mM. The total ionic strength was, therefore, kept constant at a value of 160 mM (excluding contributions from imidazole and EDTA).

Each data set, in which the concentration either of Na^+, K^+, RH421, ATP, or the pH was varied, was recorded using a single Na^+,K^+-ATPase preparation. The pH was adjusted to the value required by the addition of HCl. The buffer substances used were Bis-Tris (pH 5.8), imidazole (pH 6.2−7.8), and Tris (pH 8.2−9.0). All solutions were prepared using deionized water. The nominally K^+-free buffers were analyzed by total-reflection X-ray fluorescence spectroscopy and atomic absorption spectroscopy and were found to contain not more than 25 μM of K^+ ions.

Rapid mixing quenched-flow experiments were performed using a stepping-motor driven chemical quenched-flow device equipped with Berger ball mixers (Froehlich et al., 1976). The temperature of the reagents was maintained at 24 °C by a water jacket surrounding the syringe block and by circulating warm air from a hair drier onto the mixer blocks and intervening capillary tubing. Control, RH421-equilibrated, or BIPM-labeled pig kidney Na^+,K^+-ATPase was suspended (0.2 mg of protein/mL) in a solution containing 16 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 25 mM sucrose, and 25 mM imidazole, pH 7.4, and mixed with an equal volume of the same solution without enzyme containing 200 μM [γ-^{32}P]ATP. In experiments where RH421 was present, the dye was added to the enzyme and substrate solutions from an ethanolic stock solution (0.83 mM) to a final concentration of 5 μM. After a brief time delay (2.5−300 ms), the reaction was quenched by the addition of a solution containing 3% perchloric acid and 2 mM K_2HPO_4 (final concentrations) and a 2 mL sample of the quenched reaction mixture was collected for analysis of the acid stable [^{32}P]phosphoprotein (Fendler et al., 1993). To facilitate quantitative recovery of the phosphoprotein, 0.2 mg of bovine serum albumin was added to each sample prior

to centrifugation. The protein was washed three times with an ice-cold solution containing 5% trichloroacetic acid, 10% polyphosphoric acid, and 10 mM K_2HPO_4 and dissolved in 1 M NaOH for liquid scintillation counting (Cerenkov radiation).

The time course of dephosphorylation was investigated in two-stage mixing experiments by chasing the phosphoenzyme with a solution containing KCl and EDTA, which activate hydrolysis of the phosphoenzyme while preventing rephosphorylation of the Na^+,K^+-ATPase, respectively. Control and BIPM-labelled Na^+,K^+-ATPase were phosphorylated for 116 ms in the absence of K^+ as described above and then mixed with a buffered solution containing 30 mM KCl and 30 mM EDTA (10 mM each after mixing). After a variable period of time (0−289 ms) 3% perchloric acid was added to quench the reaction and a 2 mL sample was collected for analysis of the ^{32}P-labeled phosphoenzyme.

The origins of the various reagents used were as follows: tris((hydroxymethyl)amino)methane (99.9%, Sigma), imidazole (99+%, Sigma, or ≥99.5%, Fluka), bis[2-hydroxyethyl]iminotris[hydroxymethyl]methane (>98%, Sigma), EDTA (99%, Sigma), NaCl (Suprapur, Merck), KCl (analytical grade, Merck), $MgCl_2 \cdot 6H_2O$ (analytical grade, Merck), HCl (0.1 N Titrisol solution, Merck), dithiothreitol (95%, Reanal, Budapest, Hungary), ATP magnesium salt·$5.5H_2O$ (approx. 97%, Sigma), ATP disodium salt·$3H_2O$ (special quality, Boehringer Mannheim), ethanol (analytical grade, Merck), sodium orthovanadate (95%, Sigma), choline chloride (99+%, 3× crystallized, Sigma, or Microselect, Fluka) and [γ-^{32}P]adenosine 5′-triphosphate (222 TBq/mmol, >98%, New England Nuclear).

RESULTS

Effect of RH421 on the Kinetics of the Na^+,K^+-ATPase

It has been found that RH421 concentrations above 1 μM inhibit the steady state hydrolytic activity of the Na^+,K^+-ATPase (Frank et al., 1996). Here we wish to investigate whether the inhibition involves Na^+-dependent partial reactions of the enzyme. All experiments were therefore performed in the complete absence of K^+ ions.

An RH421 fluorescence stopped-flow transient for BIPM-labeled enzyme is shown in Figure 1A. The presence or absence of the covalent BIPM label on the enzyme was found to have no effect on the observed time course of the RH421 stopped-flow fluorescence transients, but in the presence of the BIPM label the amplitude of the fluorescence transients was found to be significantly reduced (i.e., the relative fluorescence change was approximately 20%, in comparison to almost 100% in the absence of BIPM). The enzyme preparation procedure had no significant influence on the observed kinetics. It was found that two exponential time functions were necessary to adequately fit the data. The faster phase was responsible for the majority of the fluorescence intensity change. In the case of RH421, the slower phase contributed only between one-sixth and one-tenth of the total fluorescence change. Control experiments in which 30 mM sodium orthovanadate was added to the drive syringe containing the Na^+,K^+-ATPase membrane fragments resulted in the complete disappearance of the fluorescence change. The signal was also abolished if Mg^{2+} ions were omitted from the buffer medium and MgATP was replaced by its disodium salt. These control experiments indicate that the observed fluorescence changes are in fact due to the ATP hydrolytic action of the Na^+,K^+-ATPase.

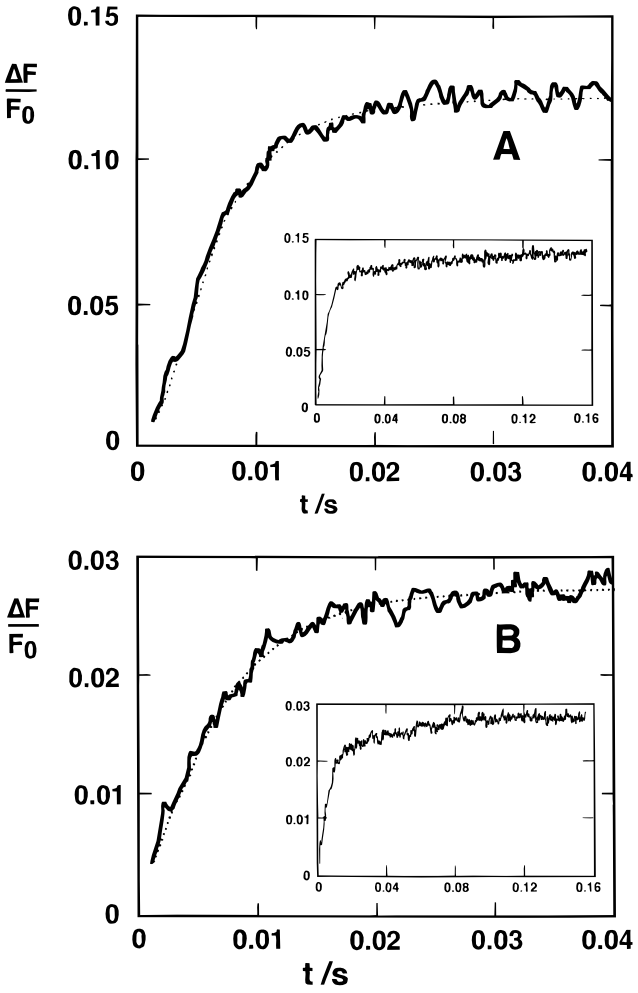

FIGURE 1: Stopped-flow fluorescence transients of BIPM-labeled Na^+,K^+-ATPase membrane fragments from pig kidney. Na^+,K^+-ATPase (10 μg/mL or 0.068 μM, after mixing) was rapidly mixed with an equal volume of MgATP (0.5 mM, after mixing). Each solution was in a buffer containing 50 mM NaCl, 105 mM choline chloride, 25 mM imidazole, 4 mM $MgCl_2$, and 1 mM EDTA; pH = 7.0, T = 24 °C. The dotted lines represent fits to a biexponential time function. (A) RH421 (75 nM, after mixing) was added to the Na^+,K^+-ATPase suspension. 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 calculated reciprocal relaxation times were 196 (±12) s^{-1} (83% of the total amplitude) and 9 (±2) s^{-1} (17%). (B) The fluoresence of BIPM covalently bound to the protein was measured using an excitation wavelength of 313 nm at emission wavelengths ≥360 nm (WG360 glass cutoff filter). The calculated reciprocal relaxation times were 195 (±17) s^{-1} (75% of the total amplitude) and 20 (±3) s^{-1} (25%). The inserts show the same experimental traces but over a longer time scale.

