Dephosphorylation Kinetics of Pig Kidney Na^+,K^+-ATPase[†]

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ABSTRACT: The kinetics of K^+-stimulated dephosphorylation of the Na^+,K^+-ATPase were investigated at pH 7.4, 24 °C, and an ATP concentration of 1.0 mM via the stopped-flow technique using the fluorescent label RH421. Two different mixing procedures were used: (a) premixing with ATP to allow phosphorylation to go to completion, followed by mixing with KCl; and (b) simultaneous mixing with ATP and KCl. Using mixing procedure (a), the dephosphorylation rate constant of enzyme complexed with K^+ ions could be determined directly to be $\leq$366 s^{-1} and the rate constant for spontaneous dephosphorylation (without K^+) $\leq$60 s^{-1}. The K^+ concentration dependence of the observed reciprocal time constant showed half-saturation at a K^+ concentration of 2.4−2.6 mM with positive cooperativity involved in the occupation of the K^+ binding sites on the E_2P conformation of the enzyme. Using mixing procedure (b), it was found that at saturating K^+ concentrations the dephosphorylation of the enzyme is rate-limited by its phosphorylation, which occurs with a rate constant of approximately 190 s^{-1} (*1*). These results show that all reactions occurring after phosphorylation and prior to dephosphorylation, i.e., the E_1P to E_2P conformational transition as well as Na^+ release and K^+ binding steps, must be fast ($>$190 s^{-1}).

The Na^+,K^+-ATPase is an enzyme found in almost all animal cell membranes. Its function is to transport Na^+ ions out of and K^+ ions into the cell, using energy derived from ATP hydrolysis. The enzyme thus generates concentration gradients for Na^+ and K^+ across the membrane, which are essential for a range of important physiological functions.

The mechanism of the enzyme is generally described by the so-called Albers−Post model (*2*−*4*). The model describes a consecutive transport of Na^+ and K^+ ions across the membrane and considers two enzyme conformations, E_1 and E_2, which can exist in phosphorylated and dephosphorylated states having differing affinities for Na^+ and K^+ ions. So far the model has been quite successful in explaining a great deal of kinetic data.

In recent times, however, the Albers−Post model has increasingly come under attack. It has been suggested that further enzyme conformations exist and, even among research groups favoring the Albers−Post formalism, widely varying rate constants have been proposed for the same individual partial reactions. With respect to the Na^+ branch of the pump cycle, the literature is particularly confusing. For example, under similar experimental conditions, rate constants for the transition between phosphorylated forms of the enzyme ($E_1P \rightarrow E_2P$) have been proposed which range between 1000 s^{-1} (*5*) and 22 s^{-1} (*6*). If the value of 22 s^{-1} were correct, this would make the $E_1P \rightarrow E_2P$ conformational change a rate-determining step of the entire reaction cycle.

A comparison between stopped-flow kinetic data using the fluorescent probe RH421 and quenched-flow measurements using radioactive ATP, in which the enzyme in the presence of Mg^{2+} and Na^+ ions was rapidly mixed with ATP, has recently shown that the phosphorylation of pig kidney Na^+,K^+-ATPase occurs at pH 7.4 and 24 °C with a rate constant of approximately 200 s^{-1} and that the subsequent conformational change, $E_1P \rightarrow E_2P$, is even faster (*1*). The RH421 stopped-flow fluorescence transients showed, however, biexponential kinetics. The majority of the fluorescence change occurred with a reciprocal time constant of close to 200 s^{-1}, but there was a minor kinetic phase with a maximum reciprocal time constant in the range 30−45 s^{-1}. At that stage, it was not clear whether the slow kinetic phase was due to a reaction of the main catalytic pathway of the enzyme. To answer this question and to investigate further the possibility of rate-determining steps in the Na^+ branch of the pump cycle, it was decided to perform stopped-flow experiments on the dephosphorylation reaction of the enzyme using RH421 as a probe.

The experiments were performed in two ways: (a) premixing the enzyme with ATP to allow phosphorylation to go to completion, then mixing with K^+ ions; (b) mixing the enzyme simultaneously with ATP and K^+ ions. Experiment (a) allows the rate of K^+-stimulated dephosphorylation to be measured directly. In the case of experiment (b), the dephosphorylation reaction will be rate-limited by any slower preceding steps. A comparison of the fluorescent transients obtained from the two types of experiments allows the identification of the rate-limiting step in the sequence of reactions, $E_1 \rightarrow E_1P \rightarrow E_2P \rightarrow E_2$. At saturating concentrations of Na^+,K^+ and ATP, it will be shown that the rate of this reaction sequence is only limited by the initial phosphorylation and that there are no subsequent slower steps. The slow phase previously detected using RH421 can,

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therefore, be attributed to a reaction which does not form part of the main catalytic pathway.

MATERIALS AND METHODS

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

Na^+,K^+-ATPase-containing membrane fragments were prepared and purified from the red outer medulla of pig kidney according to a modification (*7*) of procedure C of Jørgensen (*8*, *9*). Selected density gradient fractions were diluted 5 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 at 37 °C was approximately 1800 μmol of P_i h^{-1} (mg of protein)$^{-1}$ in 30 mM histidine (Microselect, Fluka)/HCl containing 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, and 3 mM ATP (Boehringer Mannheim), and its protein concentration was typically 2 mg/mL. The enzymatic activity in the presence of 1 mM ouabain was less than 1%. The protein concentration was determined by the Lowry method (*10*). For the calculation of the molar protein concentration a molecular mass for an $\alpha\beta$ unit of the Na^+,K^+-ATPase of 147000 g mol^{-1} (*11*) was assumed.

Stopped-flow experiments were carried out using an SF-61 stopped-flow spectrofluorometer from Hi-Tech Scientific Ltd. (Salisbury, United Kingdom). 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. 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. 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, United Kingdom). Each individual kinetic trace consisted of 512 or 1024 data points. To improve the signal-to-noise ratio, typically between 6 and 40 experimental traces were averaged before evaluating the reciprocal time constant. The errors bars shown in the figures correspond to the standard error of a fit of the averaged experimental trace of a set of measurements to an exponential time function. The time constant 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. Nonlinear least-squares fits of the reciprocal time constants 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 time constants, 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.

In one type of experiment, (a), the kinetics of K^+-stimulated dephosphorylation of the Na^+,K^+-ATPase were investigated in the stopped-flow apparatus by premixing Na^+,K^+-ATPase labeled with RH421 in one of the drive syringes with a small volume of 0.5 M Na_2ATP to a final ATP concentration of 2.0 mM. After phosphorylation of the enzyme had reached a steady state (this occurs within 1 s), the enzyme was mixed with an equal volume of a KCl solution from the other drive syringe. To avoid any change in ionic strength on mixing, the KCl solutions contained choline chloride of varying concentrations such that the total concentration of KCl plus choline chloride was constant at 40 mM, and the same total concentration of choline chloride was included in the enzyme solution. The enzyme solution and the KCl solutions were prepared in the same buffer containing, in addition to choline chloride, 30 mM imidazole, 130 mM NaCl, 5 mM $MgCl_2$, and 1 mM EDTA. The pH was adjusted to 7.4 by the addition of HCl. Control experiments at low KCl concentrations but in the absence of choline chloride showed that the inclusion of choline chloride in the buffers had no effect on the reciprocal time constant observed. To test the effect of Mg^{2+} ions on the observed reciprocal time constant, measurements were also performed in which 30 mM EDTA was included in the KCl-containing buffer solution. This high concentration of EDTA would be expected to complex the Mg^{2+} ions in solution. In this case, the buffer contained 211 mM imidazole, 130 mM NaCl, 5 mM $MgCl_2$, 30 mM EDTA, and 1.3 mM KCl. The higher concentration of imidazole was necessary to compensate for the high EDTA concentration and to allow the pH to be maintained at 7.4. To take into account any effects of the higher than normal imidazole concentration on the rates of dephosphorylation and rephosphorylation (*12*−*15*), experiments were also performed in the same buffer solution but in the absence of EDTA.

