

Allosteric Effect of ATP on Na⁺,K⁺-ATPase Conformational Kinetics[†]Ronald J. Clarke,^{*,‡} Hans-Jürgen Apell,[§] and Benjamin Y. Kong[‡]*School of Chemistry, University of Sydney, Sydney, NSW 2006, Australia, and Faculty of Biology, University of Konstanz, D-78435 Konstanz, Germany**Received November 29, 2006*

ABSTRACT: The kinetics of the E₂ → E₁ conformational change of unphosphorylated Na⁺,K⁺-ATPase was investigated via the stopped-flow technique using the fluorescent label RH421 (pH 7.4, 24 °C). The enzyme was pre-equilibrated in a solution containing 25 mM histidine and 0.1 mM EDTA to stabilize the E₂ conformation. When rabbit enzyme was mixed with 130 mM NaCl alone or with 130 mM NaCl and varying concentrations of Na₂ATP simultaneously, a fluorescence decrease was observed. In the absence of ATP, the fluorescence decrease followed a biexponential time course, but at ATP concentrations after mixing of ≥ 50 μM, the fluorescence transient could be adequately fitted by a single exponential. On the basis of the agreement between theoretical simulations and experimental traces, we propose that in the absence of bound ATP the conformational transition occurs as a two step reversible process within a protein dimer, E₂:E₂ → E₂:E₁ → E₁:E₁. In the presence of 130 mM NaCl, the sum of the forward and backward rate constants for the E₂:E₂ → E₂:E₁ and E₂:E₁ → E₁:E₁ transitions were found to be 10.4 (±1.0) and 0.49 (±0.02) s⁻¹, respectively. At saturating concentrations of ATP, however, the transition occurs in a single reversible step with the sum of its forward and backward rate constants equal to 35.2 (±0.3) s⁻¹. It was found that ATP acting at a high affinity site (*K*_d ≈ 0.25 μM), stimulated the reverse reaction, E₁ATP → E₂ATP, in addition to its known allosteric low affinity (*K*_d ≈ 71 μM) stimulation of the forward reaction, E₂ATP → E₁ATP.

Na⁺,K⁺-ATPase¹ was the first ion pump to be discovered (1), and it is one of the most fundamentally important enzymes of animal physiology. The electrochemical potential gradient for Na⁺ ions, which the enzyme maintains, is used as the driving force for numerous secondary transport systems. Examples include the Na⁺ channels in the nerve, which allow the action potential to be produced, the Na⁺-glucose cotransporter, which is responsible for glucose uptake in intestinal cells, and the Na⁺/Ca²⁺ exchanger in the heart, which plays an important role in muscle relaxation. Na⁺,K⁺-ATPase, furthermore, contributes to the osmotic regulation of cell volume and is a major determinant of body temperature. For all of these functions, the enzyme derives its energy from the hydrolysis of ATP, which is coupled to Na⁺ transport.

For many years, it has been known that ATP also has an allosteric effect on the enzyme. Under physiological conditions, ATP binds to the E₂(K⁺)₂ conformation of the enzyme

and accelerates the release of K⁺ ions to the cytoplasm without undergoing any hydrolysis. This was first demonstrated by Karlish and Yates (2), who measured the rate of the E₂ → E₁ conformational transition, which occurs simultaneously with K⁺ release, via the stopped-flow technique, utilizing the change in the enzyme's tryptophan fluorescence as the means of detection. This stimulation of the E₂ → E₁ transition by ATP has since been confirmed by a number of groups using a variety of experimental approaches (3–8). The dissociation constant of ATP for the E₂ conformation has been estimated to be 71 μM (7), 143 μM (6), 196 μM (4), 277–303 μM (8), 300 μM (3), and 450 μM (2). All of these values are significantly greater than the dissociation constant of ATP to the E₁ conformation, where in the presence of Na⁺ and Mg²⁺ ions it undergoes hydrolysis. From pre-steady-state kinetic measurements of enzyme phosphorylation, the ATP dissociation constant of the E₁ conformation has been reported to be in the range 4–14 μM (6, 7, 9–12). Because of the much higher dissociation constants found for ATP binding to the E₂ conformation, the ATP binding site of this enzyme conformation is generally referred to as the low affinity ATP site.

On the basis of a kinetic analysis of the Na⁺,K⁺-ATPase's entire pump cycle it has become clear (13) that under physiological conditions the E₂ → E₁ conformational change and the phosphorylation via ATP, that is, E₁ → E₁P, are the two major rate-determining steps of the cycle. A solid understanding of the kinetics of the E₂ → E₁ transition and the role played by ATP is, thus, crucial to understanding the function of the enzyme as a whole.

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¹ Abbreviations: Na⁺,K⁺-ATPase, sodium and potassium ion-activated adenosine triphosphatase; ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; E₁, E₂, E₁P, and E₂P, intermediates of the Na⁺,K⁺-ATPase pump cycle; RH421, *N*-(4-sulfobutyl)-4-(4-(*p*-(dipentylamino)phenyl)butadienyl)-pyridinium inner salt.

An interesting finding that has been reported by a number of researchers is that at zero or low concentrations of ATP biexponential kinetics of K^+ or Rb^+ release from E_2 are observed (3, 8, 14). In a similar fashion, in previous publications we reported that if the $\text{E}_2 \rightarrow \text{E}_1$ conformational transition is followed in a stopped-flow apparatus using the voltage-sensitive fluorescence probe RH421, a biexponential fluorescence change is observed but only at low ATP concentrations (i.e., $\leq 25 \mu\text{M}$) (7, 15). In these previous publications from our group, the biexponential behavior was reported but with no mechanistic explanation for its occurrence. The purpose of this article is, therefore, to try and offer an explanation for this behavior.

MATERIALS AND METHODS

Enzyme. Na^+, K^+ -ATPase-containing membrane fragments were prepared and purified from the red outer medulla of rabbit kidney according to procedure C of Jørgensen (16, 17). Specific ATPase activity was measured by the pyruvate kinase/lactate dehydrogenase assay (18), and the protein concentration was determined by the Lowry method (19), 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 $147,000 \text{ g mol}^{-1}$ (20) was assumed. The specific activity of the Na^+, K^+ -ATPase preparations used was in the range of $1900\text{--}2040 \mu\text{mol P}_i/\text{h}$ per mg of protein at 37°C . The protein concentration of the preparations was in the range $2.3\text{--}3.1 \text{ mg/mL}$.

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

The origins of the various reagents used were as follows: EDTA (99%, Sigma), NaCl (Suprapur, Merck), KCl (analytical grade, Merck), HCl (0.1 N Titrisol solution, Merck), ATP disodium salt $\cdot 3\text{H}_2\text{O}$ (special quality, Boehringer Mannheim), ATP tris salt $\cdot 1.5\text{H}_2\text{O}$ (95–98%, Sigma), ethanol (analytical grade, Merck), L-histidine ($\geq 99.5\%$, Fluka), and tris(hydroxymethyl)amino)methane (99.9%, Sigma).

Stopped Flow. 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. 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 fluorescence was collected at wavelengths $\geq 665 \text{ nm}$ by using an RG665 glass cutoff filter (Schott, Mainz, Germany) in front of the monochromator. Kinetic data were collected via a high-speed 12-bit analog-to-digital data acquisition board and analyzed using software developed by Hi-Tech Scientific. Each kinetic trace consisted of 1024 data points. To improve the signal-to-noise ratio, typically between 7 and 15 experimental traces were averaged before the reciprocal relaxation time was evaluated. This was done by fitting a sum (either one or two) of exponential

functions to the averaged experimental trace. The choice between a single or a double exponential fit was made on the basis of the presence or absence of any observed deviation from random fluctuation in residual plots and the values of the chi-squared parameter. 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.

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 $\text{NaCl}/\text{Na}_2\text{ATP}$ mixtures were prepared in a solution containing 25 mM histidine and 0.1 mM EDTA . 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 and stabilize an E_1 -like conformation (21–29). The conformational equilibrium of the enzyme was thus initially poised toward the E_2 conformation. Experiments were also performed in which the enzyme was pre-equilibrated in a solution containing 1 mM KCl in addition to 25 mM histidine and 0.1 mM EDTA in order to initially stabilize the $\text{E}_2(\text{K}^+)_2$ state of the enzyme. The data set, in which the ATP concentration was varied, was collected using a single Na^+, K^+ -ATPase preparation.

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 $1.7 (\pm 0.2) \text{ ms}$. 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. Interference of photochemical reactions of RH421 with the kinetics of the Na^+, K^+ -ATPase-related fluorescence transients was avoided by inserting neutral density filters in the light beam in front of the monochromator.

Steady-State Fluorescence Measurements. Steady-state fluorescence measurements were recorded with a Shimadzu RF-5301PC spectrofluorophotometer. To minimize contributions from scattering light and higher-order wavelengths, glass cutoff filters were used in front of the emission and excitation monochromators. Fluorescence emission was measured over an emission wavelength range of $665\text{--}900 \text{ nm}$ (+RG665 glass cut off filter, Coherent Scientific, Adelaide, Australia). The excitation wavelength was 577 nm (+OG570 glass cutoff filter, Coherent Scientific). These wavelengths were chosen to be as close as possible to those used in the stopped-flow experiments. Measurements of fluorescence levels on the spectrofluorophotometer before and after substrate additions allows the amplitude of fluo-

rescence changes to be easily measured. These are not as easily accessible using the stopped-flow instrument, because fast fluorescence changes sometimes occur during the deadtime of the instrument and are not observable.

Simulations. Computer simulations of the time course of fluorescence changes experimentally observed via stopped flow were performed using the commercially available program Berkeley Madonna 8.0 via the variable step-size Rosenbrock integration method for stiff systems of differential equations. The simulations yield the time course of the concentration of each enzyme intermediate involved as well as the total fluorescence. For the purposes of the simulations, each enzyme intermediate was normalized to a unitary enzyme concentration.

RESULTS

Na⁺-Induced Stopped-Flow Fluorescence Traces. On mixing rabbit kidney Na⁺,K⁺-ATPase-containing membrane fragments labeled with RH421 with a solution containing 130 mM NaCl (as described under Materials and Methods), a decrease in fluorescence occurred (Figure 1A). At low concentrations of Na₂ATP ($\leq 25 \mu\text{M}$, after mixing) two kinetic phases could be resolved, both of which resulted in a fluorescence decrease but over different timescales. The biphasic nature of the kinetic curves at low ATP concentrations can be clearly demonstrated from a comparison of mono and biexponential fits to the data. For example, in the case of the measurement at zero ATP, a monoexponential fit resulted in a chi-squared value of 2.5, whereas with a biexponential fit over the same time range, the value was more than halved to 1.16. The appropriateness of the biexponential fit is also demonstrated by its residual plot, which is completely random in comparison to the systematic deviations displayed by the monoexponential fit (Figure 2). As the Na₂ATP concentration was increased, the overall kinetics of the fluorescence change became faster, and at $\geq 50 \mu\text{M}$ after mixing, the biphasic behavior disappeared (as evidenced by a random residual plot (not shown)).

The total amplitudes of the fluorescence change (i.e., the sum of the amplitudes of both phases when two phases are present) are shown in Figure 3. At very low ATP concentrations ($\leq 1 \mu\text{M}$, after mixing), it appears as if there is a decrease in amplitude, that is, $\Delta F/F_0$ becomes less negative. At higher ATP concentrations, the magnitude of the fluorescence change increased to a saturating value of around $-(0.16 - 0.17)$.

If the enzyme was mixed with a 25 mM histidine and 0.1 mM EDTA solution containing TrisATP (up to 1 mM after mixing) but excluding NaCl, then no fluorescence change was observed in the stopped flow. Therefore, the fluorescence change observed in Figure 1A cannot be attributed to ATP binding alone. The presence of Na⁺ ions is necessary to stimulate the reactions that lead to the fluorescence change.

The observation of the fluorescence change is dependent on the composition of the solution in which the enzyme is pre-equilibrated. In the case of the measurements shown in Figure 1A, the pre-equilibration solution consisted of 25 mM histidine and 0.1 mM EDTA, which, because a very low concentration of buffer cations is present (21–29), would be expected to stabilize the E₂ conformation of the enzyme.

If 1 mM KCl was included in the pre-equilibration solution, then the fluorescence decrease was still observed, as was the biphasic behavior at low Na₂ATP concentrations. There was also no significant change in the observed reciprocal relaxation times of the two phases. On the basis of K⁺ titrations using the fluorescein-labeled enzyme (30, 31), one would expect the enzyme under these conditions to be almost totally in the E₂(K⁺)₂ state. On the basis of the results of Bugnon et al. (31), whose experimental conditions most closely approximate our own, one would expect prior to mixing a 99.5% occupancy of the E₂(K⁺)₂ state at 1 mM KCl. Therefore, whether the enzyme is in the E₂ or the E₂(K⁺)₂ state appears to make no difference in the fluorescence change observed here. However, if the pre-equilibration solution consisted of 50 mM tris and 0.1 mM EDTA at pH 7.4, then the fluorescence change of the stopped-flow experiments was almost completely abolished (Figure 4). Under these conditions, it is known that the tris buffer cations would initially stabilize an E₁-like conformation (21–29). A tris concentration of 50 mM was chosen because previous measurements (29) using the same source of enzyme showed that this was sufficient to convert the enzyme into the E₁-like conformation. Therefore, the observation of the fluorescence change in stopped-flow experiments requires that the enzyme be initially in either the E₂ or the E₂(K⁺)₂ state. It is reasonable to assume, therefore, that the kinetics that are being followed are the conversion of the enzyme to the E₁(Na⁺)₃ state, which is stabilized by interaction with Na⁺ ions. Furthermore, one can conclude that the biphasic nature of the kinetics observed at low Na₂ATP concentrations cannot be due to the enzyme in a mixture of states between E₁ and E₂.

Another possibility could be that the biphasic behavior is due to enzyme heterogeneity, that is, all of the enzyme may be present in the E₂ conformation, but the enzyme preparation contains two different isoforms of the enzyme that react differently on mixing with NaCl in the absence of ATP. If this were true, however, the two isoforms would have to have identical kinetic behavior when ATP is bound because at ATP concentrations $\geq 50 \mu\text{M}$, only a single kinetic phase was observed. Although such a coincidence could not be excluded completely, it would seem to be unlikely. A further argument against a mixture of isoforms arises from the amplitudes of the fluorescence changes of the two phases in the absence of ATP. The fast and slow phases of the fluorescence change accounted for 34% and 66% of the total fluorescence change, respectively. If the biphasic behavior were due to two isoforms, then, on the basis of these significant proportions of each phase, one would expect significant amounts of each of two isoforms to be present. In fact two different isoforms of the α subunit have been identified in the rabbit kidney. From studying the effects of monoclonal antibodies on Na⁺,K⁺-ATPase activity from sections along the rabbit nephron, Barlet-Bas et al. (32) found that the α_1 subunit is expressed in all segments of the nephron except for the collecting duct, which preferentially expresses an α_3 -like subunit. Because in the present study the enzyme was isolated from the outer medulla, whose Na⁺,K⁺-ATPase activity originates primarily from the thick ascending limb of the loop of Henle where the α_1 isoform dominates (32), it is unlikely that sufficient amounts of the α_3 isoform would be present in the preparation to account for the biphasic

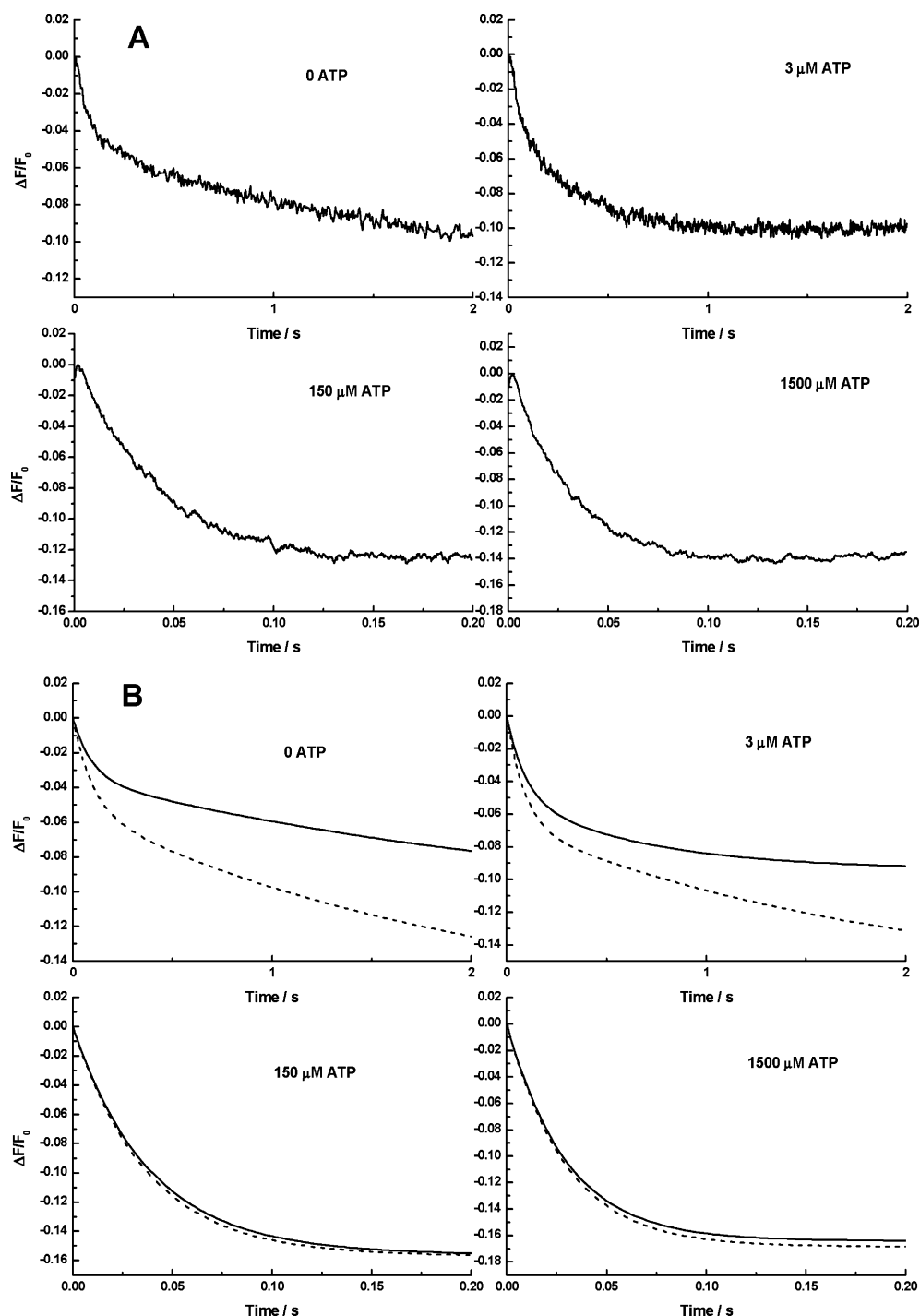


FIGURE 1: (A) Stopped-flow fluorescence transients of Na⁺,K⁺-ATPase from rabbit kidney noncovalently labeled with RH421 (75 nM, after mixing). Na⁺,K⁺-ATPase (10 $\mu\text{g}/\text{mL}$ or 0.068 μM , after mixing) was rapidly mixed with an equal volume of a solution containing 130 mM NaCl and varying concentrations of Na₂ATP (pH 7.4, 24 °C). Both the enzyme suspension and the NaCl/Na₂ATP solution were prepared in a solution containing 25 mM histidine and 0.1 mM EDTA. The fluorescence of membrane-bound RH421 was measured at an excitation wavelength of 577 nm at emission wavelengths of ≥ 665 nm (RG665 glass cutoff filter). The calculated reciprocal relaxation times were (a) 0 ATP, 10.4 (± 1.0) s⁻¹ (34% of the total amplitude) and 0.49 (± 0.02) s⁻¹ (66%); (b) 3 μM ATP, 28 (± 5) s⁻¹ (40%) and 2.1 (± 0.1) s⁻¹ (60%); (c) 150 μM ATP, 25.4 (± 0.3) s⁻¹; and (d) 1500 μM ATP, 35.2 (± 0.3) s⁻¹. (B) Kinetic simulations of the experimental fluorescence transients based on a reversible conformational change model (solid line; see Figure 5) and the rate constants, equilibrium constants, and fluorescence levels given in Table 1. The dashed line represents simulations based on the same model but with irreversible conformational changes.

kinetic behavior observed here in the absence of ATP. This conclusion is, furthermore, supported by the results of Lücking et al. (33), who showed using the competitive polymerase chain reaction that although α_1 , α_2 , and α_3 isoforms were all present in the rat kidney, the α_2 and α_3 isoforms together only amounted to $\leq 0.1\%$ of the enzyme

present in the α_1 isoform. Therefore, we conclude that it is very unlikely that the biphasic behavior is due to enzyme isoform heterogeneity.

Another possible origin of enzyme heterogeneity could arise from FXYD proteins. These are small single transmembrane segment proteins that are expressed in a tissue-

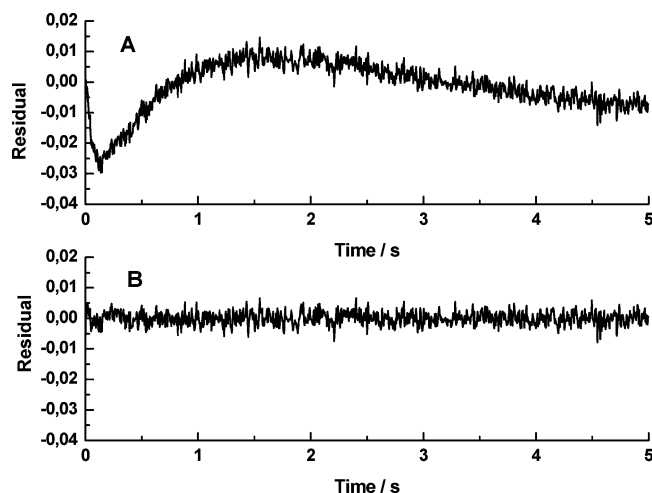


FIGURE 2: Residual plots of the best single (A) and double (B) exponential fits to the stopped-flow fluorescence transient obtained under the conditions described in Figure 1A in the absence of ATP. The chi-squared values obtained were 2.5 for the single-exponential fit and 1.16 for the double exponential fit.

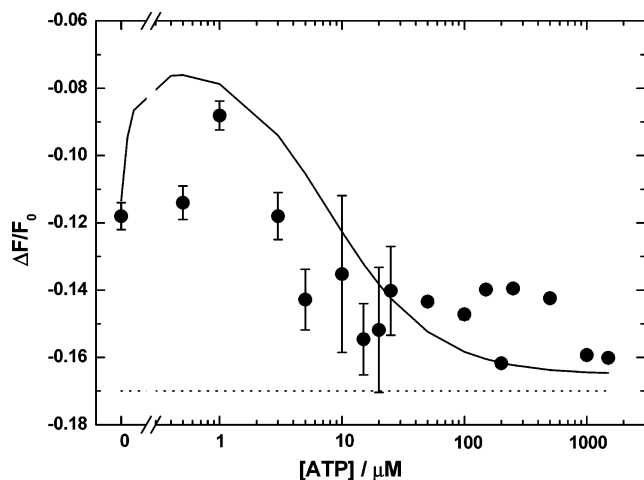


FIGURE 3: Amplitudes ($\Delta F/F_0$) of the observed fluorescence change under the conditions described in Figure 1A as a function of the ATP concentration. ΔF is the total fluorescence change (i.e., of both phases if a two-phase signal was observed), and F_0 is the initial fluorescence level prior to mixing with NaCl and ATP. The dashed line represents the prediction of simulations based on the irreversible conformational change model (see Figure 5). The solid line represents the prediction of simulations based on the reversible conformational model (see Figure 5). The values of $\Delta F/F_0$ were all determined from extrapolations to infinite time of either single or double exponential fits (whichever were more appropriate on the basis of residual plots) to the kinetic data.

specific fashion and that are thought to modulate the function of the Na^+, K^+ -ATPase, adapting its kinetic properties to the specific needs of different cells (34). In the kidney, three different FXYD proteins are present: two variants of the so-called γ subunit, γ_a and γ_b , with different N-termini (both classified as FXYD2), and the corticosteroid hormone-induced factor (CHIF), which is classified as FXYD4. These three proteins could complex with an α and a β subunit of the Na^+, K^+ -ATPase, thus potentially producing complexes with different kinetic behavior, which might account for biphasic kinetics. Experimental evidence does in fact exist that FXYD proteins do affect the $\text{E}_2 \leftrightarrow \text{E}_1$ equilibrium, shifting it toward the E_1 state (35, 36). However, in both cases this evidence was obtained from steady-state kinetic measurements of the Na^+, K^+ -ATPase, that is, under enzyme

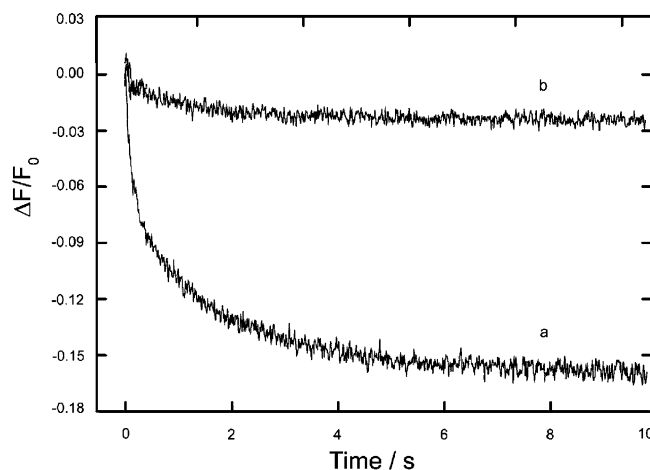


FIGURE 4: Stopped-flow fluorescence transients of rabbit kidney Na^+, K^+ -ATPase. Curve a: Enzyme was pre-equilibrated in a solution containing 25 mM histidine and 0.1 mM EDTA (pH 7.4, 24 °C). The reaction was initiated by mixing with 100 mM NaCl in 25 mM histidine and 0.1 mM EDTA solution. All other experimental conditions were as described in the caption to Figure 1. Curve b: The same as curve a, except 25 mM histidine was replaced by 50 mM Tris in the pre-equilibration solution and the mixing solution.

cycling conditions, which require the presence of ATP. In contrast, the biphasic kinetic behavior that we have observed disappears on increasing the ATP concentration (Figure 1A), that is, there is no enzyme heterogeneity at high ATP concentrations. Therefore, if one were to attribute the biphasic behavior to enzyme heterogeneity caused by the presence of FXYD proteins, this would be in contradiction to previous observations (35, 36). Furthermore, in their recent review of FXYD protein interaction with the Na^+, K^+ -ATPase, Garty and Karlish (34) stated that the effect of FXYD proteins on the kinetic parameters of the enzyme are modest, usually only about 2-fold. In contrast, the reciprocal relaxation times that we have observed for the two phases in the absence of ATP (Figure 1A) are a factor of over 20-fold different (i.e., 10.4 s^{-1} for the fast phase and 0.49 s^{-1} for the slow phase), an order of magnitude greater than FXYD protein effects. Therefore, we believe that it would be contrary to the current understanding of FXYD protein behavior to attribute our observed kinetic behavior to their action.

A further possible cause of enzyme heterogeneity could arise from modification of the membrane by sodium dodecyl sulfate (SDS), which has been used in purification of the enzyme. In this case as well as in any other explanation of biphasic kinetics based on enzyme heterogeneity, on the basis of our findings, the heterogeneity would have to be ATP dependent, that is, the heterogeneous behavior would have to disappear on increasing the ATP concentration. Such a situation seems improbable, and thus, we conclude that enzyme heterogeneity is most likely not the major reason behind the biphasic kinetic behavior reported here. If this is the case, then instead it must be due to an intrinsic property of the E_2 conformation when it is present at zero or low ATP concentrations.

The last statement does not mean, however, that the observed fluorescence change is totally due to the conformational change itself, merely that whatever reaction causes the fluorescence change, it is rate-limited by the $\text{E}_2 \rightarrow \text{E}_1$ -

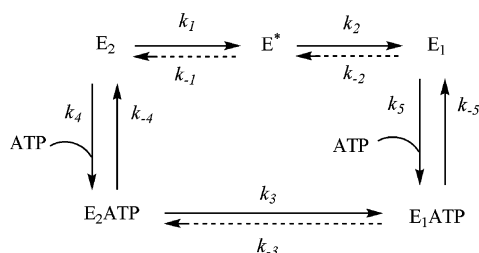


FIGURE 5: Reaction scheme of the $E_2 \rightarrow E_1$ conformational change of the Na^+, K^+ -ATPase with and without bound ATP. Na^+ ions have been omitted for simplicity. However, under the experimental conditions used (130 mM NaCl), all of the E_1 states, with or without ATP, would be completely saturated with Na^+ ions and would, thus, all have three bound Na^+ ions. The solid lines represent all those reaction steps included in initial simulations based on an irreversible conformational change. In the case of the reversible conformational model, the reactions shown with dashed lines were also taken into account in the simulations. The state intermediate between E_2 and E_1 in the absence of ATP is here temporarily symbolized by E^* . The precise nature of this intermediate state will be discussed later.

(Na^+)₃ transition. If the enzyme plus RH421 was equilibrated in a 50 mM tris and 0.1 mM EDTA buffer at pH 7.4, then fluorescence measurements in a steady-state fluorimeter showed that the addition of NaCl does in fact cause a decrease in fluorescence. The magnitude of the decrease increased with increasing NaCl concentration, saturating at a value of $-23 (\pm 1)\%$ at a NaCl concentration of ≥ 50 mM. The fact that this fluorescence change was not observed in the stopped-flow when the enzyme was preincubated in tris buffer means that it must be due to a very fast reaction, that is, Na^+ binding to enzyme in the E_1 conformation, which occurs during the dead time of the stopped-flow instrument. Therefore, it appears that a major cause of the fluorescence change is Na^+ binding to the E_1 conformation but that is preceded by the rate-limiting $E_2 \rightarrow E_1$ transition when the enzyme is preincubated in histidine solution. Further discussion of the precise origin of the fluorescence change will be presented later within the context of the mechanism of the reactions being observed.

Theoretical Simulations. On the basis of the experimental observations, we first considered a kinetic model of the $E_2 \rightarrow E_1$ transition with and without bound ATP in which the conformational transitions are all treated as irreversible (Figure 5). The major points of the model are that in the absence of ATP the conformational change is assumed to occur in two steps, whereas when ATP is bound to the enzyme, the conformational change is a single step reaction (or the second step has become so fast that it can be considered as instantaneous on the time scale of the first). Each step is assumed to produce a change in the fluorescence of the probe.

Kinetic simulations based on this irreversible model were carried out over a range of ATP concentrations as described under Materials and Methods. The ATP dissociation constant for the E_2 state of the enzyme was chosen to have a value of 71 μM , consistent with literature data (7). Both the predicted time course of the fluorescence change and its total amplitude as a function of ATP concentration were calculated. The results of these simulations are shown in Figures 1B and 3. The simulations reproduced some of the experimental observations, that is, the overall kinetics became faster with increasing ATP concentrations, and the biexpo-

ponential kinetic behavior observed at low ATP concentrations disappeared as the ATP concentration increased. There was, however, a significant difference between the simulations and the experimental traces in that experimental changes in the time course of the fluorescence change occurred at a lower ATP concentration than that predicted by the simulations. Experimentally, it was seen that the addition of 3 μM ATP after mixing caused a significant acceleration of the kinetics (Figure 1A), whereas in the case of the simulations, 3 μM ATP is predicted not to be sufficient to cause any major effect (Figure 1B). In fact, experimentally, the observed reciprocal relaxation times almost doubled even after the addition of only 0.5 μM ATP after mixing (not shown). At 0.5 μM ATP, the values of the observed reciprocal relaxation times were $17 (\pm 2) \text{ s}^{-1}$ (49% of the total amplitude) and $1.18 (\pm 0.07) \text{ s}^{-1}$ (51%), compared to the following values at zero ATP: $10.4 (\pm 1.0) \text{ s}^{-1}$ (34%) and $0.49 (\pm 0.02) \text{ s}^{-1}$ (66%).

In order to reproduce the acceleration experimentally observed in the submicromolar ATP range using this model, one would have to choose an unrealistically low ATP dissociation constant for the E_2 state, that is, also in the submicromolar range. However, if one does this, then further acceleration of kinetics into the hundreds of micromolar range is no longer observed, that is, the kinetics saturate too early.

This simple irreversible model also does not predict the ATP concentration dependence of the amplitudes (Figure 3). If the conformational transitions were all irreversible, then mixing with NaCl would have to produce total conversion of the E_2 state into E_1 . The magnitude of the fluorescence change would then always have a constant value, regardless of the ATP concentration.

Because of the discrepancies between the experimental traces and theoretical simulations, we decided to extend our kinetic model to include the possibility of reversibility for all of the conformational transitions (Figure 5). The set of differential rate equations based on this reversible model that are required to carry out simulations are given in the Appendix section. The values of all of the rate and equilibrium constants used in the simulations are given in Table 1. The simulated kinetic curves and their corresponding amplitudes are shown in Figures 1B and 3, respectively.

Comparing the results of the simulations with the experimental results (Figure 1A), it can be seen that the significant acceleration observed at 3 μM ATP is now well reproduced. The other experimental observations of continued acceleration of the $E_2 \rightarrow E_1$ transition up to 1500 μM ATP and the disappearance of biexponential kinetics on increasing ATP concentrations, which were reproduced by the simpler irreversible model, are still observed in the more complex model. Furthermore, the reversible model predicts the concentration dependence of the amplitudes (Figure 3). Therefore, this model is a more appropriate description of the experimental behavior.

DISCUSSION

The stopped-flow kinetic measurements and theoretical simulations we have presented support the conclusion that in the absence of bound ATP the $E_2 \rightarrow E_1$ conformational change occurs as a two step reversible process with an

Table 1: Values of the Rate Constants, Equilibrium Constants, and Fluorescence Parameters of the Reversible Conformational Change Model Used for the Simulations Shown in Figure 1B

parameter	reaction ^a	value	reference
k_1	$E_2 \rightarrow E^*$	6.94 s^{-1}	this work ^b
k_{-1}	$E^* \rightarrow E_2$	3.47 s^{-1}	this work ^b
k_2	$E^* \rightarrow E_1$	0.329 s^{-1}	this work ^c
k_{-2}	$E_1 \rightarrow E^*$	0.165 s^{-1}	this work ^c
k_3	$E_2\text{ATP} \rightarrow E_1\text{ATP}$	34 s^{-1}	this work ^d
k_{-3}	$E_1\text{ATP} \rightarrow E_2\text{ATP}$	1 s^{-1}	this work ^d
k_4	$E_2 + \text{ATP} \rightarrow E_2\text{ATP}$	$52 \mu\text{M}^{-1} \text{ s}^{-1}$	this work ^e
k_{-4}	$E_2\text{ATP} \rightarrow E_2 + \text{ATP}$	3692 s^{-1}	this work ^f
k_5	$E_1 + \text{ATP} \rightarrow E_1\text{ATP}$	$52 \mu\text{M}^{-1} \text{ s}^{-1}$	this work ^e
k_{-5}	$E_1\text{ATP} \rightarrow E_1 + \text{ATP}$	13 s^{-1}	49
K_A	$E_2\text{ATP} \leftrightarrow E_2 + \text{ATP}$	$71 \mu\text{M}$	7
K_A'	$E_1\text{ATP} \leftrightarrow E_1 + \text{ATP}$	$0.25 \mu\text{M}$	14, 46
f_{E_2}	fluorescence level of E_2 and $E_2\text{ATP}$	1.0	this work ^g
f_{E^*}	fluorescence level of E^*	0.9433	this work ^h
f_{E_1}	fluorescence level of E_1 and $E_1\text{ATP}$	0.83	this work ^h

^a For each reaction, Na^+ ions have been omitted for simplicity, but in each case the rate constant refers to the value in the presence of 65 mM NaCl. Under such conditions, all of the E_1 states, with or without ATP, would be completely saturated with Na^+ ions and would, thus, all have three bound Na^+ ions. ^b This value has been chosen so as to closely reproduce the observed experimental ATP dependence of the time course and the amplitudes of the fluorescence transients and to satisfy the condition $(k_1 + k_{-1}) = 10.41 \text{ s}^{-1}$, the experimentally determined reciprocal relaxation time of the fast phase observed in the absence of ATP. ^c This value has been chosen so as to closely reproduce the observed experimental ATP dependence of the time course and the amplitudes of the fluorescence transients and to satisfy the condition $(k_2 + k_{-2}) = 0.49 \text{ s}^{-1}$, the experimentally determined reciprocal relaxation time of the slow phase observed in the absence of ATP. ^d This value has been chosen so as to closely reproduce the observed experimental ATP dependence of the time course and the amplitudes of the fluorescence transients and to satisfy the condition $(k_3 + k_{-3}) = 35 \text{ s}^{-1}$, the experimentally determined reciprocal relaxation time of the fluorescence transient observed in the presence of 1500 μM ATP after mixing. ^e The rate constants for ATP binding to the E_2 state have been taken to be equal to that of binding to the E_1 state. The differences in their ATP affinities are assumed to be due to different dissociation rate constants alone. This value has been estimated by dividing the dissociation rate constant by the ATP dissociation constant, i.e., $k_5 = k_{-5}/K_A'$. ^f This value has been estimated by multiplying the ATP dissociation rate constant for the E_1 state by the ratio of the ATP dissociation constants of the E_2 and E_1 states, i.e., $k_{-4} = k_{-5}(K_A/K_A')$. ^g f_{E_2} has been arbitrarily been defined as 1.0 as a reference point for the fluorescence changes. ^h These values have been estimated on the basis of the relative amplitudes of the two phases in the absence of ATP (i.e., 34% fast phase and 66% slow phase) and by approximating the fluorescence change of saturating conversion into the E_1 state by the amplitude $(\Delta F/F_0)$ measured at high levels of ATP of approximately -0.17.

intermediate enzyme state, here temporarily designated E^* , whereas when ATP is bound, the conformational change occurs in a single step. This was evidenced by a disappearance of biexponential kinetics on increasing the ATP concentration. In fact there are two reasons why the observed stopped-flow transients become single exponential at higher ATP concentrations. First, as just stated, the $E_2\text{ATP} \rightarrow E_1\text{ATP}$ transition occurs as a single step. But second, if ATP binding to E_2 is very rapid, then any enzyme initially in the E_2 conformation could follow the pathway $E_2 + \text{ATP} \rightarrow E_2\text{ATP} \rightarrow E_1\text{ATP}$ rather than the pathway $E_2 \rightarrow E^* \rightarrow E_1 + \text{ATP} \rightarrow E_1\text{ATP}$. Because it has been found that binding of ATP causes no fluorescence change, the pathway $E_2 + \text{ATP} \rightarrow E_2\text{ATP} \rightarrow E_1\text{ATP}$ would be expected also to produce a monoexponential decrease in fluorescence. At extremely low ATP concentrations, the ATP binding steps in this sequence

might be expected to produce an initial lag phase in the kinetics, but as long as the ATP concentration is sufficiently high that ATP binding is in rapid equilibrium on the time-scale of the subsequent conformational change, the lag would be negligible and not observable.

A further important point is that a comparison of the experimental data with simulations based on models incorporating either irreversible or reversible conformational transitions indicates that reversibility is an essential component of the mechanism. This is supported by measurements of the kinetics of phosphorylation by inorganic phosphate. Cornelius et al. (37) were able to show that in the absence of alkali cations the $E_2 \rightarrow E_1$ transition of shark Na^+, K^+ -ATPase at 20 °C proceeded with a rate constant of 0.95 s^{-1} and that the backward reaction $E_1 \rightarrow E_2$ proceeded with a rate constant of 0.18 s^{-1} . These rate constants are of the same order of magnitude as that found here from the slow phase of the fluorescence transients observed in the absence of ATP and the presence of 65 mM NaCl after mixing. It appears, therefore, that apart from a nonspecific acceleration of the $E_2 \rightarrow E_1$ transition by Na^+ (15), perhaps via ionic strength, Na^+ binding to the transport sites of the Na^+, K^+ -ATPase has no major influence on the intrinsic kinetics of the conformational transitions. It is clear, however, that in the presence of K^+ ions Na^+ does have a significant indirect effect by competing for the transport sites with K^+ , which greatly stimulates the reverse $E_1 \rightarrow E_2$ transition. For the reaction $E_1(\text{K}^+)_2 \rightarrow E_2(\text{K}^+)_2$, Bugnon et al. (31) determined a rate constant of 550 s^{-1} , that is, an acceleration of around 3 orders of magnitude over that in the absence of K^+ . If Na^+ ions are present, as they are in the studies presented here, then competition between Na^+ and K^+ for two of the three transport sites will significantly reduce the rate constant. An equation for estimating the reverse rate constant at any combination of Na^+ and K^+ concentrations has been derived by us (eq 12 of ref 13). On the basis of the NaCl and KCl concentrations used here, 65 mM and 0.5 mM after mixing, the use of the derived equation shows that the K^+ -induced stimulation of the reverse reaction would be expected to be completely abolished, and one would expect a reverse rate constant identical to that in the absence of both Na^+ and K^+ ions.

The reason that the reversible model allows for concentration dependence of the amplitudes as well as an acceleration of the fluorescence transient over such a wide ATP concentration range is the perturbation of the E_2/E_1 equilibrium by ATP. The magnitude of the total fluorescence change depends on how much E_2 is converted to E_1 . If at the end of the experiment the reaction has not gone to completion and enzyme in the E_2 state is still present, then the amplitude will be reduced. This model predicts that at submicromolar ATP concentrations, high affinity ATP binding to the E_1 state will stimulate the reverse reaction $E_1\text{ATP} \rightarrow E_2\text{ATP}$, thus causing an acceleration of the relaxation of the $E_2\text{ATP} \leftrightarrow E_1\text{ATP}$ equilibrium and an increase in the fraction of enzyme present in the E_2 state at the end of the relaxation. This hence yields a decrease in the fluorescence change at submicromolar ATP concentrations relative to that obtained in the absence of ATP. If the ATP concentration is increased above 1 μM , the model predicts that low affinity ATP binding to the E_2 state will then start to occur, stimulating the forward reaction $E_2\text{ATP} \rightarrow E_1\text{ATP}$, thus causing a further acceleration

of the relaxation of $E_2\text{ATP} \leftrightarrow E_1\text{ATP}$ and an increase in the conversion of enzyme into the E_1 state at the end of the relaxation. This hence yields an increase in the fluorescence change on proceeding to ATP concentrations above 1 μM , which overrides the decrease at lower concentrations.

The important new point here is that on the basis of these results the allosteric role of ATP is not restricted to a low affinity binding accelerating the $E_2\text{ATP} \rightarrow E_1\text{ATP}$ transition. The allosteric behavior of ATP is completely symmetrical. High affinity ATP binding to the E_1 state also modifies the kinetics of the reverse $E_1\text{ATP} \rightarrow E_2\text{ATP}$ reaction. This supports the idea that the low and high affinity ATP sites are both located at the same position on the enzyme, with the affinities simply changing because of the different conformations of the enzyme in the E_2 and E_1 states.

Biexponential kinetics similar to that observed here has also been reported using a different kinetic method. A number of researchers have observed biexponential kinetics in the release of ^{42}K or ^{86}Rb from the E_2 conformation of the enzyme (3, 8, 14). Forbush (38) concluded that in the presence of ATP both of the K^+ ions are released simultaneously from their binding sites. This was because he presumed that the $E_2 \rightarrow E_1$ transition is rate-limiting, and he implicitly assumed that the conformational change occurred in a single step. However, the same may not be true in the absence of ATP. Montes et al. (14) found that whether or not biexponential kinetics were observed depended on the concentration of ATP or eosin (which binds to the ATP site) present. Biexponential Rb^+ deocclusion disappeared on increasing the ATP or eosin concentration, that is, analogous to the findings reported here for the RH421 fluorescence transients. On the basis of the comparison of these results, it is tempting to conclude that in the absence of ATP the conformational transition between $E_2(\text{K}^+)_2$ and $E_1(\text{Na}^+)_3$ occurs in two steps, with each step resulting in the release of one K^+ ion, that is, $E_2(\text{K}^+)_2 \rightarrow E^*\text{K}^+ + \text{K}^+$ and $E^*\text{K}^+ \rightarrow E_1 + \text{K}^+$. On the basis of the results presented here as well as the previously published data, however, this is probably not correct.

In the absence of ATP, it was observed here that each step in the sequence $E_2 \rightarrow E^* \rightarrow E_1$ produced a fluorescence decrease but that the fluorescence change was due to Na^+ binding subsequent to the conformational change and not to the conformational change itself. Previously published careful equilibrium titrations have shown in fact that the fluorescence decrease is due to the binding of the third Na^+ ion to the E_1 conformation, that is, the formation of the $E_1(\text{Na}^+)_3$ state (39, 40). The binding of the first and second Na^+ ions do not produce any fluorescence change. On the basis of these results, the sequence of reactions $E_2(\text{K}^+)_2 \rightarrow E^*\text{K}^+ + \text{K}^+$ and $E^*\text{K}^+ \rightarrow E_1 + \text{K}^+$ becomes untenable because in order to observe a fluorescence change, three Na^+ ions must bind to the enzyme in each step of the sequence. In the absence of K^+ , this is also not possible. In either case, the binding of three Na^+ ions at each step requires six Na^+ ions to bind to the transport sites at the end of the two step sequence. For a monomeric enzyme, this would conflict with its well-established stoichiometry, which is three Na^+ transport sites per α subunit. If one assumes that the enzyme exists as a protein dimer, however, in which each α subunit can be in an E_1 or an E_2 conformation, then one can easily imagine a sequence of reactions in which three Na^+ ions could bind

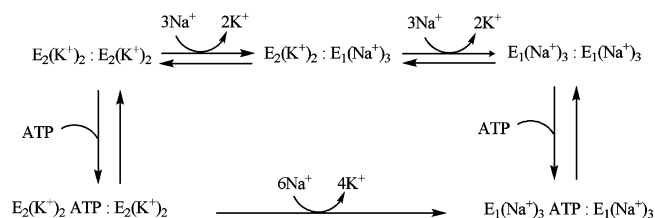


FIGURE 6: Dimer model of the $E_2 \rightarrow E_1$ conformational change of the Na^+ , K^+ -ATPase.

after each conformational change. Writing each α subunit separately, the reaction sequence could then be $E_2(\text{K}^+)_2:E_2(\text{K}^+)_2 \rightarrow E_2(\text{K}^+)_2:E_1 + 2\text{K}^+$ followed by $E_2(\text{K}^+)_2:E_1 \rightarrow E_1:E_1 + 2\text{K}^+$. Such a sequence makes an α subunit in the E_1 conformation available for the binding of three Na^+ ions at the end of each step and hence on the basis of the known voltage sensitivity of RH421 is consistent with a two phase fluorescence decrease as experimentally observed. On the basis of this scheme, the enzyme state up to now signified as E^* can be more precisely identified as a protein dimer in which one of the α subunits is in the E_2 conformation and one is in the E_1 conformation, that is, $E^* \equiv E_2:E_1$.

In the presence of saturating concentrations of ATP, only a single phase fluorescence decrease was observed. On the basis of the above argumentation, this, therefore, implies that both α subunits of the dimer must simultaneously undergo the conformational change. Including K^+ ions as above, the reaction can be written as $E_2\text{ATP}(\text{K}^+)_2:E_2(\text{K}^+)_2 \rightarrow E_1\text{ATP}:E_1 + 4\text{K}^+$. The function of ATP in this reaction appears to be not just to accelerate the conformational change of the unphosphorylated enzyme and to catalyze the release of K^+ ions, as was previously known (2–8), but also to synchronize the conformational change of both α subunits within a protein dimer.

The essential points of our new proposed mechanism of the $E_2 \rightarrow E_1$ conformational change of the Na^+ , K^+ -ATPase can now be summarized as follows:

- (1) The enzyme exists in the membrane as an $(\alpha\beta)_2$ diprotomer.
- (2) Each α subunit can independently exist in an E_1 or E_2 conformation.
- (3) In the absence of ATP, the two α subunits consecutively undergo the $E_2 \rightarrow E_1$ conformational change with different rate constants.
- (4) In the presence of ATP bound to one of the α subunits, the $E_2 \rightarrow E_1$ conformational change of each α subunit is accelerated, and the conformational changes of the two α subunits are synchronized, that is, they occur with the same rate constant.
- (5) On the time scale of the conformational change, both K^+ ions bound to any individual α subunit are always simultaneously released to the cytoplasm regardless of whether or not ATP is bound.

This entire mechanism is presented diagrammatically in Figure 6.

It is important to point out that the idea that Na^+ , K^+ -ATPase may exist as an $(\alpha\beta)_2$ diprotomer is not new. This was first proposed by Stein et al. (41) and Repke and Schon (42) in 1973 and has been a subject of debate ever since. The evidence for a diprotomer was nicely reviewed by Askari (43) in 1987. In the abstract of this paper, Askari expressed the opinion that “...any realistic approach to the resolution

of the kinetic mechanism of the sodium pump should include the consideration of the established site-site interactions of the oligomer."

Now we would like to speculate briefly on the mechanism by which the binding of ATP might cause a synchronization of the two α subunits within an $(\alpha\beta)_2$ diprotomer. The simplest way that this could occur would be if the two α subunits were completely independent of one another, that is, functionally uncoupled. Such an uncoupling could come about by an ATP-induced dissociation of the diprotomer into separate $\alpha\beta$ protomers. However, a complete spatial dissociation is not absolutely necessary. ATP binding could interfere with the interaction between two $\alpha\beta$ protomers via a minor conformational change or by the shielding of electrostatic interactions.

It is interesting to note that in the scheme shown in Figure 6 even after ATP binding one α subunit within an $(\alpha\beta)_2$ protomer is still free of ATP. Whether another ATP can bind and produce an $E_1ATP(Na^+)_3:E_1ATP(Na^+)_3$ dimer is still unclear and requires further investigation. This could potentially explain a puzzling observation regarding the amount of ATP required for the maximal rate of phosphorylation. It has been known for over 30 years that if one measures the ATP dissociation constant of enzyme in the E_1 conformation in isolation from the rest of the pump cycle, that is, under conditions where phosphorylation does not occur, then the value obtained is generally below 1 μM . In the absence of Mg^{2+} ions, the values reported include 0.12 μM (44), 0.22 μM (45), and 0.24 μM (46). However, if pre-steady-state kinetic measurements of enzyme phosphorylation via ATP are carried out, it is found that much higher concentrations of ATP are required to achieve a half-maximal rate of phosphorylation. ATP dissociation constants reported from such studies include 3.5 μM (9), 10 μM (10), 14 μM (11), 5 μM (12), 7 μM (6), and 8 μM (7). Averaging these values indicates that the ATP dissociation constant from phosphorylation studies is approximately 40 times greater than that measured in the absence of phosphorylation. It is unlikely that this difference arises from the presence or absence of Mg^{2+} ions. Campos and Beaugé (47) found that Mg^{2+} had no effect on the rate constant for ATP association and that it decreased the dissociation rate constant only by a factor of 4. However, if one accepts the dimer model described above, one could imagine a situation where the binding of one ATP molecule is sufficient for a maximum rate of the $E_2 \rightarrow E_1$ transition, but the binding of two ATP molecules is necessary for the maximum rate of phosphorylation. Then both sets of values can be accepted as correct. One simply has to take the view that each $(\alpha\beta)_2$ diprotomer binds the first ATP molecule with very high affinity (submicromolar) and the second with much lower affinity ($\sim 10 \mu M$). Cornelius (48) did in fact find two apparent ATP dissociation constants for the stimulation of the phosphorylation of shark rectal gland Na^+, K^+ -ATPase by ATP, one of 5.4 (± 0.3) μM and another of 32 (± 11) nM.

Although the results presented here support the presence of a protein dimer, it cannot be concluded that dimer formation is necessary for Na^+, K^+ -ATPase function. The enzyme preparation used in this study was from the outer medulla of the kidney, a tissue in which Na^+, K^+ -ATPase naturally occurs in very high density within the cell membrane. This could then automatically promote the

formation of protein dimers. It remains to be seen whether protein dimerization occurs in the cell membranes of tissues in which the enzyme is less tightly packed.

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APPENDIX

Simulation of Fluorescence Transients. On the basis of the reversible conformational change kinetic model (Figure 5), the differential rate equations describing the changes in concentration of all the enzyme intermediates are as follows:

$$\frac{d[E_2]}{dt} = -k_1[E_2] + k_{-1}[E^*] - k_4[E_2][ATP] + k_{-4}[E_2ATP] \quad (A1)$$

$$\frac{d[E_2ATP]}{dt} = -k_3[E_2ATP] + k_{-3}[E_1ATP] + k_4[E_2][ATP] - k_{-4}[E_2ATP] \quad (A2)$$

$$\frac{d[E^*]}{dt} = k_1[E_2] - k_{-1}[E^*] - k_2[E^*] + k_{-2}[E_1] \quad (A3)$$

$$\frac{d[E_1]}{dt} = k_2[E^*] - k_{-2}[E_1] - k_5[E_1][ATP] + k_{-5}[E_1ATP] \quad (A4)$$

$$\frac{d[E_1ATP]}{dt} = k_3[E_2ATP] - k_{-3}[E_1ATP] + k_5[E_1][ATP] - k_{-5}[E_1ATP] \quad (A5)$$

Prior to mixing with NaCl, the enzyme is assumed to be initially either in the E_2 or the E_2ATP state. If the binding and dissociation of ATP to and from the E_2 state are much faster than the conformational changes to the E^* and E_1 -ATP states, then an equilibrium between the E_2 and E_2ATP states will be established prior to any conformational change. Under these conditions, the initial distribution of enzyme between these two states is dependent on the ATP concentration and is governed by the ATP dissociation constant, K_A , for the E_2 state according to the following two expressions:

$$[E_2]_{t=0} = \frac{1}{1 + ([ATP]/K_A)} \quad (A6)$$

$$[E_2ATP]_{t=0} = \frac{([ATP]/K_A)}{1 + ([ATP]/K_A)} \quad (A7)$$

The initial concentrations of all of the other enzyme states, that is, E^* , E_1 and E_1ATP , are assumed to be zero.

The total fluorescence, F , is due to contributions from fluorescence levels, f , of the probe associated with each of the enzyme conformational states. Because the addition of ATP alone to enzyme in the E_2 state was found to cause no fluorescence change in the stopped-flow apparatus, we

assume that the fluorescence levels of an enzyme state is independent of whether or not it has ATP bound. The total fluorescence is then given by the following equation:

$$F = f_{E_2}([E_2] + [E_2\text{ATP}]) + f_{E^*}[E^*] + f_{E_1}([E_1] + [E_1\text{ATP}]) \quad (\text{A8})$$

Arbitrarily, we define the fluorescence level of the E₂ state to be 1.0, that is, $f_{E_2} = 1.0$. The total fluorescence change observed in the stopped-flow measurements on mixing with 130 mM NaCl was found to increase at high levels of ATP, from a value of approximately −12% in the absence of ATP to a value of approximately −17% at 1500 μM ATP (Figure 3). Therefore, we estimate f_{E_1} to be 0.83. In the absence of ATP, approximately 33% of the total fluorescence change was due to the fast phase and 66% to the slow phase. Therefore, the fluorescence level of the E* state should be approximately one-third of the way between the values of f_{E_2} and f_{E_1} . Therefore, we assume a value of $f_{E^*} = 0.9433$.

The values of all of the rate and equilibrium constants used in the simulations are given in Table 1. The values of ($k_1 + k_{-1}$) and ($k_2 + k_{-2}$) were taken directly from the reciprocal relaxation times derived from exponential fitting of the fast and slow phases, respectively, of the experimental stopped-flow trace measured here for conditions of zero ATP (Figure 1A). The value of ($k_3 + k_{-3}$) was derived from the reciprocal relaxation time observed for the experimental stopped-flow transient measured here at 1500 μM (Figure 1A). The values of k_4 , k_{-4} , k_5 , k_{-5} , K_A , and K_A' were taken from previous literature measurements.

REFERENCES

- Skou, J. C. (1957) The influence of some cations on an adenosine triphosphatase from peripheral nerves, *Biochim. Biophys. Acta* 23, 394–401.
- Karlish, S. J. D., and Yates, D. W. (1978) Tryptophan fluorescence of (Na⁺ + K⁺)-ATPase as a tool for study of the enzyme mechanism, *Biochim. Biophys. Acta* 527, 115–130.
- Forbush, B., III. (1987) Rapid release of ⁴²K and ⁸⁶Rb from an occluded state of the Na,K-pump in the presence of ATP or ADP, *J. Biol. Chem.* 262 (1987) 11104–11115.
- Steinberg, M., and Karlish, S. J. D. (1989) Studies on conformational changes in Na,K-ATPase labeled with 5-iodoacetamidofluorescein, *J. Biol. Chem.* 264, 2726–2734.
- Pratap, P. R., Palit, A., Grassi-Nemeth, E., and Robinson, J. D. (1996) Kinetics of conformational changes associated with potassium binding to and release from Na⁺/K⁺-ATPase, *Biochim. Biophys. Acta* 1285, 203–211.
- Kane, D. J., Fendler, K., Grell, E., Bamberg, E., Taniguchi, K., Froehlich, J. P., and Clarke, R. J. (1997) Stopped-flow kinetic investigations of conformational changes of pig kidney Na⁺,K⁺-ATPase, *Biochemistry* 36, 13406–13420.
- Clarke, R. J., Kane, D. J., Apell, H.-J., Roudna, M., and Bamberg, E. (1998) Kinetics of Na⁺-dependent conformational changes of rabbit kidney Na⁺,K⁺-ATPase, *Biophys. J.* 75, 1340–1353.
- González-Lebrero, R. M., Kaufman, S. B., Montes, M. R., Nørby, J. G., Garrahan, P. J., and Rossi, R. C. (2002) The occlusion of Rb⁺ in the Na⁺/K⁺-ATPase, *J. Biol. Chem.* 277, 5910–5921.
- Froehlich, J. P., Hobbs, A. S., and Albers, R. W. (1983) Evidence for parallel pathways of phosphoenzyme formation in the mechanism of ATP hydrolysis by electrophorus Na,K-ATPase, *Curr. Top. Membr. Transp.* 19, 513–535.
- Borlinghaus, R., and Apell, H.-J. (1988) Current transients generated by the Na⁺/K⁺-ATPase after an ATP concentration jump: dependence on sodium and ATP concentration, *Biochim. Biophys. Acta* 939 197–206.
- Fendler, K., Jaruschewski, S., Hobbs, A., Albers, W., and Froehlich, J. P. (1993) Pre-steady state charge translocation in NaK-ATPase from eel electric organ, *J. Gen. Physiol.* 102, 631–666.
- Friedrich, T., Bamberg, E., and Nagel, G. (1996) Na⁺,K⁺-ATPase pump currents in giant excised patches activated by an ATP concentration jump, *Biophys. J.* 71, 2486–2500.
- Kong, B. Y., and Clarke, R. J. (2004) Identification of potential regulatory sites of the Na⁺,K⁺-ATPase by kinetic analysis, *Biochemistry* 43, 2241–2250.
- Montes, M. R., González-Lebrero, R. M., Garrahan, P. J., and Rossi, R. C. (2004) Quantitative analysis of the interaction between the fluorescent probe eosin and the Na⁺,K⁺-ATPase studied through Rb⁺ occlusion, *Biochemistry* 43, 2062–2069.
- Humphrey, P. A., Lüpfer, C., Apell, H.-J., Cornelius, F., and Clarke, R. J. (2002) Mechanism of the rate-determining step of the Na⁺,K⁺-ATPase, *Biochemistry* 41, 9496–9507.
- Jørgensen, P. L. (1974) Isolation of (Na⁺ + K⁺)-ATPase, *Methods Enzymol.* 32, 277–290.
- Apell, H.-J., Marcus, M. M., Anner, B. M., Oetliker, H., and Läuger, P. (1985) Optical study of active ion transport in lipid vesicles containing reconstituted Na,K-ATPase, *J. Membr. Biol.* 85, 49–63.
- Schwartz, A., Nagano, K., Nakao, M., Lindenmayer, G. E., Allen, J. C., and Matsui, H. (1971) The sodium- and potassium-activated adenosinetriphosphatase, *Methods Pharmacol.* 1, 361–388.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent, *J. Biol. Chem.* 193, 265–275.
- Jørgensen, P. L., and Andersen, J. P. (1988) Structural basis for E₁-E₂ conformational transitions in Na,K-pump, *J. Membr. Biol.* 103, 95–120.
- Karlish, S. J. D. (1980) Characterization of conformational changes in (Na,K)ATPase labelled with fluorescein at the active site, *J. Bioenerg. Biomembr.* 12, 111–136.
- Skou, J. C., and Esmann, M. (1983) The effects of Na⁺ and K⁺ on the conformational transitions of (Na⁺ + K⁺)-ATPase, *Biochim. Biophys. Acta* 746, 101–113.
- Schuermans Stekhoven, F. M. A. H., Swarts, H. G. P., de Pont, J. J. H. H. M., and Bonting, S. L. (1985) Na⁺-like effect of imidazole on the phosphorylation of (Na⁺ + K⁺)-ATPase, *Biochim. Biophys. Acta* 815, 16–24.
- Schuermans, Stekhoven, F. M. A. H., Swarts, H. G. P., 't Lam, G. K., Zou, Y. S., and de Pont, J. J. H. H. M. (1988) Phosphorylation of (Na⁺ + K⁺)-ATPase; stimulation and inhibition by substituted and unsubstituted amines, *Biochim. Biophys. Acta* 937, 161–176.
- Mezele, M., Lewitzki, E., Ruf, H., and Grell, E. (1988) Cation selectivity of membrane proteins, *Ber. Bunsen-Ges.* 92, 998–1004.
- Grell, E., Warmuth, R., Lewitzki, E., and Ruf, H. (1991) Precision Titrations to Determine Affinity and Stoichiometry of Alkali, Alkaline Earth, and Buffer Cation Binding to Na,K-ATPase, in *The Sodium Pump: Recent Developments* (Kaplan, J. H., and De Weer, P., Eds.) pp 441–445, Rockefeller University Press, New York.
- Grell, E., Lewitzki, E., Ruf, H., and Doludda, M. (1994) Reassignment of Cation-Induced Population of Main Conformational States of FITC-Na⁺/K⁺-ATPase as Detected by Fluorescence Spectroscopy and Characterized by Equilibrium Binding Studies, in *The Sodium Pump: Structure Mechanism, Hormonal Control and Its Role in Disease* (Bamberg, E., and Schoner, W., Eds.) pp 617–620, Steinkopff Verlag, Darmstadt, Germany.
- Doludda, M., Lewitzki, E., Ruf, H., and Grell, E. (1994) Kinetics and Mechanism of Cation-Binding to Na⁺,K⁺-ATPase, in *The Sodium Pump: Structure Mechanism, Hormonal Control and Its Role in Disease* (Bamberg, E., and Schoner, W., Eds.) pp 629–632, Steinkopff Verlag, Darmstadt, Germany.
- Lüpfer, C., Grell, E., Pintschovius, V., Apell, H.-J., Cornelius, F., and Clarke, R. J. (2001) Rate limitation of the Na⁺,K⁺-ATPase pump cycle, *Biophys. J.* 81, 2069–2081.
- Smirnova, I. N., and Faller, L. D. (1993) Mechanism of K⁺ interaction with fluorescein 5'-isothiocyanate-modified Na⁺,K⁺-ATPase, *J. Biol. Chem.* 268, 16120–16123.
- Bugnon, P., Doludda, M., Grell, E., and Merbach, A. E. (1997) High-Pressure Stopped-Flow Spectrometer for Kinetic Studies of Fast Bioinorganic Reactions by Absorbance and Fluorescence Detection, in *High Pressure Research in the Biosciences and*

- Biotechnology* (Heremans, K., Ed.) pp 143–146, Leuven University Press, Leuven, Belgium.
32. Barlet-Bas, C., Arystarkhova, E., Cheval, L., Marsy, S., Sweadner, K., Modyanov, N., and Doucet, A. (1993) Are there several isoforms of Na,K-ATPase α subunit in the rabbit kidney? *J. Biol. Chem.* 268, 11512–11515.
 33. Lücking, K., Nielsen, J. M., Pedersen, P. A., and Jørgensen, P. L. (1996) Na-K-ATPase isoform (α_3 , α_2 , α_1) abundance in rat kidney estimated by competitive RT-PCR and ouabain binding, *Am. J. Physiol.* 271, F253–F260.
 34. Garty, H., and Karlish, S. J. D. (2006) Role of FXYD proteins in ion transport, *Annu. Rev. Physiol.* 68, 431–59.
 35. Therien, A. G., Goldshleger, R., Karlish, S. J. D., and Blostein, R. (1997) Tissue-specific distribution and modulatory role of the γ subunit of the Na,K-ATPase, *J. Biol. Chem.* 272, 32628–32634.
 36. Pu, H. X., Cluzeaud, F., Goldshleger, R., Karlish, S. J. D., Farman, N., and Blostein, R. (2001) Functional role and immunocytochemical localization of the γ_a and γ_b forms of the Na,K-ATPase γ subunit, *J. Biol. Chem.* 276, 20370–20378.
 37. Cornelius, F., Fedosova, N. U., and Klodos, I. (1998) E₂P phosphoforms of Na,K-ATPase. II. Interaction of substrate and cation-binding sites in Pi phosphorylation of Na,K-ATPase, *Biochemistry* 37, 16686–16696.
 38. Forbush, B., III. (1987) Rapid release of ⁴²K or ⁸⁶Rb from two distinct transport sites on the Na,K-pump in the presence of P_i or vanadate, *J. Biol. Chem.* 262, 11116–11127.
 39. Schneeberger, A., and Apell, H.-J. (2001) Ion selectivity of the cytoplasmic binding sites of the Na,K-ATPase: II. Competition of various cations, *J. