Kinetics of Na^+-Dependent Conformational Changes of Rabbit Kidney Na^+,K^+-ATPase

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ABSTRACT The kinetics of Na^+-dependent partial reactions of the Na^+,K^+-ATPase from rabbit kidney were investigated via the stopped-flow technique, using the fluorescent labels *N*-(4-sulfobutyl)-4-(4-(*p*-(dipentylamino)phenyl)butadienyl)pyridinium inner salt (RH421) and 5-iodoacetamidofluorescein (5-IAF). When covalently labeled 5-IAF enzyme is mixed with ATP, the two 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, $1/\tau_1 \approx 155\ s^{-1}$ for 5-IAF-labeled enzyme and $1/\tau_1 \approx 200\ s^{-1}$ for native enzyme (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 smaller amplitude slow phase, $1/\tau_2 = 30-45\ s^{-1}$, is attributed to the relaxation of the dephosphorylation/rephosphorylation equilibrium in the absence of K^+ ions ($E_2P \Leftrightarrow E_2$). The Na^+ concentration dependence of $1/\tau_1$ showed half-saturation at a Na^+ concentration of 6–8 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 8.0 (± 0.7) μM. It was found that P^3-1-(2-nitrophenyl)ethyl ATP, tripropylammonium salt (NPE-caged ATP), at concentrations in the hundreds of micromolar range, significantly decreases the value of $1/\tau_1$ observed. This, as well as the biexponential nature of the kinetic traces, can account for previously reported discrepancies in the rates of the reactions investigated.

INTRODUCTION

The enzymatic mechanism of Na^+,K^+-ATPase is often described by the so-called Albers-Post model (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 oversimplification, the Albers-Post model has so far been quite successful in explaining a great deal of kinetic data.

Recently, however, the Albers-Post model has increasingly been subject to criticism. It has been suggested that further enzyme conformations are present, and even among research groups favoring the Albers-Post formalism, widely varying rate constants have been proposed for the same individual partial reactions. The situation is particularly confusing in the case of the Na^+-related reactions of the pump cycle. As pointed out by Forbush and Klodos (1991), some of the discrepancies between rate constants reported by different groups may be associated with different sources of the enzyme and inherent species differences. Using enzyme prepared from a single source (dog kidney), however, Pratap and Robinson (1993) observed different kinetic behavior for the conformational change of the enzyme induced by ATP, depending on the probe molecule they used. For their stopped-flow experiments they employed three fluorescent probes: 5-iodoacetamidofluorescein (IAF), *N*-[*p*-benzimidazoyl)phenylmaleimide (BIPM), and *N*-(4-sulfobutyl)-4-(4-(*p*-(dipentylamino)phenyl)butadienyl)pyridinium inner salt (RH421). They found that the rate constants measured using IAF and RH421 were approximately half that measured using BIPM under the same experimental conditions. They therefore proposed that the enzyme undergoes a sequence of conformational changes and that the probes detect different steps along the reaction pathway.

Since then it has been found (Frank et al., 1996; Kane et al., 1997) that the probe RH421 (above a concentration of ~1 μM) has an inhibitory effect on the enzyme. If experiments are carried out at a sufficiently low RH421 concentration, it was shown by Kane et al. (1997), using enzyme derived from pig kidney, that the probes RH421 and BIPM yield indistinguishable rate constants. The hypothesis of Pratap and Robinson (1993) of different conformational changes, therefore, appears questionable. There still remains, however, the slower kinetics they observed with IAF (Pratap et al., 1991; Pratap and Robinson, 1993). Here mention must be made of the fitting procedure used. Kane et al. (1997) found that two exponential time functions were necessary to fit their fluorescence transients, obtained using RH421 and BIPM. It was also reported by Heyse et al. (1994) that their fluorescence signals, obtained using both RH421 and IAF, could be fitted much better by two exponentials than by one. Pratap et al. (1991), on the other hand, fitted their IAF data to a single exponential. If a double-exponential relaxation is fitted to a single exponential, this

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would result in an underestimation of the reciprocal relaxation time of the faster phase.

Concerning the discrepancies in the rate constants of individual partial reactions reported in the literature, it is important to point out the different methods used to add ATP to the enzyme. Whereas some groups have added ATP simply by rapid mixing (Steinberg and Karlish, 1989; Forbush and Klodos, 1991; Pratap et al., 1991; Pratap and Robinson, 1993; Kane et al., 1997), others have added ATP by releasing it photochemically from a caged complex (Kaplan et al, 1978; Forbush, 1984; Fendler et al., 1985, 1987, 1993; Borlinghaus et al., 1987; Nagel et al., 1987; Klodos and Forbush, 1988; Borlinghaus and Apell, 1988; Stürmer et al., 1989, 1991; Bühler et al., 1991; Friedrich et al., 1996; Friedrich and Nagel, 1997). A complication of the latter method is, however, the binding of the caged complex (NPE-caged ATP) to the ATP binding site, in competition with ATP itself (Forbush, 1984; Nagel et al., 1987; Fendler et al., 1993). Unphotolyzed NPE-caged ATP can, therefore, act as a competitive inhibitor toward ATP binding. This could easily lead to an underestimation of the rate constants of Na^+-dependent partial reactions of the enzyme, if it is not taken into account in the analysis of the kinetic transients obtained. To estimate the magnitude of this effect, stopped-flow kinetic measurements on Na^+,K^+-ATPase, using the fluorescent probe RH421, are therefore reported here for experiments in which NPE-caged ATP has been included in the reaction medium.

The aim of the present paper is, therefore, fourfold: 1) to compare kinetic data previously obtained with pig kidney enzyme (Kane et al., 1997) with data measured using enzyme obtained from rabbit kidney and, thus, to examine the possibility of species differences; 2) to compare the kinetics obtained using the probe IAF with those obtained using noninhibitory concentrations of RH421 under the same experimental conditions and using the same fitting procedure; 3) to examine further the origin of the double-exponential kinetic behavior previously observed with RH421 (Kane et al., 1997); and 4) to determine the effect of NPE-caged ATP on the experimentally observed kinetic behavior.

With respect to aim 3), it should be pointed out that the faster of the two exponential phases could already be confidently attributed to phosphorylation of the enzyme and its subsequent conformational change ($E_1 \rightarrow E_1P \rightarrow E_2P$). Based on stopped-flow studies of the dephosphorylation reaction of the enzyme, it was shown subsequently (Kane et al., 1998) that the slower exponential phase is not a reaction on the main catalytic pathway of the enzyme (i.e., in the presence of saturating concentrations of Na^+, K^+, and ATP), but its exact origin is still unclear.

MATERIALS AND METHODS

N-(4-Sulfobutyl)-4-(4-(*p*-(dipentylamino)phenyl)butadienyl)-pyridinium inner salt (RH421) and 5-iodoacetamidofluorescein (5-IAF) were obtained from Molecular Probes (Eugene, OR) and were 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. P^3-1-(2-Nitrophenyl)ethyl ATP, tripropylammonium salt (NPE-caged ATP), was prepared as described previously (Fendler et al., 1985).

Na^+,K^+-ATPase-containing membrane fragments were prepared and purified from the red outer medulla of rabbit kidney according to procedure C of Jørgensen (1974a,b). The specific ATPase activity was measured by the pyruvate kinase/lactate dehydrogenase assay (Schwartz et al., 1971), and the protein concentration was determined by the Lowry method (Lowry et al., 1951), using bovine serum albumin as a standard. For the calculation of the molar protein concentration, a molecular weight of an $\alpha\beta$ unit of the Na^+,K^+-ATPase of 147,000 g mol^{-1} (Jørgensen and Andersen, 1988) was assumed. The specific activity of the unlabeled Na^+,K^+-ATPase preparations used was in the range of 1900–2040 μmol P_i/h per mg protein at 37°C. The protein concentration of the unlabeled preparations was in the range of 2.3–3.1 mg/ml. The protein concentration and the specific activity of the 5-IAF-labeled enzyme preparation were somewhat lower, i.e., 1.1 mg/ml and 1240 μmol P_i/h per mg protein, respectively.

Labeling of the enzyme with 5-IAF was performed by incubating 200–300 μg of the enzyme for 48 h at 4°C with a solution containing 100 μM 5-IAF, 20 mM KCl, 20 mM $MgCl_2$, 1 mM EDTA, and 30 mM imidazole (Kapakos and Steinberg, 1982). The pH of the solution was adjusted to 7.4 with HCl. The labeled enzyme was separated from unbound dye by passing the reaction mixture through a 3-cm-long Sephadex G-25 column. The K^+ ions necessary for labeling of the enzyme were subsequently removed by dialysis in the buffer solution used for the stopped-flow measurements.

Stopped-flow experiments were carried out using an SF-61 stopped-flow spectrofluorimeter from Hi-Tech Scientific (Salisbury, England). The solution in the observation chamber was excited with a 100-W short-arc mercury lamp (Osram, Germany), and the fluorescence was detected at right angles to the incident light beam with 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 5-IAF-labeled enzyme, the mercury line at 435 nm was used for excitation, and the fluorescence was collected at wavelengths $\geq$ 530 nm by using an OG530 glass cutoff filter (Schott) 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. Each kinetic trace consisted of either 512 or 1024 data points. To improve the signal-to-noise ratio, in the case of measurements using RH421, typically between 8 and 10 experimental traces were averaged before the reciprocal relaxation time was evaluated. Because of the much lower fluorescence intensity changes in the case of measurements where 5-IAF fluorescence was detected, a total of 57 individual traces were averaged. The error 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 here defined 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 the stopped-flow method employed here is not 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. 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 5-IAF 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 Na^+ concentration occurred on mixing. In the case of experiments performed to test the effect of NPE-caged ATP on the observed kinetics, the enzyme was equilibrated for ~10 min with the NPE-caged ATP before mixing with ATP. The solutions in the drive syringes were equilibrated to a temperature of 24°C before 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 (± 0.2) ms. The electrical time constant of the fluorescence detection system was set to a value of not less than 10 times faster than the relaxation time of the fastest enzyme-related transient, i.e., from 0.33 ms for RH421 measurements at saturating ATP and Na^+ concentrations down to 3.3 ms in the case of measurements in the absence of Mg^{2+} ions. Interference of photochemical reactions of the fluorescence probes with the kinetics of Na^+,K^+-ATPase-related fluorescence transients was avoided by inserting neutral density filters in the light beam in front of the monochromator. The kinetics of conformational changes of unphosphorylated enzyme were investigated in the stopped-flow apparatus by mixing Na^+,K^+-ATPase labeled with RH421 with an equal volume of 130 mM NaCl containing varying concentrations of Na_2ATP. Both the enzyme suspension and the NaCl/Na_2ATP mixtures were prepared in a solution containing 25 mM histidine and 0.1 mM EDTA. In this case Mg^{2+} ions were omitted from the solution to prevent the phosphorylation reaction from occurring. The pH of the solution was adjusted to 7.4 with HCl. It should be noted that at this pH value histidine no longer functions well as a buffer. Nevertheless, its use in combination with EDTA allows the pH to be adjusted to 7.4 and prevents the introduction of buffer cations to the medium, which are known to bind to the enzyme in a fashion similar to that of Na^+ ions (Schuurmans Stekhoven et al., 1986; Grell et al., 1991, 1992, 1994; Doludda et al., 1994).

All stopped-flow experiments with the Na^+,K^+-ATPase in which the enzyme underwent phosphorylation, except those at varying Na^+ concentrations, 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 either the concentration of Na^+ or that of ATP was varied, were collected using a single Na^+,K^+-ATPase preparation. The pH was adjusted to 7.4 by the addition of HCl. 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 found to contain not more than 25 μM K^+ ions.

The origins of the various reagents used were as follows: imidazole (99+%, Sigma or ≥99.5%, Fluka), EDTA (99%, Sigma), NaCl (Suprapur, Merck), K_2SO_4 (analytical grade, Merck), $MgCl_2 \cdot 6H_2O$ (analytical grade, Merck), HCl (0.1 N Titrisol solution, Merck), ATP magnesium salt·$5.5H_2O$ (~97%, Sigma), ATP disodium salt·$3H_2O$ (special quality, Boehringer Mannheim), ethanol (analytical grade, Merck), L-histidine (≥99.5%, Fluka), and choline chloride (99+%, 3× crystallized, Sigma or microselect, Fluka). Sephadex G-25 was obtained from Serva (Heidelberg).

RESULTS

Comparison of RH421 and 5-IAF fluorescence transients

It has been found previously that RH421 concentrations above 1 μM inhibit the steady-state hydrolytic activity (Frank et al., 1996) and the transient kinetics of Na^+,K^+-dependent partial reactions of the Na^+,K^+-ATPase (Kane et al., 1997). For the stopped-flow measurements reported here, therefore, a noninhibitory RH421 concentration of 150 nM (before mixing) was used. The kinetics of the ATP-induced fluorescence transients of both 5-IAF and RH421 were measured using the same 5-IAF-labeled enzyme preparation. Measurements, in which the 5-IAF fluorescence signal was detected, were first performed in the absence of RH421. Subsequently, RH421 was added, and the experiments were repeated, but with the detection of the RH421 fluorescence signal. The detection of the RH421 fluorescence in the presence of 5-IAF is possible, because the wavelength range of fluorescence emission of RH421 is significantly red-shifted in comparison to that of 5-IAF. This method, therefore, allows a direct comparison of the 5-IAF and RH421 signals for the same preparation, under conditions that are as close to identical as possible.

The RH421 and 5-IAF fluorescence stopped-flow transients are shown in Fig. 1. In the case of RH421, reaction of the enzyme with ATP results in an increase in fluorescence of 97 (± 9)% over the value immediately after mixing, whereas in the case of 5-IAF a fluorescence decrease of 3.1 (± 0.2)% occurs. In both cases, however, 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 (88% of the total amplitude for RH421 and 81% for 5-IAF). From the fits of the experimental curves the reciprocal relaxation times of the faster phase were determined to be 164 (± 9) s^{-1} for RH421 and 149 (± 7) s^{-1} for 5-IAF. It appears, therefore, that at least for the faster phase, the two probes give very similar reciprocal relaxation times.

In the case of the slow phase, the values of the reciprocal relaxation time were 32 (± 6) s^{-1} for RH421 and 14 (± 2) s^{-1} for 5-IAF. The rate of the slow phase detected by 5-IAF, therefore, appears to be somewhat slower than that detected by RH421. It should be noted, however, that accurate determination of the reciprocal relaxation time is much more difficult for the slow phase than for the fast phase, because of its much smaller amplitude.

Because the amplitude of the overall fluorescence change observed on mixing with ATP is ~30 times greater when the RH421 fluorescence signal rather than that of 5-IAF is used, all subsequent stopped-flow measurements were performed using native Na^+,K^+-ATPase membrane fragments (i.e., in the absence of the covalent 5-IAF label), to which RH421 was added shortly before the measurements.

Inhibition by NPE-caged ATP

The effect of NPE-caged ATP on the kinetics of the ATP-induced fluorescence transients of Na^+,K^+-ATPase labeled with RH421 was investigated by equilibrating the enzyme with NPE-caged ATP before mixing with ATP. Kinetic traces obtained in the absence and presence of 125 μM NPE-caged ATP (after mixing) and at an ATP concentration

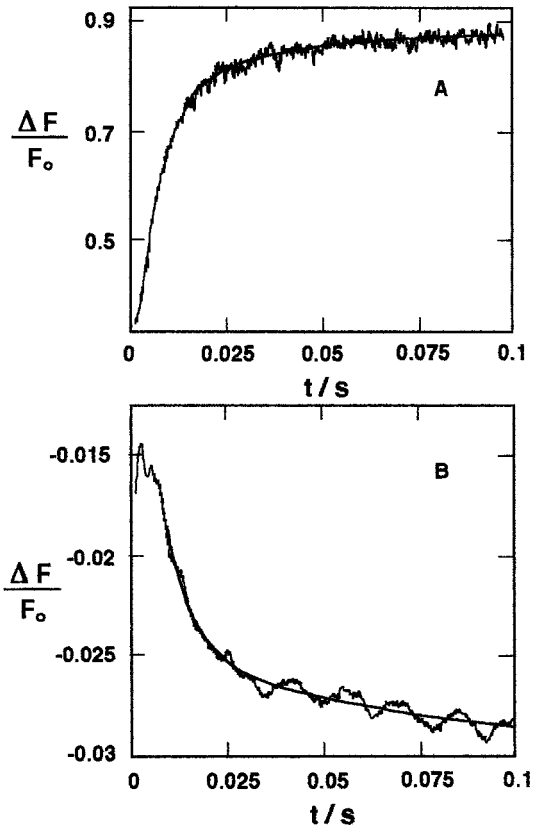

FIGURE 1 Stopped-flow fluorescence transients of 5-IAF-labeled Na^+,K^+-ATPase membrane fragments from rabbit kidney. Na^+,K^+-ATPase was rapidly mixed with an equal volume of MgATP (0.5 mM, after mixing). Each solution was in a buffer containing 130 mM NaCl, 30 mM imidazole, 5 mM $MgCl_2$, and 1 mM EDTA; pH 7.4, $T = 24°C$. The solid lines represent fits to a biexponential time function. (*A*) RH421 (75 nM, after mixing) was added to the Na^+,K^+-ATPase (11 μg/ml or 0.075 μM, after mixing) suspension. The fluorescence of membrane-bound RH421 was measured using an excitation wavelength of 577 nm at emission wavelengths $\geq$ 665 nm (RG665 glass cutoff filter). The calculated reciprocal relaxation times were 164 ($\pm$ 9) s^{-1} (88% of the total amplitude) and 32 ($\pm$ 6) s^{-1} (12%). (*B*) The fluoresence of 5-IAF covalently bound to the protein (50 μg/ml or 0.34 μM, after mixing) was measured using an excitation wavelength of 435 nm at emission wavelengths $\geq$ 530 nm (OG530 glass cutoff filter). The calculated reciprocal relaxation times were 149 ($\pm$ 7) s^{-1} (81% of the total amplitude) and 14 ($\pm$ 2) s^{-1} (19%).