The effect of the concentration of RH421 on the magnitude of the reciprocal relaxation time of the fast phase, $1/\tau_1$, is shown in Figure 2. It can be seen that there is a continual decrease in the value of $1/\tau_1$ from its maximum value of approximately 160 s^{-1} as the dye concentration increases. This is consistent with the previously observed dye inhibition of steady state enzyme activity (Frank et al., 1996). In the case of the slow phase, no significant change in the magnitude of the reciprocal relaxation time could be observed. $1/\tau_2$ was always in the range 8−30 s^{-1}. It should

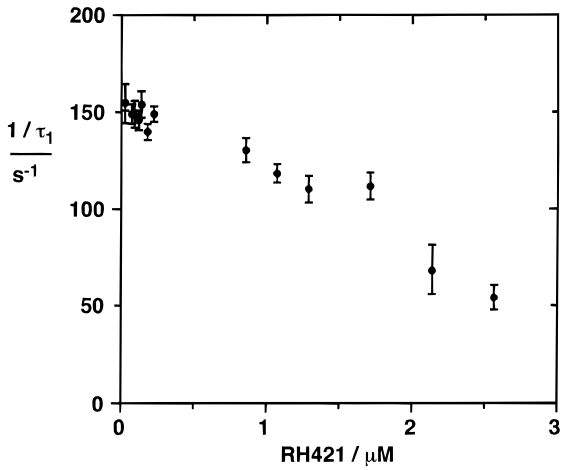

FIGURE 2: Dependence of the reciprocal relaxation time, $1/\tau_1$, of the fast phase of the RH421 fluorescence change on the concentration of RH421 (after mixing) for stopped-flow experiments in which Na^+,K^+-ATPase without the BIPM label was rapidly mixed with MgATP in the absence of K^+ ions. [Na^+,K^+-ATPase] = 10 μg/mL (≡ 0.068 μM), [NaCl] = 130 mM, [MgATP] = 1.5 mM, [imidazole] = 30 mM, [$MgCl_2$] = 5 mM, [EDTA] = 1 mM, λ_{ex} = 577 nm, $\lambda_{em} \geq 665$ nm, pH = 7.48, T = 24 °C.

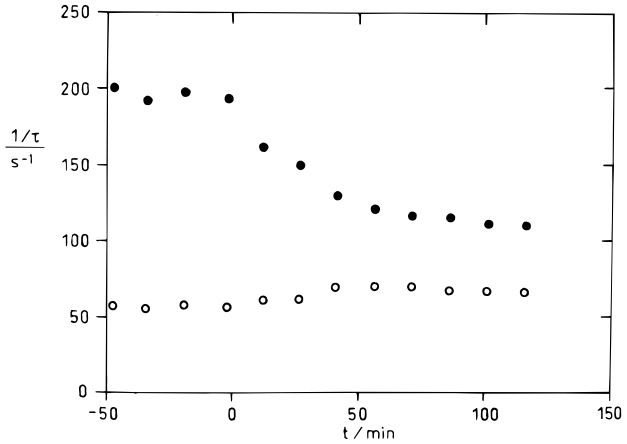

FIGURE 3: Reciprocal relaxation time, $1/\tau$, of the rising phase (●) and the decaying phase (○) of the current relaxation of Na^+,K^+-ATPase membrane fragments adsorbed to a black lipid membrane on photochemical release of ATP from its caged complex as a function of time, t, before and after the addition of RH421. At t = 0, RH421 was added to the cell to a concentration of 5.7 μM. [Na^+,K^+-ATPase] = 25 μg/mL (≡ 0.17 μM), [NaCl] = 130 mM, [caged ATP] = 100 μM, pH = 6.2, T = 24 °C.

be noted, however, that accurate determination of the reciprocal relaxation time of the slow phase is made difficult because of its much smaller amplitude compared to the fast phase. We have, therefore, decided to concentrate on a quantitative analysis of the dominant fast phase, which, because its rate is significantly higher than that of the slow phase over all the concentrations of Na^+ and ATP studied, can be considered chemically decoupled from the slow phase. In contrast, it could be possible that the relaxation of the slow phase is chemically coupled to that of the fast phase, if the two phases represent consecutive reactions on the same catalytic pathway. This possibility is presently under investigation.

The detection of the slow phase was found to depend on the excitation wavelength used. If, instead of the mercury line at 577 nm, the line at 544 nm was used, and the fluorescence was collected at wavelengths >610 nm by using an RG610 glass cutoff filter, then the observed transients could be fitted well to a single exponential with a reciprocal relaxation time indistinguishable from the value obtained for the fast phase at 577 nm excitation. The mercury line at 577 nm was chosen for excitation in our studies because it has previously been found in steady state fluorescence measurements using RH421 and Na^+,K^+-ATPase membrane fragments that the percentage fluorescence change after the addition of ATP increases significantly as the excitation wavelength increases (Bühler et al., 1991; Clarke et al., 1992).

On the basis of the results shown in Figure 2, for all subsequent stopped-flow measurements using RH421 a dye concentration of 75 nM after mixing was chosen, so that dye-induced inhibition of the Na^+,K^+-ATPase could be avoided.

RH421-induced inhibition of Na^+-dependent partial reactions of the Na^+,K^+-ATPase could also be observed electrically in a black lipid membrane after the photochemical release of ATP from its caged compound. These experiments were performed at pH 6.2, because the kinetics of ATP release in the presence of Mg^{2+} ions at neutral pH values is too slow for the time resolution necessary (Walker et al., 1988). The experimental current transients are characterized by a rapid rise in the current followed by a slower decay. Representative transients displaying this behavior can be found in Fendler et al. (1987, 1993) and Borlinghaus and Apell (1988). As the dye (final concentration 5.7 μM) is spontaneously incorporated into the adsorbed membrane fragments, there is a slow decrease in the reciprocal relaxation time characterizing the rising phase of the transient pump current from a value of almost 200 s^{-1} prior to the addition of the dye to a final value of approximately 110 s^{-1} (see Figure 3). The reciprocal relaxation time characterizing the decaying phase of the pump current was almost unaffected by the addition of dye. The rising phase of the transient signal has been attributed by Fendler et al. (1993) to an electrogenic conformational change of the enzyme ($E_1P \rightarrow E_2P$). Apell et al. (1987), on the other hand, attribute it to nonelectrogenic processes preceding phosphorylation of the enzyme, including photochemical release and binding of ATP to the protein. The finding from the stopped-flow and quenched-flow results, that RH421 inhibits conformational changes of the enzyme subsequent to phosphorylation (see below), would lend support to the interpretation of Fendler et al. (1993) for the current transients.

Effect of RH421 and BIPM on the Na^+,K^+-ATPase Phosphorylation Kinetics

Conditions have been found where RH421 does not inhibit the overall kinetics of Na^+,K^+-ATPase phosphorylation and concomitant conformational changes. If the rate constants obtained using the probe RH421 are to be compared with those measured using BIPM-labeled Na^+,K^+-ATPase, it is important to establish that under the conditions of the experiment BIPM does not inhibit the enzyme. Earlier work (Taniguchi et al., 1982) showed some inhibition of Na^+,K^+-ATPase activity by BIPM treatment, but later it was found by that labeling pig kidney Na^+,K^+-ATPase with BIPM following an *N*-ethylmaleimide pre-block to enhance the specificity of incorporation at Cys 964 does not prevent phosphorylation of the enzyme using either ATP (Nagai et

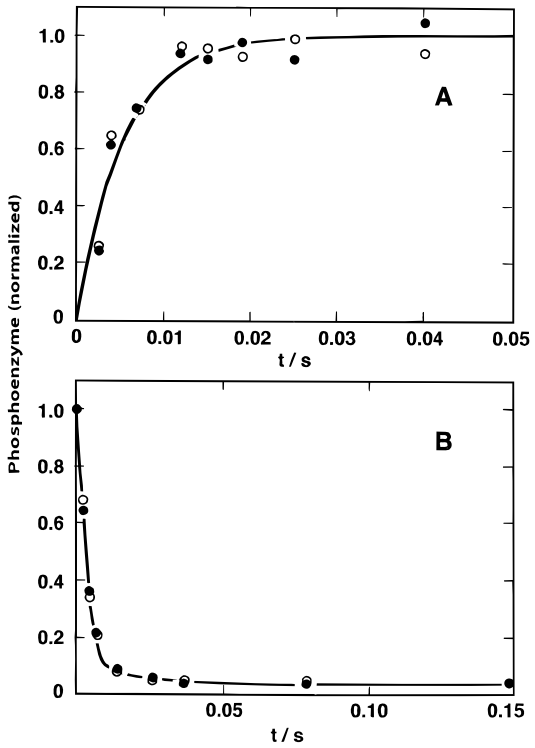

FIGURE 4: Phosphoenzyme formation (A) and dephosphorylation (B) of native (●) and BIPM-labeled enzyme (○), both prepared by NaI and deoxycholate treatment and measured by quenched-flow mixing. Enzyme suspended in 16 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 25 mM sucrose, and 25 mM imidazole, pH 7.5, was phosphorylated at 24 °C by the addition of 100 μM [γ-^{32}P]ATP (final concentration) and mixed with 3% perchloric acid at the indicated times to terminate the reaction. After 116 ms of phosphorylation, dephosphorylation was initiated by the addition of 10 mM KCl + 10 mM EDTA and allowed to proceed for the indicated times before the addition of acid. The amount of phosphoenzyme formed in each experiment has been normalized by dividing by (A) the final concentration of phosphoenzyme formed or by (B) the concentration of phosphoenzyme formed after 116 ms. Each point is the average of duplicate experiments. The solid lines in A represent fits to a monoexponential time function, where the reciprocal relaxation times were 191 (±18) s^{-1} for native enzyme and 198 (±21) s^{-1} for BIPM-labeled enzyme. The solid lines in B represent fits to a biexponential time function with reciprocal relaxation times of 277 s^{-1} (95% of the total amplitude) and 4.9 s^{-1} (5%) for the native enzyme and 271 s^{-1} (96%) and 2.4 s^{-1} (4%) for the BIPM-labeled enzyme.

al., 1986) or acetylphosphate as substrates (Taniguchi et al., 1988, 1994). To determine if covalent modification with BIPM influences the kinetics of the Na^+,K^+-ATPase partial reactions, rapid mixing acid quenched-flow experiments were performed on native and BIPM-labeled enzyme preparations. This method allows the formation of the phosphoenzyme to be determined as a function of time so that the rate constant for this reaction can be evaluated. Figure 4A compares the initial time courses of phosphorylation in the native and BIPM-labeled enzyme preparations at 100 μM [γ-^{32}P]ATP and 24 °C in the presence of 16 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 25 mM sucrose, and 25 mM imidazole, pH 7.4. Both preparations displayed monoexponential kinetics with the native enzyme yielding a phosphorylation rate constant of 191 (±18) s^{-1} and the BIPM-labeled enzyme a value of 198 (±21) s^{-1}. That the Na^+ and ATP concentrations used in the phosphorylation experiments were saturating at their respective high-affinity sites was confirmed by stopped-flow measurements under the same conditions using the BIPM-labeled preparation. These data suggest that BIPM does not alter the kinetics of phosphorylation by ATP.

To determine if modification with BIPM alters dephosphorylation, two-stage mixing experiments were carried out in which the native and BIPM-labeled Na^+,K^+-ATPase were initially phosphorylated with 10 μM ATP for 116 ms and then chased with 10 mM KCl and 10 mM EDTA for a variable period of time before quenching with acid (see Figure 4B). Although the resulting phosphoenzyme decay patterns are complex, they overlap completely after normalization, demonstrating that BIPM does not alter the kinetics of K^+-activated dephosphorylation.

The decline in the reciprocal relaxation time for the fast phase of the RH421 fluorescence signal with increasing RH421 concentration (cf. Figure 2) implies that either the reaction being monitored by this probe or a preceding reaction is becoming inhibited. To test whether phosphorylation is affected by RH421, we added 5 μM of the dye to the enzyme and substrate syringes prior to measuring phosphorylation as described above. At 100 μM ATP, the time course of phosphorylation (not shown) obeyed monoexponential kinetics with a rate constant of 202 (±20) s^{-1} at pH 7.4 and 24 °C. The close agreement between this value and that obtained using the native enzyme (191 s^{-1}) shows that the inhibitory effect of RH421 detected in the fluorescence signal and overall Na^+,K^+-ATPase activity influences a reaction downstream from phosphorylation, possibly the E_1P to E_2P conformational transition or Na^+ deocclusion. Furthermore, it is worth noting that the steady state proportion of enzyme in the phosphorylated state was about 10% higher in the presence of RH421 than in the native preparation. Because the steady state proportion of enzyme in the phosphorylated state reflects a balance between the kinetics of phosphoenzyme formation and turnover, it is likely that RH421 inhibits either the hydrolysis of enzyme in the E_2P state or a reaction just prior to hydrolysis.

Effect of pH on the Na^+,K^+-ATPase Kinetics

An analysis of the pH dependence of the reciprocal relaxation time of RH421-labeled Na^+,K^+-ATPase membrane fragments upon mixing with ATP in the stopped-flow apparatus allows the optimum pH for the Na^+-dependent partial reactions of the pump to be determined. This has not previously been possible using the method of photochemical release of ATP because of the pH dependence of the photolysis kinetics of caged ATP, as mentioned earlier. The stopped-flow experiments indicate that the value of $1/\tau_1$ reaches a maximum of approximately 188 (±6) s^{-1} at a pH of approximately 7.5 (see Figure 5). This value agrees well with values found by Apell and Marcus (1986) for the turnover rate of ion transport of the Na^+,K^+-ATPase reconsituted into lipid vesicles and by Breitwieser et al. (1987) for the ouabain-inhibitable Na^+ efflux of squid giant axons.

Effect of ATP Concentration

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 Figure 6). At a NaCl concentration of 130 mM, it was found that $1/\tau_1$ increased with increasing Na_2ATP concentration until it leveled out at a maximum value in the range 160−180 s^{-1}. Similar behavior was also found for the slow phase, except

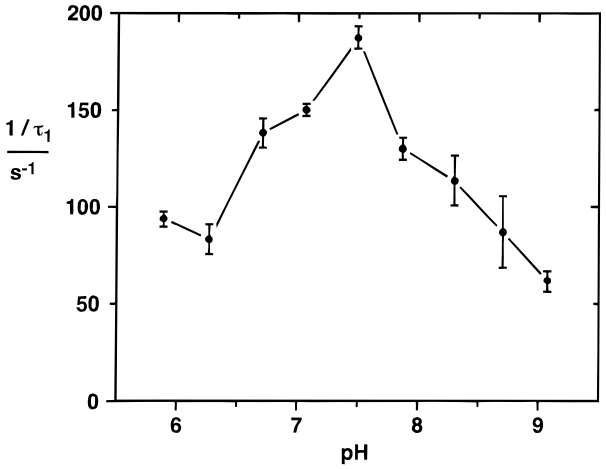

FIGURE 5: Dependence of the reciprocal relaxation time, $1/\tau_1$, of the fast phase of the RH421 fluorescence change on the pH for stopped-flow experiments in which Na^+,K^+-ATPase was rapidly mixed with MgATP in the absence of K^+ ions. [Na^+,K^+-ATPase] = 11 μg/mL (≡ 0.075 μM), [NaCl] = 130 mM, [MgATP] = 1.5 mM, [RH421] = 75 nM, [$MgCl_2$] = 3 mM, [EDTA] = 0.1 mM, T = 24 °C. The excitation and emission wavelengths were as in Figure 2. The buffer substances used were Bis-Tris (pH 5.8), imidazole (pH 6.2−7.8), and Tris (pH 8.2−9.0), each at a concentration of 25 mM. The points have been joined by lines to aid the eye of the reader.

that the maximum value was in the range 30−45 s^{-1}. The fact that the reciprocal relaxation time of the fast phase reaches a maximum value suggests that the process being observed is not simply the binding of ATP to the enzyme, since this would be expected to show a linear dependence of the reciprocal relaxation time on the ATP concentration. The simplest explanation is, therefore, that the observed process is a reaction of the enzyme occurring subsequent to ATP binding. Possible candidates are the phosphorylation of the enzyme or a conformational change (and possibly ADP and Na^+ ion release steps) induced by phosphorylation.

The total relative fluorescence change (fast and slow phases), $\Delta F/F_o$, increased with increasing ATP concentration, from a value of 0.42 at the lowest ATP concentration used (0.37 μM), until it reached a maximum value of approximately 0.94−0.97 in the ATP concentration range 15−50 μM. At higher ATP concentrations there was a decrease in the value of $\Delta F/F_o$ to 0.82 at 500 μM ATP.

Fitting the reciprocal relaxation time data for the fast phase according to the model shown in Figure 7 to eq A5 or A8 yields the following parameters:

$$k_3 = 178\ (\pm 4)\ s^{-1}$$

$$K_A = 1.42\ (\pm 0.12) \times 10^5\ M^{-1}$$

where k_3 represents the rate constant for the rate-determining step of phosphorylation and any subsequent conformational changes and K_A represents the apparent binding constant of ATP to its binding site on the enzyme. At the high Na^+ concentration used for the experiments the exact value of the Na^+ apparent binding constants, K_1 and K_2, used for the fit is unimportant, because under these conditions the final ratio in eqs A5 and A8 involving the Na^+ concentration reduces to unity. The reciprocal of K_A, i.e., 7.0 (±0.6) μM, corresponds to the apparent dissociation constant of the ATP binding site.

Effect of Na^+ Ion Concentration

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 indistinguishable from zero in the absence of Na^+ to a saturating value of 170−180 s^{-1} at 130 mM Na^+ (see Figure 8). This behavior is consistent with the idea, incorporated in the Albers−Post model, that phosphorylation of the Na^+,K^+-ATPase only occurs at a significant rate when the Na^+ ion binding sites of the enzyme are occupied. The slow phase also showed an increase in its reciprocal relaxation time with increasing Na^+ ion concentration, reaching a saturating value of 30−45 s^{-1} at 130 mM.

The total relative fluorescence change (fast and slow phases), $\Delta F/F_o$, increased with increasing Na^+ ion concentration, from a value of 0.07 at the lowest Na^+ ion concentration used (0.23 mM), until it reached a maximum value of approximately 0.94 in the Na^+ ion concentration range 8−10 mM. At higher Na^+ ion concentrations, similar to the behavior observed in the ATP titration, there was a decrease in the value of $\Delta F/F_o$ to 0.73 at a concentration of 130 mM.

The total ionic strength in these experiments was maintained at 160 mM by the addition of choline chloride. The reason for limiting the ionic strength to this value was that experiments at higher salt concentrations showed an inhibition of the enzyme activity, in agreement with electrical bilayer measurements of Nagel et al. (1987). Although choline binds to pig kidney Na^+,K^+-ATPase (Grell et al., 1992, 1994), its binding appears not to affect the investigated Na^+ concentration dependence of the reciprocal relaxation time of the measured stopped-flow transients. Because Mg^{2+} ions exhibit a corresponding effect to choline but at lower concentrations (Grell et al., 1992, 1994), presumably the comparatively high concentration of Mg^{2+} used in this study prevents any effects of choline on the enzyme conformation.

Similar behavior to that shown in Figure 8, although with significantly lower values of the saturating reciprocal relaxation time, has previously been reported by Pratap and Robinson (1993) for RH421 fluorescence transients produced by rapid mixing with ATP and by Bühler et al. (1991), who released ATP photochemically from its caged precursor using a light flash. Pratap and Robinson fitted their data phenomenologically using a Hill equation. We have preferred to fit our data to the behavior expected for a proposed reaction scheme (see Appendix). This allows the estimation of rate and equilibrium constants for the reactions involved. The reaction scheme chosen is basically the Na^+ branch of the Albers−Post model, but it includes the basic assumption that the Na^+ binding steps are so fast that they are always in equilibrium on the time scale of the phosphorylation reaction. This assumption is very likely to be justified. Heyse et al. (1994) have estimated a second-order rate constant for the binding of Na^+ to the Na^+,K^+-ATPase of $\geq 1 \times 10^5$ M^{-1} s^{-1}.

In the first instance it was attempted to fit the data shown in Figure 8 to a model in which all the Na^+ binding sites are identical and there is no interaction between them. Models incorporating one, two, or three identical sites were tested, but in all cases significant systematic positive and negative deviations of the experimental points from the fitted

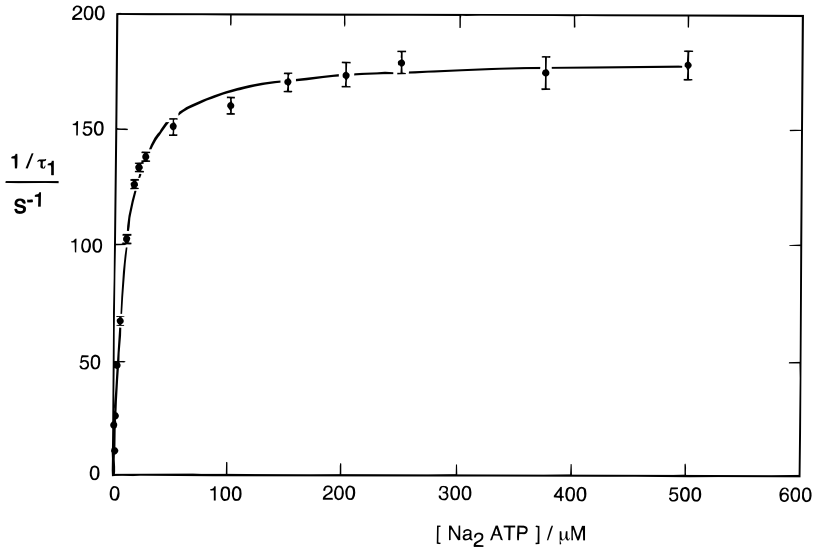

FIGURE 6: Dependence of the reciprocal relaxation time, $1/\tau_1$, of the fast phase of the RH421 fluorescence change on the concentration of Na_2ATP (after mixing) for stopped-flow experiments in which Na^+,K^+-ATPase was rapidly mixed with Na_2ATP in a nominally K^+-free buffer medium. [Na^+,K^+-ATPase] = 10 μg/mL ($\equiv$ 0.068 μM), [NaCl] = 130 mM, [RH421] = 75 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 Figure 2. The solid line represents a nonlinear least squares fit of the data to eq A5 or A8.

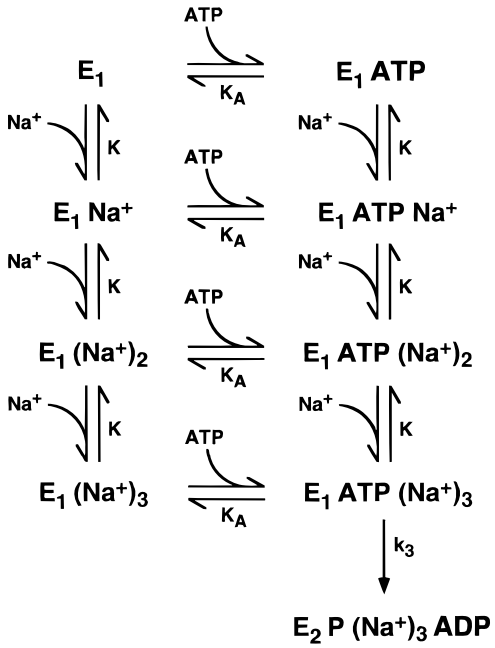

FIGURE 7: Reaction scheme describing the Na^+ and ATP binding steps of the Na^+,K^+-ATPase and its subsequent phosphorylation and conformational change.

curve were apparent. It is possible that increasing the number of Na^+ binding sites in the model to values significantly greater than three might be able to produce an improved fit to the data. Since other investigations have indicated, however, that there are only three Na^+ binding sites (Cornelius & Skou, 1988), the theoretical model has not been extended to higher stoichiometries. An identical site model was, therefore, considered to be an inappropriate description of the data.

The clear sigmoidal form of the Na^+ ion concentration dependence of $1/\tau_1$ (see Figure 8) would appear to be indicative of positive cooperativity in the binding of the Na^+ ions to the Na^+,K^+-ATPase, i.e., the binding of the first or the second Na^+ ion to the enzyme increases the apparent affinity of subsequently binding Na^+ ions for the enzyme. Such behavior has previously been suggested by a number of authors (Matsui & Homareda, 1982; Cornelius & Skou, 1988; Schulz & Apell, 1995). It was therefore decided to try and fit the experimental data to models in which the first or the first and second Na^+ ions bind weakly and, due to a modification of the enzyme structure by the weakly binding Na^+ ions, the subsequently binding Na^+ ions bind more strongly. A description of the derivations of the appropriate kinetic equations is given in the Appendix.

It was found that both models incorporating positive cooperativity gave much improved descriptions of the experimentally observed behavior over identical site models. Judging by the sum of the squares of the residuals, the best fit was obtained using a model (eq A8) involving two weakly binding sites and one strongly binding site. The fit to this model is shown in Figure 8.

The values of the parameters calculated from the fits to the positive cooperativity models are as follows. For the model incorporating one weakly binding site (apparent association constant K_1) and two strongly binding sites (apparent microscopic association constant K_2), the best fit values were $K_1 = 1.4\ (\pm 1.1) \times 10^1\ M^{-1}$, $K_2 = 4.4\ (\pm 2.0) \times 10^2\ M^{-1}$, and $k_3 = 186\ (\pm 9)\ s^{-1}$. The values of K_1 and K_2 correspond to apparent microscopic dissociation constants of 70 ($\pm$55) mM and 2.3 ($\pm$1.0) mM, respectively. For the model incorporating two weakly binding sites (apparent microscopic association constant K_1) and one strongly binding site (apparent association constant K_2), the best fit values were $K_1 = 1.2\ (\pm 0.5) \times 10^2\ M^{-1}$, $K_2 = 3.9\ (\pm 2.1) \times 10^2\ M^{-1}$ and $k_3 = 182\ (\pm 8)\ s^{-1}$. In this case the values of K_1 and K_2 correspond to apparent microscopic dissociation constants of 8.3 ($\pm$3.4) and 2.5 ($\pm$0.5) mM, respectively. For a mathematical definition of the microscopic binding constants see the Appendix. At the high ATP concentration used in the experiments the exact value of the ATP apparent binding constant, K_A, used for the fits is unimportant, because under these conditions the ratio $K_A[ATP]/(1 + K_A[ATP])$ in eqs A5 and A8 reduces to unity.

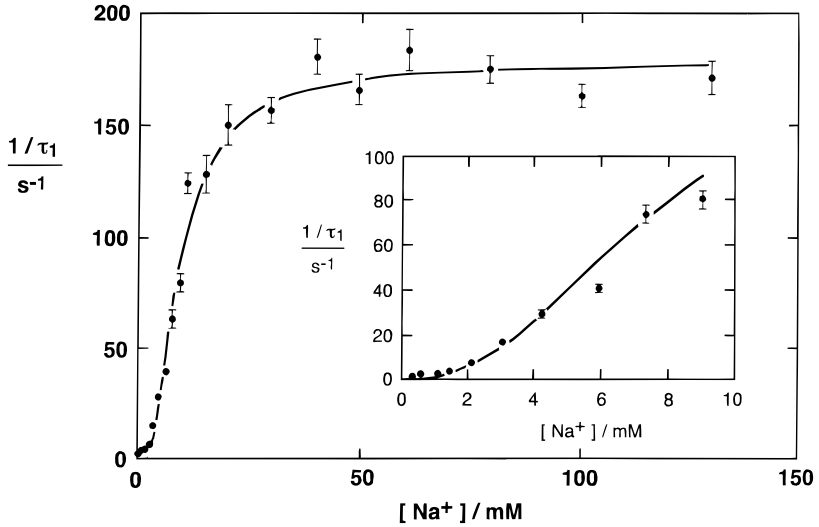

FIGURE 8: Dependence of the reciprocal relaxation time, $1/\tau_1$, of the fast phase of the RH421 fluorescence change on the concentration of Na^+ ions for stopped-flow experiments in which Na^+,K^+-ATPase was rapidly mixed with MgATP in a nominally K^+-free buffer medium. [Na^+,K^+-ATPase] = 10 μg/mL ($\equiv$ 0.068 mM), [MgATP] = 1.0 mM, [RH421] = 75 nM, [imidazole] = 30 mM, [$MgCl_2$] = 5 mM, [EDTA] = 1 mM, pH = 7.4, T = 24 °C. The total ionic strength was maintained at a constant value at NaCl concentrations below 130 mM by replacing NaCl in the solution by choline chloride, so that the total concentration of choline plus Na^+ ions was always 130 mM. The excitation and emission wavelengths were as in Figure 2. The solid line represents a a nonlinear least squares fit of the data to eq A8. The sum of the squares of the residuals between the experimental and calculated values of $1/\tau_1$ were 1518 s^{-2} (eq A5) and 1456 s^{-2} (eq A8, solid line). For comparison, a fit of the data to a model involving three identical Na^+ binding sites yielded a value for the sum of the squares of the residuals of 4047 s^{-2}.

Conformational Change of Unphosphorylated Na^+,K^+-ATPase

In order to investigate the kinetics of the conformational change and associated K^+ deocclusion of unphosphorylated Na^+,K^+-ATPase, we have carried out experiments similar to those of Karlish and Yates (1978), in which the enzyme in the presence of 1 mM KCl is rapidly mixed with 130 mM NaCl, but we used the fluorescent probe RH421 rather than the intrinsic protein fluorescence. A significant advantage of this approach is that the absorbance of ATP plays no role. In the case of tryptophan fluorescence measurements the overlap between the absorbance spectrum of ATP and the fluorescence emission spectrum of tryptophan causes fluorescence quenching and, therefore, prohibits measurements at high ATP concentrations. Stopped-flow experiments of this type have also been performed by Karlish (1980), Faller et al. (1991), Smirnova and Faller (1993), Doludda et al. (1994), and Smirnova et al. (1995) using Na^+,K^+-ATPase labelled covalently with fluorescein. The fluorescein labeling, however, blocks the high-affinity binding site for ATP and inhibits ATP hydrolysis (Scheiner-Bobis et al., 1993; Ward & Cavieres, 1996). These problems are not encountered using the probe RH421, as long as its concentration is kept sufficiently low.

The Na^+-activated reaction observed is characterised by a decrease in fluorescence, which indicates that the fluorescence intensity of dye associated with enzyme in the $E_2(K^+)_2$ conformation is higher than that of dye associated with enzyme in the $E_1(Na^+)_3$ conformation. This is in agreement with studies of Stürmer et al. (1991) on the fluorescence intensities of RH421 associated with different enzyme conformations. The kinetics of the fluorescence decrease could be closely approximated with a single exponential time function. As previously found by Karlish and Yates (1978) using the protein's tryptophan fluorescence and by Steinberg and Karlish (1989) using the 5-IAF label, the value of the reciprocal relaxation time for the observed fluorescence transient increases with increasing ATP concentration, reaching a saturating value of 28 s^{-1} (see Figure 9). The relative fluorescence change, $-\Delta F/F_o$, also increased with increasing ATP concentration, from a value of 0.052 at the lowest ATP concentration used (10 μM) to a saturating value at high ATP concentrations of approximately 0.12. 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 timescale of the conformational change and K^+ deocclusion, then it can be shown that the reciprocal relaxation time, $1/\tau$, is related to the concentration of ATP by

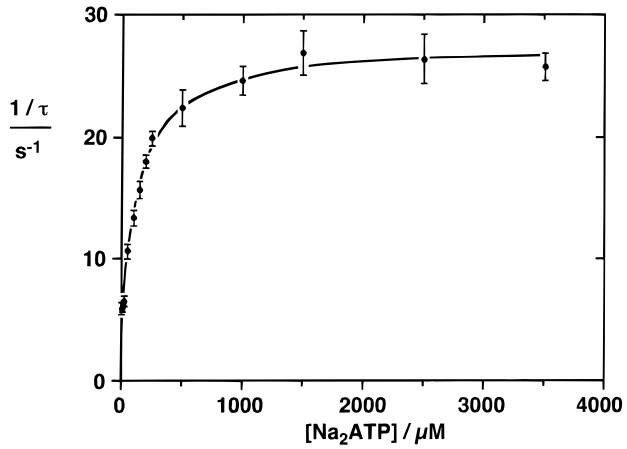

FIGURE 9: Dependence of the reciprocal relaxation time, $1/\tau$, of the RH421 fluorescence change on the concentration of Na_2ATP (after mixing) for stopped-flow experiments in which Na^+,K^+-ATPase in a buffer containing 1 mM KCl was rapidly mixed with a buffer containing 130 mM NaCl. [Na^+,K^+-ATPase] = 10 μg/mL ($\equiv$ 0.068 μM), [RH421] = 75 nM, [imidazole] = 30 mM, [EDTA] = 1 mM, pH = 7.4, T = 24 °C. The excitation and emission wavelengths were as in Figure 2. The solid line represents a nonlinear least squares fit of the data to eq 1.

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

where K_A' is the apparent binding constant of ATP to the low-affinity binding site of the enzyme, $(1/\tau)_{min}$ is the reciprocal relaxation time for the E_2 to E_1 conformational change, including K^+ deocclusion and Na^+ binding, 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 for the E_2 to E_1 conformational change: one in the absence of bound ATP and one that is ATP stimulated. Fitting the data shown in Figure 9 to eq 1 yields a value for K_A' of 7.0 ($\pm$0.8) $\times$ 10^3 M^{-1}. This corresponds to an apparent dissociation constant of 143 ($\pm$17) μM. The values of $(1/\tau)_{min}$ and $(1/\tau)_{max}$ determined from the fit were 3.6 ($\pm$0.4) s^{-1} and 27.9 ($\pm$0.7) s^{-1}, respectively.

The values of $(1/\tau)_{max}$ and the apparent dissociation constant found here may be compared with the corresponding values found by Steinberg and Karlish (1989) and by Pratap et al. (1996) for 5-IAF-labeled dog kidney Na^+,K^+-ATPase. At pH 7.0 and 20 °C, Steinberg and Karlish (1989) determined a maximum reciprocal relaxation time of between 15.9 and 28.8 s^{-1}, depending on the buffer composition, and a half-saturating ATP concentration of 196 ($\pm$36) μM. A reciprocal relaxation time of 31 s^{-1} was also found by Pratap et al. (1996) at pH 7, 20 °C, and an ATP concentration of 1 mM. When the different enzyme source and the slightly different conditions are considered, the agreement with the values presented here is surprisingly good.

DISCUSSION

The kinetics of Na^+-dependent partial reactions of the Na^+,K^+-ATPase have been investigated via the stopped-flow technique by mixing fluorescently labeled enzyme in the presence of Na^+ and Mg^{2+} ions with ATP. Two fluorescent labels were used: BIPM, which is covalently attached to the enzyme, and RH421, which is noncovalently associated with the enzyme-containing membrane fragments. The two labels delivered almost identical kinetic responses (see Figure 1). In both cases two exponential time functions were necessary to fit the data. The fast phase is the major component, contributing between 75% and 90% of the overall fluorescence change. When experiments were carried out with the same NaI/deoxycholate extracted BIPM-labeled enzyme preparation under identical experimental conditions (saturating [Na^+] and [ATP], pH 7.4 and 24 °C), no significant difference was found in the reciprocal relaxation times of the two probes: 196 ($\pm$12) s^{-1} for RH421 and 195 ($\pm$17) s^{-1} for BIPM. When experiments were carried out using RH421 on unlabeled pig kidney enzyme prepared using the Jørgensen procedure, it was found that the value varies slightly from one preparation to another in the range 150−200 s^{-1}. The origin of the slow phase is not clear at this stage. The fact that the slow phase has not previously been reported seems to be connected with the choice of excitation wavelength. In contrast to previous stopped-flow studies using RH421 (Forbush & Klodos, 1991; Pratap & Robinson, 1993), we have chosen to use a significantly longer excitation wavelength. This choice was based on the finding from steady state fluorescence measurements using RH421 and Na^+,K^+-ATPase membrane fragments that the percentage fluorescence change after the addition of ATP increases significantly as the excitation wavelength increases (Bühler et al., 1991; Clarke et al., 1992).

Based on the dependence of the observed reciprocal relaxation times on pH, ATP concentration and Na^+ concentration and taking into account the previous results of other groups, the fast kinetic phase can be interpreted as follows. Prior to the addition of ATP the enzyme can be considered to exist in an equilibrium between two conformations (E_1 and E_2). In the presence of Na^+ ions (zero added K^+), one of the conformations (E_1) is favored over the other. After the addition of ATP, enzyme in the E_1 conformation is rapidly phosphorylated and undergoes a subsequent even faster (on the time scale of the phosphorylation reaction) conformational change ($E_1(Na^+)_3$ + ATP → $E_2P(Na^+)_3$ + ADP). The experimental results can, therefore, be explained in terms of the Albers−Post model of two major enzyme conformations. No additional enzyme conformations need be assumed at this stage.

Previously it had been suggested by Pratap and Robinson (1993) that additional enzyme conformations were present. Their conclusion was based on stopped-flow kinetic data using the probes RH421, BIPM, and 5-IAF with Na^+,K^+-ATPase from dog kidney. Under saturating conditions of Na^+ and ATP they found that the reciprocal relaxation times measured using BIPM were approximately double those found for RH421 and 5-IAF. Here it has been shown that micromolar concentrations of RH421 inhibit Na^+-dependent partial reactions of the Na^+,K^+-ATPase. Since Pratap and Robinson used an RH421 concentration of 2 μM, the discrepancy they found between the kinetics observed using RH421 and BIPM could be attributed to dye-induced inhibition of the enzyme. The same explanation can be given for the slow observed rate constants of pig kidney Na^+,K^+-ATPase reported by Forbush and Klodos (1991), who used an RH421 concentration of 6 μM. Different explanations can be offered for the slow rate constants, i.e., approximately 25 s^{-1}, measured for rabbit kidney Na^+,K^+-ATPase using RH421 by Bühler et al. (1991) and Stürmer et al. (1991), who released ATP photochemically. They used an RH421 concentration of 0.7 μM, so that little dye-induced inhibition would be expected, but they used quite low concentrations of caged ATP, in the range 10−20 μM, on the basis of the assumption of an ATP dissociation constant of 0.1−1 μM (Stürmer et al., 1989). If the photochemical release efficiency is not 100%, then the amount of ATP actually available to the enzyme is likely to be significantly less. Here it has been found that the apparent dissociation constant of ATP for its high-affinity binding site is 7.0 ($\pm$0.6) μM. Therefore, assuming a similar apparent dissociation constant for their enzyme, at an ATP concentration of 10 μM the ATP binding sites would only be about 60% saturated and a rate just over half that of the maximal value would be expected. This is supported by the stopped-flow results shown in Figure 6. Further important factors which would contribute to the low rate constant found by Bühler et al. (1991) and Stürmer et al. (1991) are competition between unphotolyzed caged ATP and free ATP for the ATP binding sites (Fendler et al., 1993) and the pH and Mg^{2+}concentration dependences of the rate of photochemical release of ATP from its caged complex (Friedrich & Nagel, 1997). Walker et al. (1988) found that the release of ATP is acid catalyzed and that it is slowed by increasing Mg^{2+} concentrations. At

a pH of 7.2, a Mg^{2+} concentration of 10 mM and 20 °C, the conditions of the experiments of Bühler et al. (1991) and Stürmer et al. (1991), the rate of release can be calculated from the data of Walker et al. (1988) to be approximately 22 s^{-1}. Such a slow release would, therefore, severely limit the time resolution of the method and preclude the measurement of more rapid enzyme-related reactions. The possibility that species differences may be responsible for some of the differences in reported rate constants would seem to be unlikely, since stopped-flow measurements carried out on rabbit kidney Na^+,K^+-ATPase in an identical fashion to those described here on enzyme from pig kidney have shown very similar behavior (Clarke, Kane, Roudna, and Apell, unpublished results).

The high-affinity ATP dissociation constant of 7.0 (±0.6) μM found here can be compared with previously determined values. Using the method of photochemical release of ATP from caged ATP, but with electrical detection on a bilayer system and at a lower pH of 6.2, Fendler et al. (1993) calculated an apparent ATP dissociation constant for the Na^+,K^+-ATPase from the electric organ of eel of 14 μM. From rapid quenched-flow measurements Froehlich et al. (1983) also calculated a dissociation constant for the Na^+,K^+-ATPase from the electric organ of eel of 3.5 μM. Borlinghaus and Apell (1988) calculated a dissociation constant of ATP to the Na^+,K^+-ATPase of 10 μM from electrical bilayer measurements similar to those of Fendler et al. (1993). Much lower values, in the range 0.1−0.2 μM, have been found from equilibrium binding studies (Hegyvary & Post, 1971; Nørby & Jensen, 1971). The differences between the kinetically determined values and those from binding studies may be related to the presence or absence of Mg^{2+} and Na^+ ions, which in the case of the equilibrium binding studies were omitted to prevent phosphorylation. It has been shown by Mårdh and Post (1977) that Mg^{2+} and Na^+ can significantly modify the dissociation rate constant of ATP from the enzyme.

It is also interesting to compare the Na^+ affinity of the enzyme found here with other values in the literature. Here it was found that a model incorporating the positive cooperative binding of three Na^+ ions provided the best description of the experimental behavior. From stopped-flow experiments using RH421 similar to those reported here, Pratap and Robinson (1993) also found evidence for positive cooperativity and half-saturation of their observed rate constant at a Na^+ concentration of 6 mM. This agrees quite well with our data, which show half-saturation at 8−10 mM. Much higher Na^+ affinities have been found, however, from equilibrium binding studies in the *absence* of Mg^{2+} ions. Ruf et al. (1994) determined a dissociation constant at pH 7.4 of approximately 0.2 mM. Fitting similar data to a two-site model, Grell et al. (1992, 1993) derived two macroscopic dissociation constants, both with values of 0.2 mM. From kinetic stopped-flow studies, but also in the absence of Mg^{2+} ions, Smirnova et al. (1995) calculated a microscopic (intrinsic) dissociation constant for a two-site model of 0.38 mM. In this case it is clear that the differences in apparent affinities can be attributed to the presence or absence of Mg^{2+} ions. Grell et al. (1992, 1993) have presented evidence based on equilibrium binding studies for a competition between Mg^{2+} and Na^+ ions for sites on the E_1 conformation of the enzyme, and Schwappach et al. (1994) have demonstrated a clear reduction in the apparent Na^+ affinity from fluorescence titrations using RH421 after the addition of Mg^{2+} ions to the same enzyme preparation.

Let us now consider the origin of the fluorescence signal observed using RH421. Using chymotrypsin-modified Na^+,K^+-ATPase, which is still able to be phosphorylated but is no longer able to transport Na^+ ions (Jørgensen & Petersen, 1985), Stürmer et al. (1991) found that the fluorescence changes of RH421 previously induced by the addition of ATP to native Na^+,K^+-ATPase were abolished, thus indicating that the phosphorylation reaction alone is insufficient to produce a fluorescence change. Furthermore, Pratap and Robinson (1993) found that after treatment with oligomycin, an inhibitor which has no effect on the rate of phosphorylation and is thought to act by blocking the conversion of E_1P to E_2P (Glynn, 1985), the rate of the fluorescence change of RH421-labelled Na^+,K^+-ATPase membrane fragments induced by mixing with ATP decreased by 2 orders of magnitude. Likely causes of the RH421 signal are, therefore, the conformational change in which Na^+ ions are transported across the membrane ($E_1P(Na^+)_3 \rightarrow E_2P(Na^+)_3$) or a subsequent Na^+ release from $E_2P(Na^+)_3$. It is possible that these steps may involve charge movement, which could be detected by the dye as a change of local electric field strength (Bühler et al., 1991; Clarke & Kane, 1997), but since the mechanism of interaction of RH421 with the Na^+,K^+-ATPase is not clear at this stage, the attribution of RH421 fluorescence changes to electrogenic reaction steps has been avoided here. Additional support for the assignment of the RH421 signal to the reactions $E_1P(Na^+)_3 \rightarrow E_2P(Na^+)_3$ or Na^+ release derives from a comparison of the BIPM and RH421 signals which were kinetically indistinguishable in the present study. Taniguchi et al. (1982, 1984) found that the BIPM signal consists of an initial downward deflection which is stabilized by very high concentrations of NaCl ($K_{1/2}$ = 0.6 M). This behavior suggests that the fluorescence decrease corresponds to the formation of the $E_1P(Na^+)_3$ conformation, which has been shown by Post and Suzuki (1991) to be stabilized by high chloride concentrations. The subsequent increase in BIPM fluorescence, which predominates at low concentrations of NaCl, is then likely to represent the formation of an E_2P state, either with or without bound Na^+ ions.

At pH 7.4 and at saturating Na^+ and ATP concentrations, the reciprocal relaxation time of the fast phase (150−200 s^{-1} at 24 °C) is indistinguishable from the limit of around 180 s^{-1} set by the rate of the phosphorylation reaction for the Na^+ branch of the pump cycle (Mårdh & Zetterqvist, 1974; Hobbs et al, 1988). This limitation on the kinetics of the fast phase was confirmed in the present study by quenched-flow measurements of the phosphorylation reaction in the presence of RH421 and in BIPM-labeled and unlabeled Na^+,K^+-ATPase (see Figure 5); at pH 7.5 and a saturating ATP concentration of 100 μM a rate constant of 190−200 s^{-1} was obtained. This means that, if it is accepted that RH421 is detecting the conformational change ($E_1P(Na^+)_3 \rightarrow E_2P(Na^+)_3$), the rate of this reaction must be much faster. If RH421 is actually detecting the release of Na^+ from $E_2P(Na^+)_3$, then both the conformational change and the Na^+ release must be much faster. A lower limit of the conformational change rate constant can be obtained if one assumes that the overall reaction leading to $E_2P(Na^+)_3$ consists of essentially two irreversible steps with the first step corresponding to the phosphorylation reaction with a rate constant of 190−200 s^{-1} and the second step corresponding to the

conformational change. The ATP binding reaction is considered to be in rapid equilibrium and is, therefore, included in the phosphorylation step. Under these conditions one would expect an initial induction period before any enzyme in the $E_2P(Na^+)_3$ conformation is formed (Laidler, 1987), so that there should be a slight lag at the beginning of the fluorescence stopped-flow traces. Since no significant lag is observed, the induction period must be shorter than the dead-time of the mixing cell (1.7 ms). Simulations of the time course of the stopped-flow transients based on the assumption of two irreversible reaction steps show that the close agreement between the reciprocal relaxation times of the stopped-flow and quenched-flow experiments can only be observed if the rate constant of the conformational change is ≥ 600 s^{-1}. The simulations were carried out by solving the corresponding series of differential rate equations describing the change in concentrations of the species $E_1(Na^+)_3$, $E_1P(Na^+)_3$, and $E_2P(Na^+)_3$ for the following reaction scheme:

$$E_1(Na^+)_3 \xrightarrow{k_a} E_1P(Na^+)_3 \xrightarrow{k_b} E_2P(Na^+)_3$$

In accordance with the results of the quenched-flow measurements, k_a was kept constant at a value of 200 s^{-1} and k_b was varied until the reciprocal relaxation time for the growth of E_2P reached the maximum value of approximately 190 s^{-1} found by stopped-flow. An important conclusion from these studies is that the phosphoenzyme conformational transition is very fast and that at pH 7.4 this step as well as the reaction(s) monitored by RH421 and BIPM are controlled by the phosphorylation reaction which is rate-limiting in the Na^+ translocation sequence, $E_1(Na^+)_3 + ATP \rightarrow E_2P(Na^+)_3 + ADP$, at saturating ATP concentrations (≥ 100 μM; see Figure 6).

A fast $E_1P(Na^+)_3$ to $E_2P(Na^+)_3$ transition is not consistent with computer simulations of Läuger and Apell (1988) and Stürmer et al. (1989) on the basis of their caged ATP experiments with electrical detection or with fluorescence detection using 5-IAF. Similarly it does not agree with the calculations of Wuddel and Apell (1995) based on combined caged ATP and charge-pulse experiments. They propose a rate constant of around 20 s^{-1} at 20 °C and suggest that it is the rate-limiting process of the Na^+ translocation. However, as discussed above, this low value can be explained by the underestimation (Stürmer et al., 1989) by these workers of the ATP dissociation constant of the enzyme and by the slow rate of photochemical release of ATP under their experimental conditions (pH 7.2 and 10 mM $MgCl_2$). Our conclusion of a rapid $E_1P(Na^+)_3$ to $E_2P(Na^+)_3$ transition is closer to results obtained from voltage jump measurements. Using cardiac cells, Nakao and Gadsby (1986) reported a reciprocal relaxation time at zero holding potential and 37 °C of approximately 250 s^{-1}, while Rakowski (1993) found a value of approximately 180 s^{-1} at 22 °C using *Xenopus* oocytes. Using giant cardiac membrane patches and, therefore, in the absence of all constituents of the cell cytoplasm, Hilgemann (1994) determined a reciprocal relaxation time of 400 s^{-1} at 37 °C, while Friedrich and Nagel (1997) found a value of 200 s^{-1} at 24 °C. When allowances are made for the differences in temperature, these values are similar to the rate of phosphorylation at saturating ATP, 190 s^{-1}, measured at 24 °C, as well to the reciprocal relaxation time of the fast phase of the stopped-flow RH421 and BIPM signals. This similarity leads one to suspect that the relaxation measured in the voltage jump experiments might in fact be due to a phosphorylation equilibrium, which is coupled to a much faster electrogenic equilibrium, presumably the $E_1P(Na^+)_3 \leftrightarrow E_2P(Na^+)_3$ conformational change or the subsequent release of Na^+ ions. This could, however, only be the case if ADP was present in the medium. Rakowski (1993) was of the opinion that at least in his voltage jump experiments sufficient ADP was present to allow for reversibility of the phosphorylation reaction. An analysis of the ATP concentration dependence of the reciprocal relaxation times measured via voltage jump may be able to resolve the present confusion and provide a more reliable estimate of the rate of the conformational change. Recent estimates of the rate of the conformational change, based on simulations of phosphoenzyme formation and inorganic phosphate release in the presence of K^+ in the eel and pig kidney Na^+,K^+-ATPases, indicate a value of ca. 3000 s^{-1} at pH 7.4 and 24 °C (Froehlich et al., 1997). The lower limit of 600 s^{-1} obtained in the present study is based on the assumption that RH421 monitors the sequence of steps ending with the formation of $E_2P(Na^+)_3$. If the reaction monitored by RH421 was separated from the conformational transition by an additional step, i.e., deocclusion of Na^+ from $E_2P(Na^+)_3$, then the rate constant for the conformational transition could be significantly higher.

One final interesting point is the origin of the inhibition found when using RH421. Here it was found that micromolar concentrations of RH421, although inhibiting the overall ATPase reaction (Frank et al., 1996) and the fast phase of the RH421 signal observed in the stopped-flow experiments (see Figure 2), did not affect the kinetics of phosphorylation. Furtheremore, 5 μM RH421 was found to increase the steady state level of phosphoenzyme (by about 10%). These findings indicate that a step between E_1P formation and the hydrolysis of enzyme in the E_2P state is inhibited, possibly the $E_1P(Na^+)_3 \rightarrow E_2P(Na^+)_3$ conformational change or Na^+ release from the $E_2P(Na^+)_3$ state. RH421 possesses a positively charged moiety, which can insert into the bilayer and change the local electric field strength (Malkov & Sokolov, 1996; Clarke & Kane, 1997). This could increase the potential energy barrier for Na^+ transport or release. Such an inhibition mechanism has been suggested for the positively charged tetraphenylphosphonium ion (Cornelius, 1995; Klodos et al., 1995).

In conclusion, the measurements reported here using the probes RH421 and BIPM are consistent with the conclusion that at pH 7.4 and saturating ATP and Na^+ concentrations, the phosphorylation reaction of the Na^+,K^+-ATPase occurs at a rate of approximately 200 s^{-1} at 24 °C and that the subsequent conformational change ($E_1P(Na^+)_3 \rightarrow E_2P(Na^+)_3$) is even faster. The rate-determining step of the Na^+,K^+-ATPase under steady-state conditions is most likely to be the conformational change and associated K^+ deocclusion and Na^+ binding of unphosphorylated enzyme ($E_2(K^+)_2 + 3Na^+ \rightarrow E_1(Na^+)_3 + 2K^+$), which, on the basis of stopped-flow measurements, occurs in the absence of Mg^{2+} with a rate constant of ≤ 28 s^{-1}. Further investigations are necessary in order to show whether or not this reaction is significantly accelerated by the presence of Mg^{2+} ions.

APPENDIX

Reciprocal Relaxation Time for ATP Mixing Experiments

In order to describe the ATP and Na^+-concentration dependence of $1/\tau_1$ the reaction scheme shown in Figure 7 has been used. The reaction scheme incorporates the following essential points:

(1) a single ATP binding site per enzyme molecule in the state E_1, whose binding constant is independent of the number of Na^+ ions bound,
(2) three Na^+ binding sites,
(3) the Na^+ and ATP binding steps are always in equilibrium on the time scale of the phosphorylation reaction,
(4) phosphorylation and any subsequent more rapid conformational changes and ADP and Na^+ release steps occur only after the binding of ATP and three Na^+ ions,
(5) the phosphorylation reaction is irreversible.

These points are based predominantly upon the experimental findings reported here, but they also take into account previously established kinetic information about the Na^+,K^+-ATPase reported elsewhere.

In order to derive an equation for $1/\tau_1$ containing the equilibrium and rate constants for the various steps as well as the total ATP and Na^+ concentrations we have applied the theory of relaxation kinetics, as developed by Eigen (1968) and applied by Kirschner and co-workers (1966, 1971a,b) 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 Na^+ and ATP over enzyme, the theory is also applicable to stopped-flow data.

The rate of change of the deviation of the concentration of $E_2P(Na^+)_3$ from its final equilibrium value is given by

$$-\frac{d\Delta E_2P(Na^+)_3}{dt} = k_3\Delta E_1ATP(Na^+)_3 \qquad (A1)$$

The binding constant, K_A, of ATP to its binding site on the enzyme is given by

$$K_A = \frac{E_1ATP}{E_1{\cdot}ATP} = \frac{E_1ATP\ Na^+}{E_1Na^+{\cdot}ATP} = \frac{E_1ATP(Na^+)_2}{E_1(Na^+)_2{\cdot}ATP} = \frac{E_1ATP(Na^+)_3}{E_1(Na^+)_3{\cdot}ATP} \qquad (A2)$$

For a positive cooperative model in which the first Na^+ ion binds weakly with an association constant K_1 and the two subsequent Na^+ ions bind strongly with a microscopic (or intrinsic) association constant K_2, K_1 and K_2 are defined by the following two equations:

$$K_1 = \frac{E_1Na^+}{E_1{\cdot}Na^+} = \frac{E_1ATP\ Na^+}{E_1ATP{\cdot}Na^+} \qquad (A3)$$

$$K_2 = \frac{E_1(Na^+)_2}{2E_1Na^+{\cdot}Na^+} = \frac{E_1ATP(Na^+)_2}{2E_1ATP\ Na^+{\cdot}Na^+} = \frac{2E_1(Na^+)_3}{E_1(Na^+)_2{\cdot}Na^+} = \frac{2E_1ATP(Na^+)_3}{E_1ATP(Na^+)_2{\cdot}Na^+} \qquad (A4)$$

The factors of 2 in eq A4 are statistical coefficients which take into account the number of free and occupied binding sites per enzyme molecule (Läuger & Apell, 1986; Cantor & Schimmel, 1980; Tanford, 1961). 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 A2−A4 and the law of conservation of mass, an expression for $\Delta E_1ATP(Na^+)_3$ in terms of $\Delta E_2ATP(Na^+)_3$. Substituting this expression into eq A1, it can be shown upon integration of the differential rate equation that the reciprocal relaxation time, $1/\tau$, is given by

$$\frac{1}{\tau} = k_3\frac{K_A[ATP]}{1+K_A[ATP]} \times \frac{K_1K_2^2[Na^+]^3}{1+K_1[Na^+]+2K_1K_2[Na^+]^2+K_1K_2^2[Na^+]^3} \qquad (A5)$$

τ represents here the time necessary for the difference in the concentration of the species $E_2P(Na^+)_3$ from its final equilibrium value to decay to 1/e of its initial value. The experimental values of τ obtained at different Na^+ and ATP concentrations can now be fitted to eq A5 to see if the postulated mechanism adequately explains the data and to derive values of the parameters k_3, K_A, K_1, and K_2.

Now let us consider a further case of positive cooperativity, in which the first two Na^+ ions bind weakly with a microscopic association constant K_1 and the third Na^+ ion binds strongly with an association constant K_2. In this case K_1 and K_2 are defined by

$$K_1 = \frac{E_1Na^+}{2E_1{\cdot}Na^+} = \frac{E_1ATP\ Na^+}{2E_1ATP{\cdot}Na^+} = \frac{2E_1(Na^+)_2}{E_1Na^+{\cdot}Na^+} = \frac{2E_1ATP(Na^+)_2}{E_1ATP\ Na^+{\cdot}Na^+} \qquad (A6)$$

$$K_2 = \frac{E_1(Na^+)_3}{E_1(Na^+)_2{\cdot}Na^+} = \frac{E_1ATP(Na^+)_3}{E_1ATP(Na^+)_2{\cdot}Na^+} \qquad (A7)$$

Proceeding as before, as in the derivation of eq A5, yields eq A8 for the reciprocal relaxation time.

$$\frac{1}{\tau} = k_3\frac{K_A[ATP]}{1+K_A[ATP]} \times \frac{K_1^2K_2[Na^+]^3}{1+2K_1[Na^+]+K_1^2[Na^+]^2+K_1^2K_2[Na^+]^3} \qquad (A8)$$

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