In a second type of experiment, (b), the Na^+,K^+-ATPase labeled with RH421 in one of the drive syringes was mixed with an equal volume of a 2.0 mM Na_2ATP solution containing varying concentrations of KCl from the other drive syringe. Prior to the mixing with KCl, the enzyme was incubated in buffer containing 130 mM NaCl and in the nominal absence of K^+ ions, so that the enzyme can be considered to be initially fully in the state $E_1(Na^+)_3$, as in the case of the previously published study (*1*) of the kinetics of phosphorylation and subsequent conformational changes. For both types of experiment, the solutions in the drive syringes were equilibrated to a temperature of 24 °C prior to each experiment, and in both cases, the pH was 7.4. 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 fluores-

cence detection system was 0.33 ms. Each data set in which the concentration of K^+ was varied, unless specifically stated otherwise, was performed using a single Na^+,K^+-ATPase preparation.

Steady-state fluorescence measurements were recorded with a Hitachi F-4500 fluorescence spectrophotometer. To minimize contributions from scattering of the exciting light and higher order wavelengths, glass cutoff filters were used in front of the excitation and emission monochromators where appropriate. The temperature of the cuvette holder was themostatically controlled. To determine the fluorescence change of RH421 associated with the addition of KCl to phosphorylated enzyme, enzyme (6.4 μL of a 2.34 mg/mL suspension), RH421 (1.25 μL of a 60 μM ethanolic solution), and Na_2ATP (2 μL of a 0.5 M aqueous solution) were added consecutively to 491 μL of buffer (30 mM imidazole, 5 mM $MgCl_2$, 1 mM EDTA, 40 mM choline chloride, pH 7.4) in a quartz cuvette. After waiting for the fluorescence to reach a constant value (F_o), 5 μL of a concentrated stock solution of KCl (0.015−3 M) was further added, and the associated fluorescence change (ΔF) was measured. For each individual KCl concentration, a new cuvette containing enzyme, RH421, ATP, and buffer was prepared. The small constant dilution due to the addition of KCl of 1% has been neglected in the calculation of the relative fluorescence change ($\Delta F/F_o$).

The origins of the various reagents used were as follows: tris(hydroxymethyl)aminomethane (99.9%, Sigma), imidazole (99+%, Sigma; or ≥99.5%, Fluka), 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), ATP disodium salt$\cdot 3H_2O$ (special quality, Boehringer Mannheim), ethanol (analytical grade, Merck), ouabain octahydrate (95+%, Sigma), and choline chloride (99+%, 3× crystallized, Sigma; or microselect, Fluka).

RESULTS

K^+-Stimulated Dephosphorylation of the Na^+,K^+-ATPase. The kinetics of the dephosphorylation reaction of the Na^+,K^+-ATPase were investigated as a function of the K^+ concentration using experimental procedure (a) described under Materials and Methods. After mixing RH421-labeled enzyme with ATP, the enzyme is rapidly (200 s^{-1}) phosphorylated, and it undergoes an even faster conformational change (*1*). These reactions are accompanied by an increase in the fluorescence of the probe. In the absence of K^+ ions, the subsequent dephosphorylation reaction occurs at a much slower rate than the phosphorylation, i.e., 4 s^{-1} according to quenched-flow measurements of Hobbs et al. (*16*) on enzyme from eel electric organ. As long as excess ATP is present, a steady state will, therefore, be reached in which the enzyme accumulates in its phosphorylated form (E_2P). Subsequent mixing of the phosphorylated enzyme with KCl accelerates significantly the rate of dephosphorylation (*16*), so that there will be an increase in the proportion of enzyme in a dephosphorylated state and a decay in the concentration of enzyme in the E_2P state until a new steady state is reached. This can be detected as a decrease in the fluorescence of RH421. The time course of the RH421 fluorescence decrease, therefore, allows the determination of rate constants for the dephosphorylation reaction and K^+ dissociation constants.

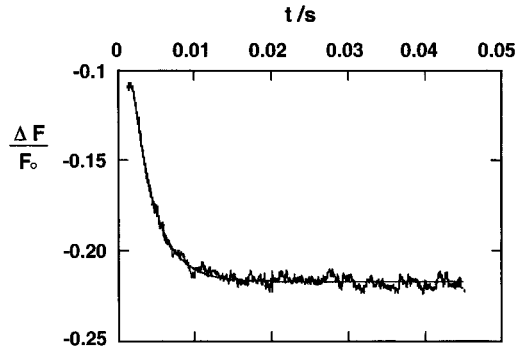

FIGURE 1: Sequential mixing stopped-flow fluorescence transient of Na^+,K^+-ATPase membrane fragments from pig kidney, noncovalently labeled with RH421. Na^+,K^+-ATPase (60 μg/mL or 0.41 μM) was premixed with a small volume of 0.5 M Na_2ATP (2.0 mM after mixing). After 10 s, the enzyme was mixed with an equal volume of a KCl solution (6.1 mM after mixing). The final Na^+,K^+-ATPase concentration after mixing was 30 μg/mL or 0.20 μM, and the RH421 concentration after mixing was 150 mM. The enzyme and KCl solutions were in a buffer containing 30 mM imidazole, 130 mM NaCl, 5 mM $MgCl_2$, and 1 mM EDTA, pH 7.4, 24 °C. The total ionic strength was maintained at a constant value by the addition of choline chloride to the enzyme and KCl solutions ([choline chloride] + [KCl] = 40 mM). The solid line represents a fit to a monoexponential time function. The experimental transient is an average of 10 individual measurements. 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 time constant was 345 (±7) s^{-1}, and the relative fluorescence change ($\Delta F/F_o$) was 0.219 (±0.008).

A typical stopped-flow transient is shown in Figure 1. Control experiments in which 1.5 mM ouabain, a specific inhibitor of the Na^+,K^+-ATPase, was added to the phosphorylated enzyme prior to mixing with KCl resulted in the complete disappearance of the fluorescence change, thus indicating that the fluorescence change can confidently be attributed to the action of the Na^+,K^+-ATPase.

Measurements over a range of K^+ concentrations showed an increase in the reciprocal time constant of the fluorescence decrease with increasing K^+ concentration, from a value of 50−60 s^{-1} at infinitely low K^+ concentrations to a maximum value in the range 320−370 s^{-1} at high K^+ concentrations (see Figure 2). The fact that the reciprocal time constant reaches a maximum value suggests that the process being observed is not simply the initial binding of K^+ to the enzyme, since this would be expected to show a linear dependence of the reciprocal time constant on the K^+ concentration. The simplest explanation is, therefore, that the observed process is a reaction of the enzyme occurring after K^+ binding. Possible candidates are a conformational change of the enzyme, the dephosphorylation of the enzyme, or PO_4^{3-} and K^+ release steps induced by dephosphorylation. Except for the comparatively high intercept value, the sigmoidal dependence of $1/\tau$ on the KCl concentration found here is very similar to behavior reported (*17*−*19*) under similar experimental conditions for the kinetics of K^+ interaction with unphosphorylated pig kidney Na^+,K^+-ATPase labeled with fluorescein.

Now let us consider the origin of the intercept of 50−60 s^{-1} at infinitely low K^+ concentrations. If one accepts that the observed fluorescence transient is arising because of a

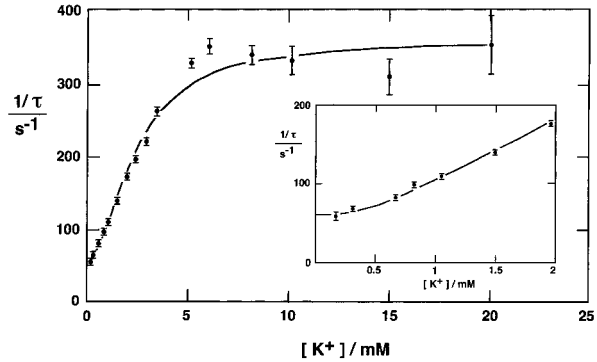

FIGURE 2: Dependence of the reciprocal time constant, $1/\tau$, of the RH421 fluorescence change on the concentration of K^+ (after mixing) for sequential mixing stopped-flow experiments in which Na^+,K^+-ATPase was premixed with Na_2ATP (2.0 mM after mixing) for 10 s to allow for phosphorylation and was subsequently rapidly mixed with KCl. [Na^+,K^+-ATPase] = 30 μg/mL (0.20 μM), [NaCl] = 130 mM, [RH421] = 150 nM, [imidazole] = 30 mM, [$MgCl_2$] = 5 mM, [EDTA] = 1 mM, pH = 7.4, T = 24 °C. The excitation and emission wavelengths were as in Figure 1. The solid line represents a nonlinear least-squares fit of the data to eq A9.

dephosphorylation of the enzyme, a likely interpretation of the intercept is the spontaneous dephosphorylation of enzyme in the absence of bound K^+ ions. For the dephosphorylation of eel electric organ enzyme in the absence of KCl, Hobbs et al. (*16*) measured a rate of approximately 4 s^{-1}. This value was obtained from quenched-flow measurements in which enzyme prephosphorylated with [^{32}P]ATP was mixed with excess of unlabeled ATP. The excess of unlabeled ATP effectively prevents rephosphorylation of the enzyme by radioactive ATP and hence any further incorporation of ^{32}P into the enzyme. Under the experimental conditions of the experiments presented in Figure 2, the rephosphorylation reaction can still occur. The fact that the value of the intercept in Figure 2 is significantly higher than 4 s^{-1} could, therefore, possibly be accounted for by the rephosphorylation of the enzyme.

To examine this possibility, experiments were performed in buffers with and without 30 mM EDTA. The other components of the buffers were 211 mM imidazole, 130 mM NaCl, 5 mM $MgCl_2$, and 1.3 mM KCl. In each case, the pH was 7.4. The high concentration of EDTA would be expected to complex the Mg^{2+} ions in solution (*20*). Since Mg^{2+} ions are a cofactor for the phosphorylation of the enzyme (*21*), the inclusion in the buffer of an EDTA concentration exceeding that of the Mg^{2+} concentration should decrease significantly the rate of rephosphorylation. Experimentally this was indeed found to be the case. In the absence of EDTA, the reciprocal time constant was measured to be 50 ($\pm$2) s^{-1}, whereas in the presence of EDTA the value was 7.3 ($\pm$0.2) s^{-1}. The latter value is of the same order of magnitude as the rate constant for spontaneous dephosphorylation of 4 s^{-1} reported by Hobbs et al. (*16*). This result is consistent with the interpretation that the value of the intercept of Figure 2 consists of contributions from spontaneous dephosphorylation of the enzyme as well as rephosphorylation. Subtracting the value of 7.3 s^{-1} from the intercept value of 50−60 s^{-1}, therefore, allows the rephosphorylation rate constant in the absence of K^+ ions to be estimated to be 43−53 s^{-1}. It should be mentioned that the reciprocal time constant of 50 s^{-1} measured in the absence of EDTA is significantly lower than the value

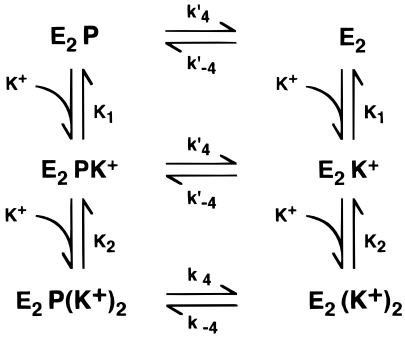

FIGURE 3: Reaction scheme describing the K^+ binding equilibria to the E_2P and E_2 conformations of the Na^+,K^+-ATPase as well as the coupled dephosphorylation and rephosphorylation reaction steps of enzyme with all its K^+ binding sites occupied (k_4 and k_{-4}) and enzyme with either vacant or partially occupied sites (k_4' and k_{-4}').

measured for the same K^+ ion concentration in the experiments shown in Figure 2. This can be attributed to an inhibitory effect of the high imidazole concentration necessary for the EDTA control experiments (*12*−*15*).

To exclude the possibility that the spontaneous dephosphorylation could be accelerated by a K^+-like effect of the relatively high concentrations of choline chloride, which was included in the buffers at low KCl concentrations to maintain a constant ionic strength, control experiments at two KCl concentrations (0.31 and 0.66 mM) were performed in which choline chloride was omitted. The other components of the buffers were 30 mM imidazole, 130 mM NaCl, 5 mM $MgCl_2$, and 1 mM EDTA. The pH was adjusted to 7.4 using HCl. The reciprocal time constants for both KCl concentrations were not significantly different from the corresponding values for buffers with choline chloride. Therefore, any effect of choline chloride on the rate of spontaneous dephosphorylation must be negligible in the concentration range employed here.

In light of the above findings, we, therefore, propose the reaction scheme shown in Figure 3 as a description of the experimentally observed behavior. K_1 and K_2 represent the association constants for the binding of the first and second K^+ ions to the E_2P or the E_2 state of the enzyme, k_4 and k_{-4} represent the rate constants for dephosphorylation and rephosphorylation, respectively, of enzyme with both K^+ binding sites occupied, and k_4' and k_{-4}' represent the corresponding values for enzyme in which the K^+ binding sites are vacant or only partially occupied. The reaction scheme chosen is basically a normal sequence of reactions as incorporated in the Albers−Post model, but it includes the basic assumption that the K^+ binding steps are so fast that they are always in equilibrium on the time scale of the dephosphorylation reaction. It should be noted that the model shown in Figure 3 assumes that the change in the dephosphorylation and rephosphorylation rate constants comes about entirely by the binding of the second K^+ ion to the enzyme. A more general model in which the rate constants for enzyme with a single bound K^+ bound could assume intermediate values between those of enzyme with completely vacant and fully occupied K^+ binding sites can, however, not be excluded. The accuracy of the experimental data unfortunately does not allow a reliable determination of the intermediate rate constants, and, therefore, we have chosen to use the simplified model shown in Figure 3. Fitting to the more general model is likely to change the

calculated values of K_1 and K_2, but it would not significantly alter the calculated values of $(k_4 + k_{-4})$ or $(k_4' + k_{-4}')$.

In the first instance, it was attempted to fit the data shown in Figure 2 to the model shown in Figure 3 assuming that both of the K^+ binding sites are identical and there is no interaction between them. For such a model, the expected dependence of the reciprocal time constant on the K^+ concentration is given by eq A11 (see Appendix). Fitting the experimental data to this equation, the best fit values obtained were $K = 7.5\ (\pm 1.3) \times 10^2\ M^{-1}$, $(k_4' + k_{-4}') = 48\ (\pm 10)\ s^{-1}$, and $(k_4 + k_{-4}) = 420\ (\pm\ 24)\ s^{-1}$. K represents here the apparent microscopic association constant of the K^+ binding sites (see Appendix). The value of K corresponds to an apparent microscopic dissociation constant of 1.33 (±0.22) mM. It was found, however, that significant systematic positive and negative deviations of the experimental points from the fitted curve were apparent. It is possible that increasing the number of K^+ binding sites in the model to values significantly greater than 2 might be able to produce an improved fit to the data. Since other investigations (*17*, *18*) have indicated, however, that there are only two K^+ binding sites and since it is generally accepted that the Na^+/K^+ coupling ratio per hydrolyzed ATP molecule for the normal pumping cycle of the enzyme is 3/2 (*21*−*23*), the theoretical model has not been extended to higher stoichiometries. An attempt was also made to fit the data to a model considering two independent K^+ binding sites with differing K^+ binding affinities. In this case, however, the two association constants converged to the same value obtained for the identical site model, thus indicating no improvement over the identical site model.

If it is accepted that there are two K^+ binding sites on the E_2P conformation of the Na^+,K^+-ATPase, the possibility exists that the binding may be cooperative. A model incorporating positive cooperativity in the K^+ binding would predict a more pronounced sigmoidicity of the K^+ ion concentration dependence of $1/\tau$ than an identical site model, and, therefore, may be able to provide a better description of the experimental data. Positive cooperativity implies that the binding of the first K^+ ion to the enzyme increases the apparent affinity of the subsequently binding K^+ ion for the enzyme. Ordered binding of K^+ ions to the E_2P form of the enzyme and the involvement of positive cooperativity have previously been discussed by a number of authors (*24*, *25*). A description of the derivation of the appropriate kinetic equation (eq A9) is given in the Appendix. It should be noted that similar behavior to that shown in Figure 2, i.e., a sigmoidal dependence of $1/\tau$ on the K^+ concentration, has also been found from stopped-flow measurements in which fluorescein 5′-isocyanate-modified enzyme was mixed with KCl (*17*−*19*). These results were interpreted as indicating a rapid ordered binding of two K^+ to the unphosphorylated form of the enzyme followed by a slower conformational change.

In the case of the results presented here, it was found that the model incorporating positive cooperativity gave a much improved description of the experimentally observed behavior over an identical site model. The fit to the positive cooperative model is shown in Figure 2. Unfortunately, because of the relatively large number of free parameters involved in the model, it was found that widely varying values of K_1 and K_2 were able to give equally good fits to the experimental data, so that only limiting values can be given. In contrast, the values of $(k_4 + k_{-4})$ and $(k_4' + k_{-4}')$ are reasonably well defined by the saturating value of $1/\tau$ at high K^+ concentrations and by the intercept. The values of the parameters calculated from the fit were as follows: $k_4 + k_{-4} = 366\ (\pm 35)\ s^{-1}$; $k_4' + k_{-4}' = 60\ (\pm 6)\ s^{-1}$; $K_1 \leq 1.3 \times 10^2\ M^{-1}$; $K_2 \geq 1.5 \times 10^3\ M^{-1}$. The values of K_1 and K_2 correspond to apparent dissociation constants of ≥7.5 mM and ≤0.65 mM, respectively. These values must be considered as *apparent* dissociation constants because of the presence of 130 mM NaCl in the reaction mixture and because the possibility of competition between K^+ and Na^+ ions for the same binding sites is not included in the model shown in Figure 3. The presence of 130 mM NaCl is necessary to ensure the complete phosphorylation of the enzyme. Based on the experiments shown in the next section as well as electrical measurements of Wagg et al. (*26*), it can be concluded that the release of Na^+ ions from the E_2P form of the enzyme is very rapid and is not likely to limit the rate of dephosphorylation, although the rate could be influenced by an unfavorable preequilibrium constant.

At the highest K^+ concentrations used for the measurements shown in Figure 2, a significant proportion of the overall fluorescence change on mixing with KCl is over during the dead-time of the stopped-flow instrument. For example, in the case of a reciprocal time constant of 350 s^{-1} (i.e., approximately the saturating value in Figure 2), this amounts to approximately 45%. A more accurate determination of the amplitude of the fluorescence change caused by mixing with KCl as a function of the KCl concentration can be obtained by measurements in a fluorescence spectrophotometer, since this allows the direct measurement of the fluorescence level, F_o, prior to the addition of KCl. In order for the relative fluorescence changes, $\Delta F/F_o$, calculated from measurements in a fluorescence spectrophotometer to be a meaningful estimation for the amplitudes of the stopped-flow measurements, however, it is important to establish that the exponential kinetic phase resolved by the stopped-flow spectrofluorometer is the only kinetic phase contributing to the fluorescence change. To do this, a stopped-flow experiment was performed in which the initial fluorescence level prior to the reaction with KCl was determined by mixing the phosphorylated enzyme with buffer excluding KCl. The final fluorescence level and the total change in fluorescence due to the reaction with KCl were then determined by mixing the same phosphorylated enzyme suspension with buffer containing 14.1 mM KCl. If $\Delta F/F_o$ was calculated by taking the dead-time into consideration and extrapolating the fit of the observed fluorescence transient to $t = 0$ to determine F_o, a value of 0.39 was obtained. On the other hand, if the value of F_o obtained from mixing with buffer excluding KCl was used, $\Delta F/F_o$ was determined to be 0.38. The good agreement between these two values indicates that there is only a single kinetic phase contributing to the fluorescence change and that the estimation of relative fluorescence amplitudes determined by measurements in a fluorescence spectrophotometer is valid. It would appear, therefore, based on the kinetic model shown in Figure 3, that the initial binding of K^+ ions to the E_2P conformation of the enzyme does not produce a significant fluorescence change.

The relative fluorescence change, $\Delta F/F_o$, calculated from measurements in a fluorescence spectrophotometer, as de-

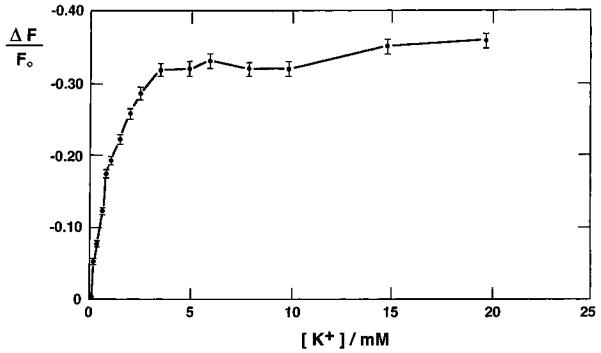

FIGURE 4: Relative fluorescence change, $\Delta F/F_o$, as measured in a fluorescence spectrophotometer, of RH421 after the addition of KCl to ATP-phosphorylated Na^+,K^+-ATPase membrane fragments as a function of the K^+ concentration: [Na^+,K^+-ATPase] = 30 μg/mL (0.20 μM), [NaCl] = 130 mM, [RH421] = 150 nM, [imidazole] = 30 mM, [$MgCl_2$] = 5 mM, [EDTA] = 1 mM, [Na_2ATP] = 2.0 mM, pH 7.4, 24 °C, λ_{ex} = 577 nm (+OG550 cutoff filter), λ_{em} = 670 nm (+RG645 cutoff filter), bandwidths = 10 nm (ex), 20 nm (em). F_o refers to the fluorescence of RH421 associated with phosphorylated enzyme prior to the addition of KCl. The individual points have been joined by lines to aid the eye of the reader.

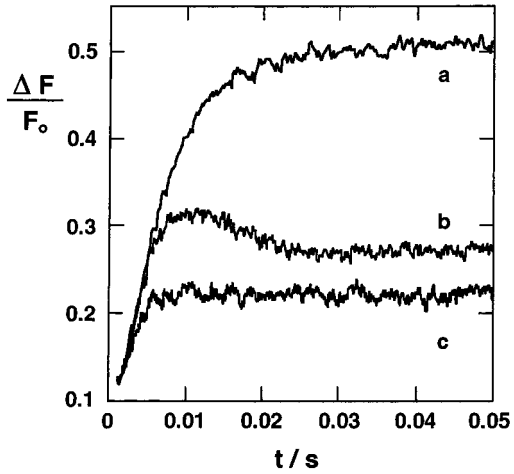

FIGURE 5: Simultaneous mixing stopped-flow fluorescence transients of Na^+,K^+-ATPase membrane fragments from pig kidney, noncovalently labeled with RH421. Na^+,K^+-ATPase (30 μg/mL or 0.20 μM, after mixing) was rapidly mixed with an equal volume of a solution containing 2.0 mM Na_2ATP and KCl in the concentration given below. Each solution was in a buffer containing 30 mM imidazole, 130 mM NaCl, 5 mM $MgCl_2$, and 1 mM EDTA; pH 7.4, 24 °C. The total ionic strength was maintained at a constant value by the addition of choline chloride to the enzyme and ATP/KCl solutions ([choline chloride] + [KCl] = 40 mM). The excitation and emission wavelengths were as in Figure 1. The curves refer to the following KCl concentrations after mixing: (a) 0.31 mM, (b) 2.44 mM, and (c) 7.05 mM.

scribed under Materials and Methods, is shown in Figure 4 as a function of the KCl concentration. It was found that $\Delta F/F_o$ increased with increasing KCl concentration until a saturating value of approximately 0.35 was reached. Half-saturation was found at a KCl concentration of approximately 0.8 mM. Similar behavior to that observed in Figure 4 has been found for enzyme from rabbit kidney (*25*) and for enzyme from both pig kidney and shark rectal gland (Cornelius, private communication). It can be explained simply by the occupation of the K^+ binding sites on the E_2P state of the enzyme, which leads to an acceleration of the dephosphorylation rate and hence an increasing perturbation of the initial steady state prior to mixing with KCl.

Simultaneous ATP and KCl Mixing Experiments. The experiments performed by procedure (b) (see Materials and Methods), in which the enzyme is simultaneously mixed with ATP and KCl, would be expected to produce the same final steady state as in the case of the consecutive mixing procedure of experiment (a). The difference is that in the case of simultaneous mixing the fluorescence changes observed arise from the phosphorylation of the enzyme as well as its dephosphorylation and the observed rate of the fluorescence changes depends on the rate constants of all reactions of the reaction sequence:

$$E_1(Na^+)_3 \rightarrow E_1P(Na^+)_3 \rightarrow E_2P(Na^+)_3 \rightarrow E_2P \rightarrow E_2P(K^+)_2 \rightarrow E_2(K^+)_2 \quad (1)$$

At very low KCl concentrations, i.e., less than or equal to approximately 0.3 mM, the simultaneous mixing with ATP and KCl produces a fluorescence increase (see Figure 5, curve a), similar to the behavior previously observed on mixing with ATP alone (ref *1*). At intermediate KCl concentrations, i.e., 1.0−3.5 mM, there is an initial rapid fluorescence increase, which is followed by a slower smaller fluorescence decrease (see Figure 5, curve b). Previously reported experiments in the absence of KCl (ref *1*) have shown that phosphorylation of the enzyme and the formation of the E_2P conformation cause an increase in fluorescence of membrane-bound RH421. The initial fluorescence increase observed here can, therefore, be attributed to the phosphorylation of the enzyme. The fluorescence decrease can be attributed to the subsequent dephosphorylation of the enzyme. The change in direction of the fluorescence transient is, therefore, due to a transient accumulation of enzyme in the E_2P conformation. At high KCl concentrations, i.e., greater than or equal to approximately 5 mM, the change in direction of the fluorescence transient disappears, and one observes a single phase fluorescence increase but with a smaller amplitude than that found at very low KCl concentrations (see Figure 5, curve c).

The fact that at very low and high KCl concentrations a fluorescence increase is observed implies that, if one accepts the reaction sequence (eq 1), the fluorescence intensity of RH421 associated with enzyme in both the E_2P and the E_2 conformations must be greater than that of RH421 associated with enzyme in the $E_1(Na^+)_3$ conformation. The fact that at intermediate KCl concentrations there is a transient fluorescence increase followed by a decrease, and the fact that the amplitude of the net fluorescence increase falls significantly as the KCl concentration is increased, implies that the fluorescence of RH421 associated with enzyme in the E_2P conformation must be greater than that of RH421 associated with enzyme in the $E_2(K^+)_2$ conformation. These findings concerning the fluorescence amplitudes are qualitatively in agreement with the relative fluorescence amplitudes of RH421 associated with different states of the Na^+,K^+-ATPase reported by Stürmer et al. (*27*).

Based on these conclusions concerning the relative amplitudes of RH421 associated with the $E_1(Na^+)_3$, E_2P, and $E_2(K^+)_2$ conformations of the enzyme, it can be shown via computer simulations of the reaction sequence (eq 1) that the disappearance of the overshoot in the fluorescence change at high KCl concentrations (see Figure 5) is consistent with the interpretation that at high KCl concentrations the rate of

dephosphorylation (including all ion binding and release steps after phosphorylation and preceding dephosphorylation) exceeds that of the phosphorylation, so that enzyme formed in the E_2P conformation is immediately dephosphorylated to the E_2 conformation. This finding agrees with the rate constant determined experimentally for the phosphorylation at saturating Na^+ and ATP concentrations of 190 s^{-1} (ref *1*) and with the K^+ concentration dependence of the rate constant for the dephosphorylation reaction (see Figure 2). There it can be seen that above a K^+ concentration of approximately 3 mM, the reciprocal time constant for the dephosphorylation reaction exceeds that of the phosphorylation reaction.

Similar behavior to that shown in Figure 5 has been reported by Stürmer et al. (*27*) using the technique of photochemical release of ATP. A transient accumulation of phosphorylated enzyme has also been observed by Hobbs et al. (*16, 28*), who measured the formation of phosphorylated enzyme using a rapid quenched flow technique. Both Stürmer et al. (*27*) and Hobbs et al. (*16, 28*) explained their data using the reaction sequence (eq 1) as described here. The fluorescence transients observed by Stürmer et al. (*27*) were, however, much slower than those observed here.

In a previous publication (ref *1*), the rate-determining step in the conversion of enzyme from the E_1 conformation to the E_2P conformation was found, by comparison with quenched-flow measurements, to be the phosphorylation reaction with a rate constant of 190 s^{-1}. If after phosphorylation and prior to dephosphorylation there were any slower steps (<190 s^{-1}), this would cause a transient accumulation of enzyme in the E_1P conformation and a significantly slower fluorescence relaxation ($1/\tau$ < 190 s^{-1}) even at saturating K^+ concentrations. The apparent reciprocal time constant of the fluorescence transient shown in Figure 5 (curve c), however, is 464 (±36) s^{-1}, i.e., even faster than the theoretical limit of 190 s^{-1} set by the rate of phosphorylation. This can be explained by the fact that the total fluorescence measured is due to contributions from RH421 associated with enzyme in both the E_2P and the E_2 conformations, and, since the fluorescence yield of RH421 associated with enzyme in the E_2P conformation is higher than that associated with the E_2 conformation, the fluorescence intensity levels out at a constant value faster than would be expected based on the phosphorylation reaction alone. The results presented here, therefore, show that at saturating concentrations of Na^+, K^+, and ATP, the rate-determining step in the reaction sequence leading from E_1 to E_2 (reaction sequence 1) is still the phosphorylation reaction and that no slower steps exist between phosphorylation and dephosphorylation of the enzyme.

DISCUSSION

The kinetics of K^+-stimulated dephosphorylation of the Na^+,K^+-ATPase have been investigated via the stopped-flow technique by mixing enzyme fluorescently labeled with RH421 in the presence of Na^+ and Mg^{2+} ions with ATP and KCl. Two modes of ATP and KCl mixing have been employed: sequential and simultaneous.

The sequential mixing experiments involved first mixing with ATP to allow phosphorylation of the enzyme to go to completion and subsequently mixing with KCl to stimulate dephosphorylation. The fluorescence change of membrane-bound RH421 accompanying the dephosphorylation was measured as a function of time. At high concentrations of KCl, the reciprocal relaxation time, of the fluorescence transient reached a saturating value of approximately 320−370 s^{-1} (see Figure 2). From the KCl concentration dependence of the reciprocal relaxation time the sum of the rate constants for the dephosphorylation and rephosphorylation reactions of enzyme with fully occupied K^+ binding sites could be determined to be 366 (±35) s^{-1} and for enzyme with fully vacant K^+ binding sites 60 (±6) s^{-1}.

Based on the stopped-flow measurements reported here as well as previously presented quenched-flow measurements (ref *1*), in which EDTA was included in the reaction mixture to inhibit rephosphorylation, estimates can also be given for the individual rate constants k_4, k_{-4}, k_4' and k_{-4}'. Stopped-flow measurements at a very low K^+ concentration and in the presence of excess EDTA (over Mg^{2+} ions) allowed the rate constant of spontaneous dephosphorylation, k_4', to be estimated to be 7 s^{-1}. Subtracting this value from the value for the sum of the rate constants for dephosphorylation and rephosphorylation, 60 s^{-1}, allows the rate constant for the rephosphorylation in the absence of K^+, k_{-4}', to be estimated to be approximately 50 s^{-1}. Quenched-flow measurements at saturating KCl concentrations on pig kidney enzyme in the presence of excess EDTA and at the same temperature and pH as the results presented here (ref *1*) showed that the rate of K^+-stimulated dephosphorylation, k_4, was 277 s^{-1}. Subtracting this value from the value for the sum of the rate constants for K^+-stimulated dephosphorylation and rephosphorylation, 366 s^{-1}, the rate constant for rephosphorylation of enzyme with fully occupied K^+ binding sites, k_{-4}, can be estimated to be approximately 90 s^{-1}.

It should be noted that the rephosphorylation refers to the re-formation of enzyme in the $E_2P(K^+)_2$ state from the E_2-$(K^+)_2$ state, but that 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. At this stage, a clear distinction between these two possible pathways cannot be made.

A kinetic analysis of the rate of exchange of ^{18}O between P_i and H_2O catalyzed by the Na^+,K^+-ATPase using ^{31}P NMR (*29*) has enabled the rate of P_i dissociation from the enzyme to be determined to be at least 80 000 s^{-1}. Despite this very high rate of P_i dissociation from the enzyme, it is possible, however, that under the conditions of our experiments sufficient P_i may still be bound to the enzyme after dephosphorylation to allow a direct rephosphorylation from inorganic phosphate. Apell et al. (*30*) have reported an apparent K_m of the E_2P conformation of the enzyme for P_i of 23 μM based on fluorescence titrations using RH421. Since prior to mixing with KCl the enzyme is hydrolyzing ATP at a rate of approximately 4 s^{-1} (Hobbs et al., 1980a), within a minute after the addition of ATP to the enzyme (0.2 μM) approximately 50 μM P_i should be present in solution. According to the results of Apell et al. (*30*), one might expect this concentration of P_i to saturate the P_i binding sites of the enzyme, so that the maximal rate of rephosphorylation by P_i would occur. The rate constants estimated above for the rephosphorylation of enzyme with fully vacant

and fully occupied K^+ binding sites of 50 and 90 s^{-1}, respectively, agree well with the minimum value of 60 s^{-1} for a direct phosphorylation by bound inorganic phosphate calculated by Karlish et al. (*31*) from measurements of rubidium exchange on Na^+,K^+-ATPase reconstituted into phospholipid vesicles. It does not agree, however, with the minimum rate constant reported by Dahms and Miara (*29*) of 2500 s^{-1}. It should be pointed out, however, that this value refers to conditions in the absence of ATP hydrolysis.

Now let us consider the possibility of rephosphorylation from ATP. In this case the rate-determining step for rephosphorylation would be expected to be the conformational change of enzyme in the $E_2(K^+)_2$ state to the $E_1(Na^+)_3$ state. Stopped-flow measurements using the same enzyme source but in the absence of Mg^{2+} ions showed previously (ref *1*) that this reaction occurs under similar experimental conditions to those employed here at a rate of $\leq$28 s^{-1}, i.e., significantly less than the estimated rates of rephosphorylation of enzyme with fully vacant and fully occupied K^+ binding sites of 50 and 90 s^{-1}, respectively. It cannot be excluded, however, that the presence of Mg^{2+} ions may accelerate the $E_2(K^+)_2$ to $E_1(Na^+)_3$ conformational change. Stopped-flow measurements of the rate of phosphorylation of enzyme in the E_2 conformation by inorganic phosphate are, therefore, necessary in order to be able to attribute the rate constants measured here to a particular rephosphorylation pathway.

It is interesting to note the similarity in the kinetic behavior shown here in Figure 2 to that found for unphosphorylated enzyme labeled with fluorescein on mixing with KCl (*17*−*19*). These workers have found that the binding of two K^+ ions to the enzyme is followed by a conformational change. Values reported for the rate constant of the conformational change are 142 s^{-1} (*17*), 150 s^{-1} (*32*), 90 s^{-1} (*18*) and 93 s^{-1} (*19*) in the presence of choline and 550 s^{-1} (*19*) in the absence of choline. Doludda et al. (*18*) proposed that the conformational change leads to an occlusion of K^+ ions within the enzyme. By analogy, it is, therefore, tempting to speculate that the slowest step (i.e., approximately 280 s^{-1}) involved in the dephosphorylation of the enzyme may in fact be the occlusion of K^+ ions within the phosphorylated enzyme.

Now let us discuss the experiments in which ATP and KCl were mixed with the enzyme simultaneously. There it was found that at low concentrations of KCl (1.0−3.5 mM) there was a transient accumulation of enzyme in the E_2P conformation (apparent as an overshoot in the fluorescence transients), indicating that under these conditions the rate of phosphorylation is greater than or equal to the rate of dephosphorylation. At higher KCl concentrations, however, there was a monophasic increase in fluorescence, indicating that the rate of dephosphorylation (including all ion binding and release steps after phosphorylation and preceding dephosphorylation) now exceeds that of the phosphorylation reaction. This result is in agreement with a previous study (ref *1*), in which a comparison of stopped-flow measurements with direct quenched-flow measurements of the phosphorylation reaction showed that the phosphorylation reaction has a rate constant at pH 7.4 and 24 °C of 190 s^{-1} and that the subsequent $E_1P(Na^+)_3 \rightarrow E_2P(Na^+)_3$ transition is very fast with a rate constant of $\geq$600 s^{-1}. It is also consistent with the electrical measurements of Wagg et al. (*26*) showing reciprocal relaxation times of $\geq$1000 s^{-1}, which they attributed to the release of Na^+ ions from the phosphorylated enzyme. In the simultaneous mixing experiments of the dephosphorylation reaction presented here, it, therefore, appears that at saturating K^+ concentrations the dephosphorylation of the enzyme is rate-limited by its phosphorylation. The results show, furthermore, that all reactions occurring after phosphorylation and prior to dephosphorylation, i.e., the $E_1P(Na^+)_3$ to $E_2P(Na^+)_3$ conformational transition, as well as Na^+ release and K^+ binding steps must be fast ($>$180 s^{-1}).

The rate constant found here for K^+-stimulated dephosphorylation should now be compared to values previously reported in the literature. Based on rapid quenched-flow measurements, Mårdh and Zetterqvist (*33*) estimated a lower limit of the rate constant of 230 s^{-1} for bovine brain Na^+,K^+-ATPase at pH 7.5 and 21 °C. An exact determination was not possible, however, because of the limited time resolution of their instrument. Similarly, using a rapid filtration apparatus Forbush and Klodos (*34*) were not able to resolve the kinetics of dephosphorylation, but they estimated a lower limit of 100 s^{-1} at 20 °C. For enzyme from eel electric organ, however, Hobbs et al. (*16*, *28*) were able to determine from quenched-flow measurements a rate constant of 350 s^{-1} at pH 7.5 and 21 °C. Using the same technique, Kane et al. (ref *1*) measured reciprocal relaxation times for the dephosphorylation of pig kidney enzyme at pH 7.5 and 24 °C of 277 s^{-1} (95% of the total amplitude) and 4.9 s^{-1} (5%). Similar experiments carried out by Cornelius (*35*) on shark Na^+,K^+-ATPase at pH 7.0 and 0 °C yielded a triple exponential decay of the phosphoenzyme concentration but with a major phase (71%) with a reciprocal relaxation time of 170 s^{-1}. Without knowledge of the activation energy of the dephosphorylation reaction, however, this value cannot be directly compared to the value obtained here because of the very different temperatures used. From electrical measurements of charge translocation by the Na^+,K^+-ATPase adsorbed to a planar lipid membrane, Gropp et al. (*36*) determined a rate constant of approximately 100 s^{-1} for the reaction $E_2P \rightarrow E_1$ at pH 6.2 and 24 °C. This value includes both the dephosphorylation step and the E_2 to E_1 conformational change, which is likely to be the major rate-determining step (*1*, *37*), so that the rate constant for the dephosphorylation step alone is possibly significantly higher. In addition, the lower pH used by Gropp et al. (*36*) in comparison to that used here may have an effect on the magnitude of the rate constant. Based on computer simulations of RH421 fluorescent transients following the photochemical release of ATP from a caged precursor, Heyse et al. (*6*) estimated a value for the dephosphorylation rate constant of enzyme from rabbit kidney at 20 °C of $\geq$1000 s^{-1}. Therefore, apart from the value given by Heyse et al. (*6*), the dephosphorylation rate constant found here of $\leq$366 s^{-1} is in agreement with previously reported values in the literature.

The K^+ concentration dependence of the dephosphorylation rate measured here can also be compared to other measurements, in which K^+ binding to the E_2P conformation could be detected. Here it was found that half-saturation of the K^+ binding sites occurred at a K^+ concentration in the range 2.0−2.4 mM. The K^+ concentration dependence of the reciprocal relaxation time for dephosphorylation (see

Figure 2) was best described by a positive cooperative model in which the K^+ ions bind sequentially to the enzyme. Bühler and Apell (*25*) observed, from steady-state RH421 fluorescence titrations of ATP-phosphorylated enzyme from rabbit kidney, half-saturation of the K^+-induced fluorescence change at a K^+ concentration of 0.19 mM at pH 7.2 and 20 °C. They fitted their data with a Hill equation and obtained a Hill coefficient of 1.33, indicating some cooperativity in the binding of the K^+ ions. For both K^+ ions, they reported apparent dissociation constants in the range 0.2−0.3 mM. Similar measurements reported here (see Figure 4) showed half-saturation of the fluorescence change at a KCl concentration of approximately 0.8 mM. It is worth noting that this concentration is significantly lower than the K^+ concentration necessary for half-saturation of the dephosphorylation rate (see Figure 2). A probable explanation for this is that the dephosphorylation reaction as well as subsequent reactions pull the K^+ binding equilibrium over toward the complexed state $[E_2P(K^+)_2]$. The effect of the dephosphorylation reaction and subsequent reactions will be to cause the K^+ binding sites and hence the fluorescence change to saturate at a lower K^+ concentration than would be expected for an isolated K^+ binding equilibrium. In this case the half-saturating K^+ concentration and the dissociation constants measured from steady-state RH421 fluorescence titrations should be considered as under-estimates of the true dissociation constants (i.e., the K^+ affinity of the enzyme is actually lower than that estimated on the basis of the fluorescence titrations). In the case of the dissociation constants derived from kinetic data, however, the effect of the dephosphorylation reaction or any conformational change occurring after K^+ binding has already been taken into consideration.

From measurements of charge translocation transients by Na^+,K^+-ATPase from shark rectal gland, Gropp et al. (*36*) observed half-saturation of K^+-activated dephosphorylation at a concentration of 4.5 mM at pH 6.2 and 24 °C. They fitted their data to an identical site model, excluding positive cooperativity. They found that half-saturation of the stationary current (i.e., enzyme in the steady state) occurred at a K^+ concentration of 0.95 mM. Similar measurements carried out by Nagel et al. (*38*) on pig kidney enzyme at pH 7.5, i.e., the same enzyme source and a very similar pH to that used here, showed half-saturation of the stationary current at 0.7 mM K^+. The half-saturating K^+ concentrations found by Gropp et al. (*36*) and Nagel et al. (*38*) when the enzyme is cycling in its steady state agree, therefore, very well with the corresponding value found here of 0.8 mM. The significantly higher apparent affinity found by Bühler and Apell (*25*) may be related to the different enzyme source or different experimental conditions.

Finally, it is interesting to consider again the biexponential kinetics found previously for the RH421 fluorescence transients observed at certain wavelengths when mixing enzyme with ATP in the absence of K^+ ions (ref *1*). There it was found that there was a major fast phase with a reciprocal time constant at saturating NaCl and ATP concentrations of approximately 180 s^{-1}, which is attributable to the phosphorylation reaction, but there was also a minor kinetic phase with a maximum reciprocal time constant in the range 30−45 s^{-1}. Very similar behavior has since been found with enzyme derived from rabbit kidney (Clarke, Kane, Roudna, Apell, and Bamberg, unpublished results). In light of the findings reported here, it is apparent that at saturating Na^+, K^+, and ATP concentrations there is no possibility of a slow reaction with a rate constant in the range 30−45 s^{-1} occurring after phosphorylation, since in that case it would limit the rate of dephosphorylation in the simultaneous mixing experiments. The slow phase can, therefore, not be attributed to a reaction lying on the main catalytic pathway of the enzyme. Possible causes of the slow phase could be: (1) a subpopulation of slowly reacting enzyme molecules, (2) phosphorylation of the enzyme on a different site than that which normally catalyzes the ion transport, or (3) an enzymatic pathway after phosphorylation which no longer occurs in the presence of K^+ ions. With respect to possibility (1), it should be pointed out that kinetic heterogeneity of the Na^+,K^+-ATPase has been suggested by Klodos et al. (*39*) to arise from the effect of different lipid phase environments on the enzyme. The possibility that the slow phase is due to an enzymatic pathway which only occurs in the absence of K^+ ions [i.e., possibility (*3*)] also deserves further attention. A likely pathway might be a relaxation of the dephosphorylation/rephosphorylation equilibrium of the enzyme in the absence of bound ions, whose maximum reciprocal time constant was shown here to have a value of 60 s^{-1}. If the fluorescence change which occurs on mixing with ATP is attributed to the release of Na^+ ions from the E_2P conformation of the enzyme, as suggested by Stürmer et al. (*27*) and Klodos (*39*), the dephosphorylation of E_2P, via its coupling to the binding equilibrium of Na^+ ions to E_2P, should cause the release of more Na^+ ions to the solution and hence a fluorescence increase, as experimentally observed.

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APPENDIX

Reciprocal Time Constant for Sequential Mixing Experiments. To describe the K^+ concentration dependence of the reciprocal time constant, $1/\tau$, the reaction scheme shown in Figure 3 has been used. The reaction scheme incorporates the following essential points: (1) two K^+ binding sites on the E_2P and E_2 conformations of the enzyme; (2) the K^+ binding steps are always in equilibrium on the time scale of the dephosphorylation and rephosphorylation reactions; (3) a significant change in the rates of dephosphorylation and rephosphorylation occurs only after the binding of two K^+ ions. 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. It should be mentioned that the assumption that the rate constants for dephosphorylation and rephosphorylation do not change after the occupation of a single K^+ binding site is not entirely necessary, but that the sigmoidal dependence of $1/\tau$ on the

K^+ concentration (see Figure 2) would suggest that the major change in rate constants occurs after binding of the second K^+ ion.

To derive an equation for $1/\tau$ containing the equilibrium and rate constants for the various steps as well as the total K^+ concentrations, we have applied the theory of relaxation kinetics, as developed by Eigen (*41*) and applied by Kirschner and co-workers (*42*−*44*), 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 K^+ over enzyme, the theory is also applicable to stopped-flow data.

The total concentrations of enzyme in the E_2P and E_2 states are given by:

$$\sum E_2P = E_2P + E_2PK^+ + E_2P(K^+)_2 \qquad \text{(A1)}$$

$$\sum E_2 = E_2 + E_2K^+ + E_2(K^+)_2 \qquad \text{(A2)}$$

The reaction scheme shown in Figure 3 can now be simplified to an overall reaction between the E_2P and E_2 states:

$$\sum E_2P \Leftrightarrow \sum E_2$$

The rate of change of the deviation of the total concentration of enzyme in the E_2P states from its final equilibrium value is given by

$$-\frac{\mathrm{d}\Delta\sum E_2P}{\mathrm{d}t} = k_4'\Delta E_2P - k_{-4}'\Delta E_2 + k_4'\Delta E_2PK^+ - k_4'\Delta E_2K^+ + k_4\Delta E_2P(K^+)_2 - k_{-4}\Delta E_2(K^+)_2 \qquad \text{(A3)}$$

To integrate this differential rate equation and derive an expression for the reciprocal time constant, equations relating ΔE_2P, ΔE_2, ΔE_2PK^+, ΔE_2K^+, $\Delta E_2P(K^+)_2$, and $\Delta E_2(K^+)_2$ to $\Delta\sum E_2P$ must first be derived. For this, we make use of the assumption that the K^+ binding steps are all in equilibrium on the time scale of the relaxation of the dephosphorylation reaction. For a positive cooperative model in which the first K^+ ion binds weakly with an association constant K_1 and the subsequent K^+ ion binds strongly with an association constant K_2, K_1 and K_2 are defined by the following two equations:

$$K_1 = \frac{E_2PK^+}{E_2P\cdot K^+} = \frac{E_2K^+}{E_2\cdot K^+} \qquad \text{(A4)}$$

$$K_2 = \frac{E_2P(K^+)_2}{E_2PK^+\cdot K^+} = \frac{E_2(K^+)_2}{E_2K^+\cdot K^+} \qquad \text{(A5)}$$

At excess concentrations of K^+ over enzyme, it can be assumed that the free concentration of K^+ is constant for the duration of the stopped-flow experiment. Under these conditions, one can derive from eqs A4 and A5 and the laws of conservation of mass (eqs A1 and A2) the following expressions for the deviations of the concentrations of the various enzyme species from their equilibrium values in terms of $\Delta\sum E_2P$:

$$\Delta E_2P = -\Delta E_2 = \frac{\Delta\sum E_2P}{1 + K_1[\mathrm{K}^+] + K_1K_2[\mathrm{K}^+]^2} \qquad \text{(A6)}$$

$$\Delta E_2PK^+ = -\Delta E_2K^+ = \frac{K_1[\mathrm{K}^+]\cdot\Delta\sum E_2P}{1 + K_1[\mathrm{K}^+] + K_1K_2[\mathrm{K}^+]^2} \qquad \text{(A7)}$$

$$\Delta E_2P(K^+)_2 = -\Delta E_2(K^+)_2 = \frac{K_1K_2[\mathrm{K}^+]^2\cdot\Delta\sum E_2P}{1 + K_1[\mathrm{K}^+] + K_1K_2[\mathrm{K}^+]^2} \qquad \text{(A8)}$$

Now substituting eqs A6−A8 into eq A3 for ΔE_2P, ΔE_2, ΔE_2PK^+, ΔE_2K^+, $\Delta E_2P(K^+)_2$, and $\Delta E_2(K^+)_2$, it can be shown upon integration of the differential rate equation that the reciprocal time constant, $1/\tau$, is given by

$$\frac{1}{\tau} = \frac{(1 + K_1[\mathrm{K}^+])\cdot(k_4' + k_{-4}') + K_1K_2[\mathrm{K}^+]^2\cdot(k_4 + k_{-4})}{1 + K_1[\mathrm{K}^+] + K_1K_2[\mathrm{K}^+]^2} \qquad \text{(A9)}$$

τ represents here the time necessary for the difference in the sum of the concentrations of all E_2P species (i.e., $\Delta\sum E_2P$) from its final equilibrium value to decay to 1/e of its initial value. The experimental values of τ obtained at different K^+ concentrations can now be fitted to eq A9 to see if the postulated mechanism adequately explains the data and to derive values of the parameters ($k_4' + k_{-4}'$), ($k_4 + k_{-4}$), K_1, and K_2.

Now let us consider a slightly less general model in which there is no cooperativity involved in the binding of the K^+ ions and both K^+ binding sites are identical. For such a model, the microscopic association constant, K, of the binding sites is defined by

$$K = \frac{E_2PK^+}{2E_2P\cdot K^+} = \frac{E_2K^+}{2E_2\cdot K^+} = \frac{2E_2P(K^+)_2}{E_2PK^+\cdot K^+} = \frac{2E_2(K^+)_2}{E_2K^+\cdot K^+} \qquad \text{(A10)}$$

The factors of 2 in eq A10 are statistical coefficients which take into account the number of free and occupied binding sites per enzyme molecule (*45*−*47*). Proceeding as in the case of the derivation of eq A9, it can be shown that for this model the reciprocal time constant is given by

$$\frac{1}{\tau} = \frac{(1 + 2K[\mathrm{K}^+])(k_4' + k_{-4}') + K^2[\mathrm{K}^+]^2(k_4 + k_{-4})}{(1 + K[\mathrm{K}^+])^2} \qquad \text{(A11)}$$

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