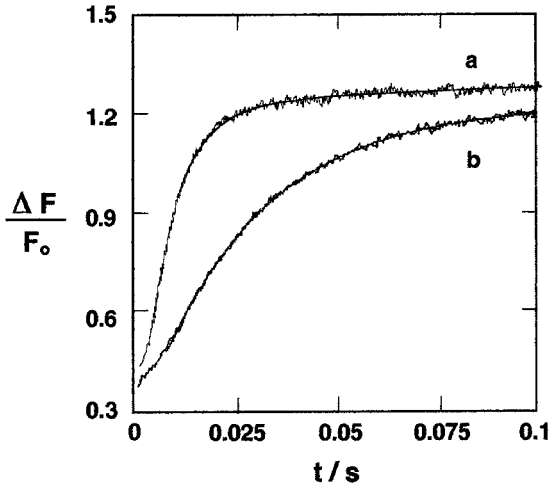

FIGURE 2 Stopped-flow fluorescence transients of native Na^+,K^+-ATPase membrane fragments from rabbit kidney noncovalently labeled with RH421 (75 nM, after mixing). Na^+,K^+-ATPase (10 μg/ml or 0.068 mM, after mixing) was rapidly mixed with an equal volume of Na_2ATP (25 μM, after mixing). Each solution was in a buffer containing 130 mM NaCl, 30 mM imidazole, 5 mM $MgCl_2$, and 1 mM EDTA; pH 7.4, $T = 24°C$. The fluorescence of membrane-bound RH421 was measured using an excitation wavelength of 577 nm at emission wavelengths $\geq$ 665 nm (RG665 glass cutoff filter). The solid lines represent fits to a sum (either one or two) of exponential time functions. (*a*) In the absence of NPE-caged ATP. The calculated reciprocal relaxation times were 137 ($\pm$ 3) s^{-1} (93% of the total amplitude) and 17 ($\pm$ 4) s^{-1} (7%). (*b*) Before mixing with ATP, the enzyme was equilibrated with NPE-caged ATP (125 μM, after mixing). The calculated reciprocal relaxation time was 37 ($\pm$ 1) s^{-1}.

of 25 μM (after mixing) are shown in Fig. 2. These concentrations were chosen to be comparable with the experimental conditions of previously published data (Stürmer et al., 1989, 1991; Bühler et al., 1991; Heyse et al., 1994). In the absence of NPE-caged ATP it was again found that two exponential time functions were necessary to fit the experimental curves. The reciprocal relaxation times determined were 137 ($\pm$ 3) s^{-1} for the dominant fast phase and 17 ($\pm$ 4) s^{-1} for the slow phase. In the presence of 125 μM NPE-caged ATP it was found that the observed kinetic transient was significantly slower. In this case the curve could be fitted adequately by a single exponential time function, and the reciprocal relaxation time was determined to be 37 ($\pm$ 1) s^{-1}. Experiments performed at a higher NPE-caged ATP concentration (250 μM after mixing), but at the same ATP concentration, showed a further retardation of the transient. In this case the reciprocal relaxation time was 26 ($\pm$ 1) s^{-1}.

It is therefore evident that the presence of unphotolyzed NPE-caged ATP can cause a significant inhibition of the Na^+-dependent partial reactions of the Na^+,K^+-ATPase induced by the addition of ATP.

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 Fig. 3). 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 of 180–220 s^{-1}. The fact that the reciprocal relaxation time reaches a maximum value suggests 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. The simplest explanation is, therefore, that the observed process is a reaction of the enzyme occurring subsequent to ATP binding, whereby at low ATP concentrations the reciprocal relaxation time is slowed by the equilibration of the ATP binding step. Possible candidates for the reaction are the

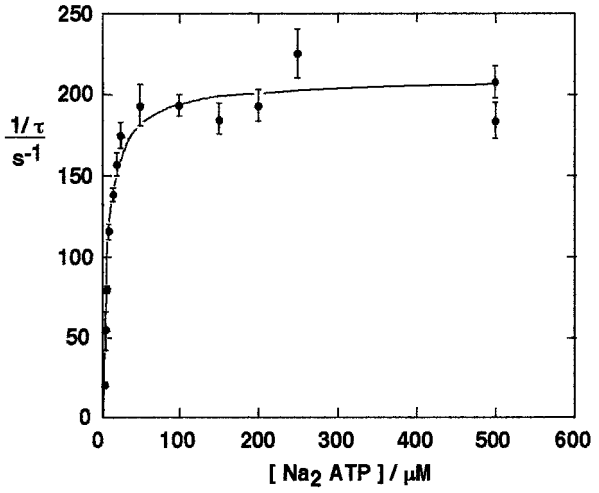

FIGURE 3 Dependence of the reciprocal relaxation time, $1/\tau$, of the fast phase of the RH421 fluorescence change on the concentration of Na_2ATP (after mixing) for stopped-flow experiments in which Na^+,K^+-ATPase was rapidly mixed with Na_2ATP in a nominally K^+-free buffer medium. [Na^+,K^+-ATPase] = 11 μg/ml ($\equiv$ 0.075 μM), [NaCl] = 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.

phosphorylation of the enzyme or a conformational change (and, possibly, ADP and Na^+ ion release steps) induced by phosphorylation. The reaction scheme shown in Fig. 4 is, therefore, proposed. According to this scheme, it can be shown (Kane et al., 1997) that, at saturating Na^+ concentrations, the ATP concentration dependence of the reciprocal relaxation time for the fast phase is described by the following equation:

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

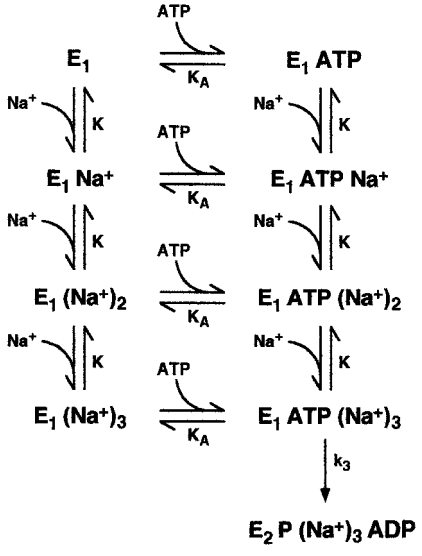

FIGURE 4 Reaction scheme describing the Na^+ and ATP binding steps of the Na^+,K^+-ATPase and its subsequent phosphorylation and conformational change.

The total relative fluorescence change (fast and slow phases), $\Delta F/F_o$, increased with increasing ATP concentration, from a value of 1.05 at the lowest ATP concentration used (1.0 μM), until it reached a maximum value of $\sim$1.9–2.3 in the ATP concentration range of 15–50 μM. At higher ATP concentrations there was a decrease in the value of $\Delta F/F_o$ to $\sim$1.2 at 500 μM ATP.

Fitting the reciprocal relaxation time data according to the model shown in Fig. 4 to Eq. 1 yields the following parameters:

$$k_3 = 208\ (\pm 5)\mathrm{s}^{-1}$$

$$K_A = 1.25\ (\pm 0.12) \cdot 10^5\ \mathrm{M}^{-1}$$

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., 8.0 ($\pm$ 0.7) 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 $\sim$200 s^{-1} at 130 mM Na^+ (see Fig. 5). This behavior is consistent with the idea, incorporated in the Albers-Post model, that phosphorylation of Na^+,K^+-ATPase only occurs at a significant rate when all of the Na^+ ion binding sites of the enzyme are occupied (i.e., as described by the reaction scheme shown in Fig. 4). The slow phase also showed an increase in its reciprocal relaxation time with increasing Na^+ ion concentration, reaching a saturation value of 30–45 s^{-1} at Na^+ concentrations $\geq$ 10 mM.

The total relative fluorescence change (fast and slow phases), $\Delta F/F_o$, increased with increasing Na^+ ion concentration, from a value of 0.51 at the lowest Na^+ ion concentration used (0.56 mM), until it reached a maximum value of $\sim$1.3 in the Na^+ ion concentration range of 10–80 mM. At higher Na^+ ion concentrations $\Delta F/F_o$ decreased to $\sim$1.0 at a concentration of 130 mM.

The total ionic strength in these experiments was maintained at 160 mM by the addition of choline chloride. This avoided any jump in the ionic strength on mixing. The reason for limiting the ionic strength to 160 mM was that both stopped-flow (Kane et al., 1997) and electrical bilayer measurements (Nagel et al., 1987) on Na^+,K^+-ATPase from pig kidney showed an inhibition of the enzyme activity at higher salt concentrations.

In the first instance it was attempted to fit the data shown in Fig. 5 to the model shown in Fig. 4, assuming that all of 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 sys-

tematic positive and negative deviations of the fitted curve from the experimental points were apparent. It is possible that increasing the number of Na^+-binding sites to values significantly greater than three might produce an improved fit to the data. Because other investigations have indicated, however, that there are only three Na^+-binding sites (Cornelius and 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 sigmoidal form of the Na^+ ion concentration dependence of $1/\tau_1$ (see Fig. 5) would appear to be indicative of positive cooperativity in the binding of the Na^+ ions to 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. 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, because of a modification of the enzyme conformation by the weakly binding Na^+ ions, the subsequently binding Na^+ ions bind more strongly. In the case of a model in which there is one weakly binding site and two strongly binding sites, the appropriate equation is

$$\frac{1}{\tau_1} = k_3 \cdot \frac{K_A[ATP]}{1 + K_A[ATP]} \cdot \frac{K_1 K_2^2 [Na^+]^3}{1 + K_1[Na^+] + 2K_1K_2[Na^+]^2 + K_1K_2^2[Na^+]^3} \quad (2)$$

K_1 represents here the association constant of the weakly binding site, and K_2 represents the microscopic (or intrinsic) association constant of the strongly binding sites. The derivation of Eq. 2 is given elsewhere (Kane et al., 1997). In the case of a model in which there are two weakly binding sites and one strongly binding site, the appropriate equation is

$$\frac{1}{\tau_1} = k_3 \cdot \frac{K_A[ATP]}{1 + K_A[ATP]} \cdot \frac{K_1^2 K_2 [Na^+]^3}{1 + 2K_1[Na^+] + K_1^2[Na^+]^2 + K_1^2K_2[Na^+]^3} \quad (3)$$

In Eq. 3, K_1 represents the microscopic association constant of the weakly binding sites, and K_2 represents the association constant of the strongly binding site. The derivation of Eq. 3 can also be found in Kane et al. (1997).

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. 3) involving two weakly binding sites and one strongly binding site. The fit to this model is shown in Fig. 5.

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 $K_1 = 1.8\ (\pm 1.3) \times 10^1\ M^{-1}$, $K_2 = 5.5\ (\pm 1.9) \times 10^2\ M^{-1}$, and $k_3 = 208\ (\pm 7)\ s^{-1}$. The values of K_1 and K_2 correspond to apparent microscopic dissociation constants of 56 ($\pm$ 42) mM and 1.8 ($\pm$ 0.6) mM, respectively. For the model incorporating two weakly binding sites (apparent microscopic association constant K_1) and one strongly binding site (association constant K_2), the best fit values were $K_1 = 1.3\ (\pm 0.5) \times 10^2\ M^{-1}$, $K_2 = 5.4\ (\pm 2.7) \times 10^2\ M^{-1}$, and $k_3 = 204\ (\pm 5)\ s^{-1}$. In this case the values of K_1 and K_2 correspond to apparent microscopic dissociation constants of 8 ($\pm$ 3) mM and 1.8 ($\pm$ 0.9) mM, respectively. The latter model is in reasonable agreement with equilibrium binding studies carried out with the same enzyme (Schulz and Apell, 1995). 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. 2 and 3 reduces to unity.

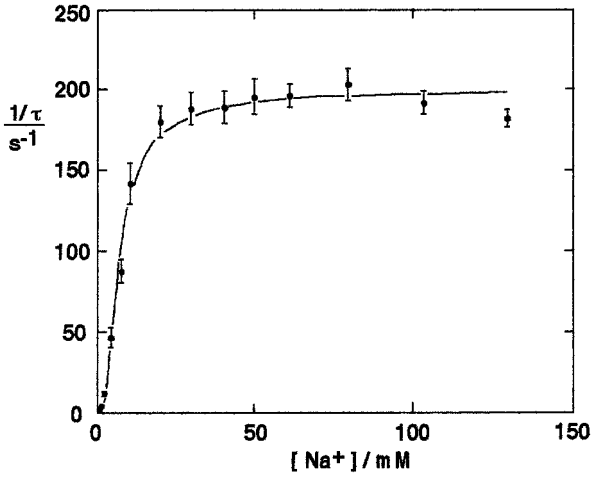

FIGURE 5 Dependence of the reciprocal relaxation time, $1/\tau$, of the fast phase of the RH421 fluorescence change on the concentration of Na^+ ions for stopped-flow experiments in which Na^+,K^+-ATPase was rapidly mixed with MgATP in a nominally K^+-free buffer medium. [Na^+,K^+-ATPase] = 11 μg/ml ($\equiv$ 0.075 μM), [MgATP] = 0.5 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 Fig. 3. The solid line represents a nonlinear least-squares fit of the data to Eq. 3. The sum of the squares of the residuals between the experimental and calculated values of $1/\tau$ were 897 s^{-2} (Eq. 2) and 709 s^{-2} (Eq. 3, *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 3173 s^{-2}.

Slow phase kinetics

The biexponential nature of the RH421 stopped-flow kinetic traces obtained on mixing enzyme in the presence of Na^+ with ATP was first identified by Kane et al. (1997). There

the dominant faster phase was attributed to phosphorylation of the enzyme and a subsequent conformational change ($E_1ATP(Na^+)_3 \rightarrow E_2P(Na^+)_3 + ADP$). The origin of the smaller amplitude slower phase was not considered in detail. It has then been shown (Kane et al., 1998) that the slower phase cannot be due to a reaction lying on the main catalytic pathway of the enzyme, because it does not cause any rate limitation of the K^+-stimulated dephosphorylation reaction. Kane et al. (1998) suggested that the slower phase could possibly be associated with an enzymatic pathway that only occurs in the absence of K^+ ions, in particular a relaxation of the dephosphorylation/rephosphorylation equilibrium of the enzyme in the absence of bound ions. Here we would like to consider this possibility in more detail by carrying out computer simulations of appropriate reaction models and by presenting the results of further experimental investigations.

Let us first consider the following reaction model:

$$E_1ATP(Na^+)_3 \xrightarrow{k_a} E_2P \underset{k_{-b}}{\overset{k_b}{\Leftrightarrow}} E_2 \qquad (4)$$

k_a represents here the overall rate constant for the phosphorylation of the enzyme, its subsequent conformational change, and release of Na^+ ions. As stated above, these reactions are attributed to the fast phase of the RH421 signal. Based on the measurements reported here, k_a is assumed to have a value of 200 s^{-1}. The rate of the backward reaction, i.e., dephosphorylation of the E_2P state via ADP, is assumed to be negligible, because the concentration of ADP present in solution is only the small amount produced by ATP hydrolysis over the time scale of an experiment. For an enzyme concentration of 0.075 μM and assuming the enzyme is hydrolyzing ATP at a rate of $\sim$5 s^{-1} (Hobbs et al., 1980; Campos and Beaugé, 1992; Apell et al., 1996; Kane et al., 1998), it can be shown that after 0.1 s (the time range of the experiments shown in Fig. 1) only $\sim$0.04 μM ADP is produced.

k_b represents the rate constant for spontaneous dephosphorylation of enzyme in the E_2P state. Quenched-flow measurements on enzyme derived from eel electric organ (Hobbs et al., 1980) yielded a value for k_b of 4 s^{-1} at 21°C and pH 7.5. Similar measurements carried out by Campos and Beaugé (1992) yielded a value of 2 s^{-1} for pig kidney enzyme at 20°C and pH 7.4. Stopped-flow measurements using enzyme from pig kidney (Kane et al., 1998) yielded a value of 7 s^{-1} for k_b at 24°C and pH 7.4. From measurements of RH421 fluorescence transients after the photochemical release of inorganic phosphate from a caged compound, Apell et al. (1996) found a value of 3 s^{-1} for enzyme from rabbit kidney at 21°C and pH 7.1. For the purposes of the simulations of the experiments described here at 24°C and pH 7.4, a value for k_b of 5 s^{-1} has been chosen.

k_{-b} represents the rate constant for rephosphorylation of the enzyme, i.e., the reformation of enzyme in the E_2P state from the E_2 state. It should be noted that, in principle, there are two possible pathways by which this could occur: 1) a direct back reaction in which the enzyme is phosphorylated by inorganic phosphate, and 2) an indirect back reaction involving a conformational change of the enzyme to the E_1 state, followed by phosphorylation by ATP. Pathway 1) can be considered to be very unlikely, however, because the concentration of inorganic phosphate present is negligible. The concentration of inorganic phosphate produced after 0.1 s can be estimated, as in the case of ADP above, to be only $\sim$0.04 μM. This is far below the reported apparent K_m of the E_2 conformation of rabbit kidney enzyme for inorganic phosphate of 23 μM (Apell et al., 1996), as well as the dissociation constants of 32 μM (Campos and Beaugé, 1994) and 29 μM (Fedosova et al., unpublished results) reported for pig kidney enzyme. Therefore, it would seem that only pathway 2), i.e., rephosphorylation by ATP via the E_1 state, need be taken into consideration. This pathway consists of two basic steps: first, the conformational change of enzyme from the E_2 to the E_1 state, and second, the phosphorylation of the enzyme by ATP and its conversion to the E_2P state, which has been found here to have a rate constant of $\sim$200 s^{-1}.

To obtain kinetic information on the rate of the E_2 to E_1 transition, stopped-flow mixing experiments have previously been performed in which the enzyme was preequilibrated with a small amount (1–5 mM) of KCl, so as to stabilize the $E_2(K^+)_2$ form of the enzyme, and then mixed with an excess of NaCl (50–130 mM). This induces the transition $E_2(K^+)_2 \rightarrow E_1(Na^+)_3$. The results obtained (Steinberg and Karlish, 1989; Pratap et al., 1996; Kane et al., 1997) showed that this reaction occurs with a rate constant of $\leq$30 s^{-1}. In the case of the experiments reported here, however, no K^+ ions were present. Therefore, to judge the feasibility of pathway 2), in which rephosphorylation is assumed to occur by ATP via the E_1 state, it is necessary to determine the kinetics of the reaction $E_2 \rightarrow E_1(Na^+)_3$. This has been performed by rapidly mixing enzyme, labeled with RH421, in the absence of Na^+ ions with 130 mM NaCl solution. To investigate the effect of ATP on this reaction, various concentrations of Na_2ATP were added to the NaCl solution.

It was found that on mixing with NaCl a decrease in fluorescence occurred. At low concentrations of Na_2ATP ($\leq$25 μM, after mixing), two kinetic phases could be resolved, a slow fluorescence decrease and a more rapid fluorescence decrease with a reciprocal relaxation time in the range 10–32 s^{-1}. The amplitude of the rapid kinetic phase, however, decreased significantly with increasing Na_2ATP concentration, until at concentrations of $\geq$50 μM after mixing, only a single kinetic phase could be resolved. The origin of the rapid phase observed at low Na_2ATP concentrations is not clear at this stage. Because the double-exponential behavior of the RH421 and IAF fluorescence transients of phosphorylation experiments (see Fig. 1) is observed even at high ATP concentrations, we shall concentrate here on the phase that is present over the whole Na_2ATP concentration range. Similar to the behavior found for experiments in which the enzyme was preequilibrated

with KCl (Karlish and Yates, 1978; Steinberg and Karlish, 1989; Pratap et al., 1996; Kane et al., 1997), the value of the reciprocal relaxation time for the observed fluorescence transient increases with increasing Na_2ATP concentration, reaching a saturating value of 39 s^{-1} (see Fig. 6). The total relative fluorescence change, $-\Delta F/F_o$, also increased with increasing Na_2ATP concentration, from a value of ~0.11 in the absence of Na_2ATP to a saturating value of ~0.15. 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 to 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 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 to $E_1(Na^+)_3$: one in the absence of bound ATP and one that is ATP stimulated. Fitting the data shown in Fig. 6 to Eq. 5 yields a value for K_A' of 1.41 ($\pm$ 0.14) $\cdot$ 10^4 M^{-1}. This corresponds to an apparent dissociation constant of 71 ($\pm$ 7) μM. The values of $(1/\tau)_{min}$ and $(1/\tau)_{max}$ determined from the fit were 0.8 ($\pm$ 0.2) s^{-1} and 39 ($\pm$ 1) s^{-1}, respectively.

These data indicate that in the absence of K^+ ions and at saturating ATP concentrations, the reaction $E_2 \rightarrow E_1(Na^+)_3$ occurs with a rate constant of $\leq$ 39 s^{-1}. Because this is much slower than the phosphorylation of the enzyme by ATP and its conversion to the E_2P state, which have been shown above to occur with a rate constant of ~200 s^{-1}, the reaction $E_2 \rightarrow E_1(Na^+)_3$ can be considered rate-determining for the rephosphorylation reaction via pathway 2), i.e., by ATP via the E_1 state. k_{-b} in model (4) can, therefore, be approximated to be 30 s^{-1}.

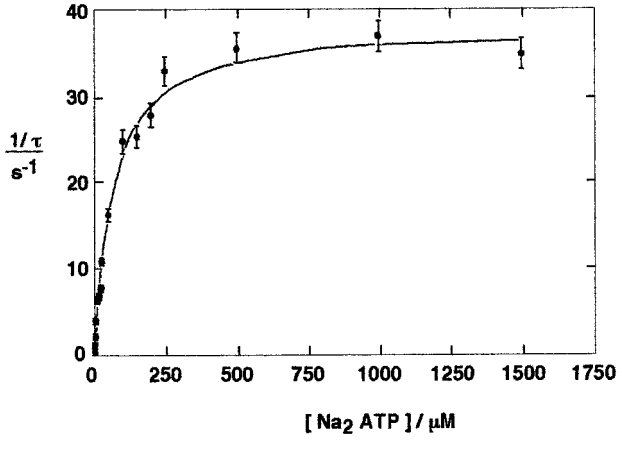

FIGURE 6 Dependence of the reciprocal relaxation time, $1/\tau$, of the RH421 fluorescence change on the concentration of Na_2ATP (after mixing) for stopped-flow experiments in which Na^+,K^+-ATPase in a solution containing 25 mM histidine and 0.1 mM EDTA was rapidly mixed with the same histidine/EDTA solution containing 130 mM NaCl. [Na^+,K^+-ATPase] = 10 μg/ml ($\equiv$ 0.068 μM), [RH421] = 75 nM; pH 7.4, T = 24°C. The excitation and emission wavelengths were as in Fig. 3. The solid line represents a nonlinear least-squares fit of the data to Eq. 5.

Using the values given above, i.e., k_a = 200 s^{-1}, k_b = 5 s^{-1} and k_{-b} = 30 s^{-1}, it can be shown via computer simulation that reaction scheme (4) is able to reproduce the experimentally observed biexponential behavior of the RH421 kinetic traces, as long as it is assumed that the fluorescence of dye associated with enzyme in its various states increases in the order $E_1ATP(Na^+)_3 < E_2P < E_2$. The assumption of a higher fluorescence level of dye associated with the E_2P state in comparison to the $E_1ATP(Na^+)_3$ state is in agreement with previous experimental observations (Bühler et al., 1991; Stürmer et al., 1991; Pratap and Robinson, 1993; Klodos, 1994; Kane et al., 1997). However, there is as yet no experimental justification for the assumption of a higher fluoresecence level of the E_2 state compared to the E_2P state. In the presence of K^+ ions it has been found that dephosphorylation of the enzyme leads to a significant decrease in fluorescence (Stürmer et al., 1991; Bühler and Apell, 1995; Kane et al., 1998). This has been interpreted as being due to the formation of enzyme in the $E_2(K^+)_2$ state. In the absence of K^+ and Na^+ ions it has also been found that the fluorescence of RH421 associated with unphosphorylated enzyme is lower than that of dye associated with phosphoenzyme formed by the addition of inorganic phosphate (Fedosova et al., 1995; Apell et al., 1996). Whether these findings concerning the direction of the fluorescence change on phosphorylation are relevant to the experiments reported here, where phosphorylation was initiated by the addition of ATP in the presence of Na^+ ions, is questionable, however, because it has been shown recently by Fedosova et al. (1997) that the E_2P enzyme forms produced on phosphorylation by ATP and inorganic phosphate are not identical, and the presence or absence of Na^+ and K^+ ions is known to cause changes in enzyme conformation, at least in the case of unphosphorylated enzyme (Karlish, 1980; Grell et al., 1992; Smirnova and Faller, 1993; Doludda et al., 1994; Smirnova et al., 1995; Bugnon et al., 1997; Kane et al., 1997). In the absence of K^+ ions and the presence of Na^+ ions, therefore, the direction of any fluorescence change induced by the dephosphorylation of phosphoenzyme produced by ATP phosphorylation is difficult to predict. Although the origin of the fluorescence changes of RH421 associated with Na^+,K^+-ATPase are unclear at this stage, Stürmer et al. (1991) and Klodos (1994) have suggested that they may arise from ion binding to and release from the enzyme rather than from phosphorylation alone. If this is true, then it might be expected that dyes associated with the E_2P and E_2 states of the enzyme may have very similar fluorescence levels (Apell et al., 1996). In this case

reaction scheme (4) would no longer be an adequate description of the experimentally observed behavior.

To accommodate the idea that the fluorescence changes in RH421 arise from changes in the occupancy of the ion-binding sites we therefore propose the following alternative reaction scheme:

$$E_1ATP(Na^+)_3 \xrightarrow{k_a} E_2P(Na^+)_3 \underset{v_{-1}}{\overset{v_1}{\Leftrightarrow}} E_2P \underset{k_{-b}}{\overset{k_b}{\Leftrightarrow}} E_2 \qquad (6)$$

In this case we assume that the fluorescence level of dye associated with enzyme in the $E_1ATP(Na^+)_3$ and $E_2P(Na^+)_3$ states is zero, whereas the fluorescence level of dye associated with enzyme in the E_2P and E_2 states is 100%, i.e., the entire fluorescence change is attributed to the release of Na^+ ions from the enzyme. v_1 and v_{-1} represent here the rates of dissociation and binding, respectively, of the Na^+ ions from or to the E_2P form of the enzyme. It should be noted that the binding of each Na^+ ion to the E_2P form of the enzyme is a second-order reaction, so that the absolute value of v_{-1} is dependent on the Na^+ concentration. Both steps are assumed to be very fast, so that on the time scale of the phosphorylation reaction, the species $E_2P(Na^+)_3$ and E_2P are always in equilibrium with each other. Stopped-flow measurements on Na^+,K^+-ATPase from pig kidney (Kane et al., 1998) have indicated that the release of Na^+ ions from the E_2P form of the enzyme is fast, i.e., at least $>180\ s^{-1}$, and electrical measurements of Wagg et al. (1997) showed reciprocal relaxation times of $\geq 1000\ s^{-1}$, which they also attributed to the release of Na^+ ions from the phosphorylated enzyme. We have therefore chosen a value of v_1 of $1000\ s^{-1}$. It is generally accepted (Glynn, 1985; Cornelius, 1991; Läuger, 1991) that the E_2P form of the enzyme has a lower affinity for Na^+ ions than the E_1 form. In the case of the E_1 form it has been found here that half-saturation occurs in the Na^+ concentration range 6–8 mM. Therefore, assuming a dissociation constant of the Na^+ binding sites of the E_2P form in the range of tens of millimolar and the value of $1000\ s^{-1}$ for v_1, it can be shown that, at a NaCl concentration of 130 mM, v_{-1} can be estimated to have a value in the range of 1,000–10,000 s^{-1}.

If one chooses the following values, $k_a = 200\ s^{-1}$, $k_b = 5\ s^{-1}$, $k_{-b} = 30\ s^{-1}$, $v_1 = 1000\ s^{-1}$, and $v_{-1} = 1000\ s^{-1}$, which have been shown to be experimentally justified above, and one assumes the fluorescence levels given above (i.e., zero for enzyme species with Na^+ bound and 100% for species free of Na^+ ions), computer simulations based on reaction scheme (6) are able to reproduce the biexponential behavior of the RH421 kinetic traces (see Fig. 7). The biexponential character of the simulated curve (*solid line*) can easily be seen from the deviations of the simulation from one in which the final dephosphorylation/rephosphorylation step has been omitted from the model (*dotted line*). In the latter case a pure single exponential relaxation is obtained. It should be noted that the exact choice of the values of v_1 and v_{-1} is not critical. The two values must merely be much greater than $200\ s^{-1}$ and be of a similar order of magnitude, so that the Na^+ ion binding and release are always in equilibrium on the time scale of the phosphorylation reaction, and that there are sufficient amounts of enzyme in the $E_2P(Na^+)_3$ and E_2P states before relaxation of the dephosphorylation/rephosphorylation equilibrium.

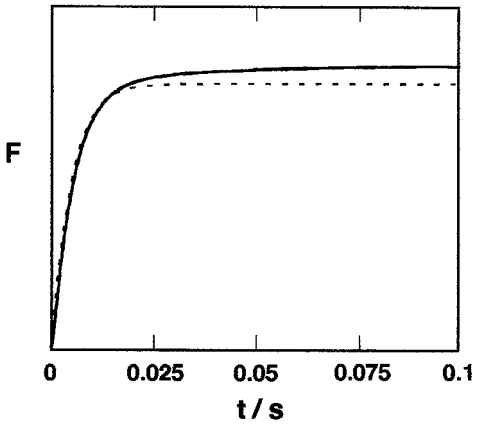

FIGURE 7 Computer simulation (*solid line*), based on the reaction model (6), of an RH421 stopped-flow kinetic transient for an experiment in which Na^+,K^+-ATPase membrane fragments are mixed with ATP. The values of the rates and rate constants chosen were $k_a = 200\ s^{-1}$, $v_1 = 1000\ s^{-1}$, $v_{-1} = 1000\ s^{-1}$, $k_b = 5\ s^{-1}$, and $k_{-b} = 30\ s^{-1}$. The total fluorescence intensity is assumed to arise solely from dye associated with enzyme in the E_2P and E_2 forms. The relative fluorescence intensities of dye associated with each of these forms are assumed to be equal. For comparison, a computer simulation (*dotted line*) is shown in which the final dephosphorylation/rephosphorylation step of reaction model (6) has been omitted. In this case a pure single exponential curve is obtained.

Although reaction scheme (6) is able to explain the observed kinetic behavior, it should be kept in mind that it is a somewhat simplified scheme, because E_2P species with one and two bound Na^+ ions are also likely to be present, and the Na^+ ions would presumably be released sequentially from the enzyme. In fact, there is some experimental evidence to suggest that model (6) may not provide a complete description of the experimental behavior. If one determines from the experiments shown in Fig. 4 the percentage of the total amplitude accounted for by the slow phase as a function of the Na^+ concentration, then it is found that there is a drop in the relative amplitude of the slow phase with increasing Na^+ concentration. At a Na^+ concentration of $\leq$10 mM, the slow phase accounts for $\sim$20% of the overall signal, whereas at a concentration of 130 mM the value is only $\sim$8%. This behavior would not be expected according to reaction scheme (6), which would predict an increase in the percentage of the slow phase with increasing Na^+ concentration until it reached a saturating value. Such a behavior would, however, be expected on the basis of reaction scheme (4), because high concentrations of Na^+ ions would be expected to stabilize the enzyme in the $E_2P(Na^+)_3$ state and hence lead to a decrease in the proportion of enzyme undergoing dephosphorylation. Alternatively, the assumption of reaction scheme (6) that the total fluorescence change arises from the release of all three Na^+

ions from the enzyme may not be justified. It is possible that the release of one or two Na^+ ions from the enzyme may be sufficient to induce the fluorescence change detected using RH421. Nevertheless, regardless of which of the two reaction schemes is closer to the truth, the simulations and the experiments described here indicate that, under the experimental conditions used, relaxation of the dephosphorylation/rephosphorylation equilibrium via ATP and the E_1 state as described by models (4) and (6) can be expected to occur and can be considered as the most likely cause for the biexponential kinetic behavior observed using RH421.

DISCUSSION

The kinetics of Na^+-dependent partial reactions of the Na^+,K^+-ATPase from rabbit kidney 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: IAF, which is covalently attached to the enzyme, and RH421, which is noncovalently associated with the enzyme-containing membrane fragments. The two labels delivered very similar kinetic responses (see Fig. 1). In both cases two exponential time functions were necessary to fit the data. The fast phase is the major component, contributing between 80% and 90% of the overall fluorescence change. When experiments were carried out with the same IAF-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: 164 ($\pm$ 9) s^{-1} (for RH421) and 149 ($\pm$ 7) s^{-1} (for IAF). When experiments were carried out using RH421 on rabbit kidney enzyme not labeled with IAF, it was found that the value was in the range of 200–210 s^{-1}. The differences in the reciprocal relaxation times obtained for IAF-labeled enzyme and enzyme not labeled with IAF can be explained by the differences in the specific activities of the two preparations.

Based on the dependence of the observed reciprocal relaxation times on ATP concentration and Na^+ concentration and taking into account previously published values of the rate constants of the various partial reactions of Na^+,K^+-ATPase, the two kinetic phases can be interpreted as follows. Before 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, undergoes a rapid conformational change, and releases, depending on the Na^+ concentration in solution, some or all of its Na^+ ions ($E_1(Na^+)_3 + ATP \rightarrow E_2P(Na^+)_3 \leftrightarrow E_2P + 3Na^+$). This accounts for the dominant fast phase of the fluorescence transients. Subsequently, the enzyme can undergo a dephosphorylation, which in the absence of K^+ ions is very slow ($\sim$5 s^{-1}), a conformational change back to the E_1 form (at a rate of $\sim$30 s^{-1}), and rephosphorylation via ATP ($E_2P \rightarrow E_2 \rightarrow E_1 + 3Na^+ \rightarrow E_1(Na^+)_3 + ATP \rightarrow E_2P(Na^+)_3 \leftrightarrow E_2P + 3Na^+$). The slow phase is attributed to the relaxation of the dephosphorylation/rephosphorylation equilibrium. The experimental results can, therefore, all be explained in terms of the Albers-Post model of two major enzyme conformations.

The very similar reciprocal relaxation times observed with RH421 and IAF on IAF-labeled enzyme suggests that the two probes are following the kinetics of the same enzyme conformational change. Previously it had been suggested by Pratap and Robinson (1993) that the three probes, BIPM, RH421, and IAF, each report on a different step in a sequence of enzyme conformational changes. Their conclusion was based on stopped-flow kinetic data using the three probes 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 IAF. In a more recent publication (Kane et al., 1997) it was shown that RH421 and BIPM gave almost identical kinetic responses. There it was suggested that the slower kinetics Pratap and Robinson (1993) observed with RH421 could perhaps be attributed to the relatively high concentration of probe they used of 2 μM, because it was found (Kane et al., 1997) that concentrations of RH421 in the micromolar range can inhibit Na^+-related partial reactions of the enzyme. Here we wish to consider the possible reason for the slower kinetics Pratap and co-workers (Pratap et al., 1991; Pratap and Robinson, 1993) observed using IAF compared to BIPM. In light of the results presented here, it would seem that an important contributing factor is the biexponential nature of the kinetic curves. Pratap and co-workers fitted their kinetic curves at saturating Na^+ and ATP concentrations, using all three probes, to a single exponential function. The relaxations presented here and elsewhere (Kane et al., 1997), however, clearly require two exponential time functions to obtain an adequate fit. Evidence for biexponential kinetic behavior can also be seen in the time course of the IAF fluorescence decay observed by Pratap et al. (1991) at 155 mM NaCl, which appears to show significant deviation from a single exponential, particularly at long times. If a biexponential relaxation is fitted to a single exponential, this results in an underestimation of the reciprocal relaxation time.

Based on the results presented here and in a previous publication (Kane et al., 1997) using the probes RH421, BIPM and IAF there appears to be no justification for the assumption made by Pratap and Robinson (1993) of a series of enzyme conformational changes. If one takes into account the inhibitory action of RH421 (at micromolar concentrations) and the biexponential behavior of the traces, then the three probes give very similar kinetic responses. A single enzyme conformational change, therefore, appears to be determining the rate of the major kinetic phase of all three probes.

Now let us consider the question of species differences. Experiments very similar to those presented here on enzyme derived from rabbit kidney have previously been reported (Kane et al., 1997) for enzyme derived from pig kidney. This allows a direct comparison of the parameters derived and an analysis of whether any significant kinetic or mechanistic differences exist between the two sources.

As stated above, in the case of rabbit kidney Na^+,K^+-ATPase, the reciprocal relaxation time for the dominant fast phase of the RH421 fluorescent signal at saturating ATP and Na^+ concentrations was found to be in the range of 200–210 s^{-1}. Analogous experiments carried out using pig kidney (Kane et al., 1997) yielded a value of ~180 s^{-1}. The apparent high-affinity dissociation constant for ATP was found here for rabbit kidney enzyme to be 8.0 (± 0.7) μM. The corresponding value for pig kidney enzyme was 7.0 (± 0.6) μM. From the Na^+ concentration dependence of the reciprocal relaxation time, it was found here for rabbit kidney enzyme that half-saturation occurs at a Na^+ concentration of 6–8 mM with positive cooperativity involved in the occupation of the Na^+ binding sites. The results obtained for pig kidney enzyme also indicated positive cooperativity, with half-saturation occurring at a Na^+ concentration of 8–10 mM. Thus it appears that there are only minor differences in the kinetic and equilibrium properties measured here between the Na^+,K^+-ATPase from rabbit and pig kidney. Any more significant differences reported in the literature for these two enzyme sources, therefore, cannot be attributed to species differences.

Using the stopped-flow method and the fluorescent probe RH421, it has been shown here that unphotolyzed NPE-caged ATP can cause a significant inhibition (e.g., a drop in $1/\tau$ of the fast phase of 73% at a NPE-caged ATP concentration of 125 μM) of the Na^+-dependent partial reactions leading from the form $E_1(Na^+)_3$ to E_2P. A similar inhibition has also been reported by Fendler et al. (1993) for the phosphorylation reaction alone, using the technique of rapid acid quenching after mixing with radioactive ATP. This inhibition can be attributed to competition between unphotolyzed NPE-caged ATP and ATP for the same binding sites (Forbush, 1984; Nagel et al., 1987; Borlinghaus and Apell, 1988; Fendler et al., 1993). To avoid significant underestimation of the rate constants for the ATP-induced partial reactions leading to the formation of enzyme in the E_2P state, any kinetic measurements in which ATP is released photochemically must therefore take into account the competition between NPE-caged ATP and ATP. Based on the results presented in Figs. 2 and 3, it is possible to estimate an association constant, K_C, of NPE-caged ATP for the enzyme. If one assumes that NPE-caged ATP and ATP compete for the same binding site and that the NPE-caged ATP binding and dissociation steps are always in equilibrium on the time scale of the phosphorylation reaction and subsequent conformational changes of the enzyme, it can be shown that the reciprocal relaxation time for the dominant fast phase of the RH421 fluorescence signal is given by the following modified form of Eq. 1:

$$\frac{1}{\tau_1} = k_3 \cdot \frac{K_A[\mathrm{ATP}]}{1 + K_A[\mathrm{ATP}] + K_C[\text{caged ATP}]} \qquad (7)$$

Using the values of k_3 and K_A calculated from the data shown in Fig. 3 of 208 (± 5) s^{-1} and 1.25 (± 0.12) · 10^5 M^{-1}, as well as the values of $1/\tau_1$ of 37 (± 1) s^{-1} and 26 (± 1) s^{-1} for NPE-caged ATP concentrations of 125 μM and 250 μM, respectively, and an ATP concentration of 25 μM, K_C can be estimated from Eq. 7 to have a value of 9.6 (± 2.6) · 10^4 M^{-1}. The reciprocal of K_C, i.e., 10 (± 3) μM, corresponds to the apparent dissociation constant of NPE-caged ATP to the ATP binding site. Comparison with the ATP dissociation constant of 8.0 (± 0.7) μM shows that NPE-caged ATP binds to the enzyme almost as strongly as ATP itself. The value of $1/K_C$ given here is on the same order of magnitude as previous estimates determined for dog kidney Na^+,K^+-ATPase at pH 7.2 of 43 μM (Forbush, 1984) and for Na^+,K^+-ATPase from eel electric organ at pH 6.2 of 35 μM (Fendler et al., 1993). It is, however, a factor of 50 times lower than the previous tentative estimate of $1/K_C$ for rabbit kidney Na^+,K^+-ATPase at pH 7.0 of 500 μM (Borlinghaus and Apell, 1988).

Here it has been shown that at pH 7.4 and saturating Na^+ and ATP concentrations, the Na^+-dependent partial reaction, $E_1(Na^+)_3 \rightarrow E_2P$, occurs at a rate of ~200 s^{-1} at 24°C. According to the effect that NPE-caged ATP has on the observed kinetics (see Fig. 2), the much slower RH421 and IAF fluorescence transients and the significantly lower rate constant of 18–30 s^{-1} previously reported in the literature (Stürmer et al., 1989, 1991; Bühler et al., 1991; Heyse et al., 1994) for the same reaction of enzyme from rabbit kidney based on experiments in which ATP was released photochemically can be attributed, at least in part, to competitive inhibition from unphotolyzed NPE-caged ATP.

The partial reaction, $E_1(Na^+)_3 \rightarrow E_2P$, can in fact be considered to occur in two composite steps: $E_1(Na^+)_3 \rightarrow E_1P(Na^+)_3 \rightarrow E_2P + 3Na^+$. If the rate constant of the initial phosphorylation reaction were known, it would therefore be possible, based on computer simulations of the stopped-flow traces, to estimate a rate constant for the conformational transition and associated Na^+ release step, $E_1P(Na^+)_3 \rightarrow E_2P + 3Na^+$. Up to now no direct measurements of the phosphorylation rate of rabbit kidney Na^+,K^+-ATPase have been performed. The following phosphorylation rate constants determined for other enzyme sources can, however, be found in the literature: 183 s^{-1} (bovine brain cortex, pH 7.4, 21°C; Mårdh and Zetterqvist, 1974), 180 s^{-1} (eel electric organ, pH 7.5; Hobbs et al., 1988), 199 s^{-1} (pig kidney, pH 7.4, 20°C; Campos and Beaugé, 1992), 460 s^{-1} (sheep kidney, pH 7.4, 25°C; Keillor and Jencks, 1996), and 190 s^{-1} (pig kidney, pH 7.5, 24°C; Kane et al., 1997). If one takes the highest of these values, i.e., 460 s^{-1}, as an upper limit of the phosphorylation rate constant, it is possible to estimate a lower limit for the rate constant of the conformational change and associated Na^+ release step,

$E_1P(Na^+)_3 \rightarrow E_2P + 3Na^+$, for the rabbit kidney enzyme of 260 s^{-1}.

Recently Sokolov et al. (1998) reported kinetic measurements of charge movements by rabbit kidney Na^+,K^+-ATPase using the principle of capacitative coupling on black lipid membranes (Fendler et al., 1985; Borlinghaus et al., 1987) in combination with a new caged ATP complex, the P^3-[1-(3′, 5′-dimethoxyphenyl)-2-phenyl-2-oxo] ester of ATP (DMB-caged ATP) (Thirlwell et al., 1994; Corrie et al., 1992). According to Sokolov et al. (1998), DMB-caged ATP is superior to NPE-caged ATP because of its faster photochemical release kinetics and because any binding of DMB-caged ATP to the ATP-binding site of Na^+,K^+-ATPase does not affect its kinetic behavior. Although Sokolov et al. (1998) used the same enzyme preparation as that employed here, it must be pointed out that their interpretation of the two phases of the current transient they observed leads to a value of the rate constant for the formation of enzyme in the E_2P conformation significantly different from that reported here. According to Sokolov et al. (1998), the reaction $E_1(Na^+)_3 \rightarrow E_2P$ occurs with a rate constant of 25 s^{-1}, whereas the stopped-flow results described here indicate a value of 200 s^{-1}. The reason for the artificially low value reported by Sokolov et al. (1998) is not clear at this stage.

Finally, it is interesting to discuss the possible rate-determining step of the Na^+,K^+-ATPase under steady-state conditions. In a previous publication (Kane et al., 1997) this was attributed to 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 on pig kidney enzyme, occurs at saturating ATP concentrations with a rate constant of $\leq$28 s^{-1}. The stopped-flow experiments reported here (see Fig. 6) for rabbit kidney enzyme indicate that the conformational change and associated Na^+ binding of unphosphorylated enzyme ($E_2 + 3Na^+ \rightarrow E_1(Na^+)_3$) occur at saturating ATP concentrations with a rate constant of $\leq$39 s^{-1}. This reaction has previously been investigated in the absence of ATP by Grell et al. (1992) and Doludda et al. (1994) for pig kidney enzyme. In agreement with the results presented here, at saturating Na^+ concentrations but in the absence of ATP, these authors found the reaction to be very slow, occurring with a reciprocal relaxation time of ~1 s^{-1}. A 10-fold lower value has been reported by Apell et al. (1996), who measured the rate of conversion of rabbit kidney enzyme phosphorylated by inorganic phosphate back to the E_1 state by the addition of Na^+ ions. In the presence of saturating ATP concentrations (millimolar range), however, it has been shown here that the rate of the reaction $E_2 + 3Na^+ \rightarrow E_1(Na^+)_3$ is accelerated over 30-fold. This result would seem at first glance to be in contradiction to the findings of Apell et al. (1990), who showed, using reconstituted vesicles, that in the presence of Na^+ ions but in the absence of K^+ ions, the low-affinity ATP stimulation of the steady-state ATPase activity disappears and only the high-affinity ATP stimulation remains. Similar to suggestions previously made by Apell et al. (1990), this apparent discrepancy, however, can easily be reconciled, if one assumes that 1) the rate-determining steps of the Na^+/K^+ exchange mode and the Na^+-only mode of the enzyme cycle are different, and 2) the rate-determining step of the Na^+-only mode is not ATP-stimulated. There is, in fact, overwhelming experimental support for a change in the rate-determining step, because it has been shown clearly that in the absence of K^+ ions the rate of the dephosphorylation is drastically reduced from a value of ~300 s^{-1} in the presence of K^+ ions to a value of ~5 s^{-1} in its absence (Mårdh and Zetterqvist, 1974; Hobbs et al., 1980; Campos and Beaugé, 1992; Kane et al., 1997, 1998), i.e., significantly slower than the upper limit of 39 s^{-1} found here for the reaction $E_2 + 3Na^+ \rightarrow E_1(Na^+)_3$. It is interesting to note that Jencks and co-workers (Keillor and Jencks, 1996; Ghosh and Jencks, 1996), based on quenched-flow measurements on sheep kidney enzyme with and without preincubation with Na^+, recently proposed that phosphorylation of Na^+,K^+-ATPase is rate-limited by a Na^+-induced conformational change of the enzyme. The rate constant they reported, 460 s^{-1}, is, however, much too high for the reaction to be rate-determining for the complete Na^+/K^+ exchange enzyme cycle.

Although care must be taken in comparing different enzyme sources (Forbush and Klodos, 1991), the very similar behavior found up to now for the pig kidney and rabbit kidney preparations would seem to justify a comparison of the rate constants found for the formation of enzyme in the $E_1(Na^+)_3$ state on preequilibration with K^+ ions ($\leq$28 s^{-1}) (Kane et al., 1997) and in the absence of K^+ ions ($\leq$37 s^{-1}) at saturating ATP concentrations. The very similar rate constants found for the two reactions would seem to suggest that, at least in the presence of saturating ATP concentrations, the K^+ deocclusion step is relatively fast ($>$37 s^{-1}), i.e., at least as fast as the formation of the $E_1(Na^+)_3$ state in the absence of K^+ ions. To give a more accurate estimate of the rate constant for K^+ deocclusion, stopped-flow measurements with and without K^+ ions would have to be repeated, using the same enzyme preparation. Using a rapid filtration apparatus, Forbush (1987) determined a rate constant of ~45 s^{-1} for the release of $^{42}K^+$ ions from dog kidney enzyme at 4 mM ATP, pH 7.2, and 20°C.

The major rate-determining step of the Na^+,K^+-ATPase at saturating concentrations of Na^+, K^+, and ATP under steady-state conditions is, therefore, most likely to be the conformational change and associated Na^+ binding of unphosphorylated enzyme ($E_2 + 3Na^+ \rightarrow E_1(Na^+)_3$), which, based on the stopped-flow measurements reported here, occurs in the absence of Mg^{2+} with a rate constant of $\leq$37 s^{-1}. Further investigations are necessary to show whether this reaction is significantly accelerated by the presence of Mg^{2+} ions.

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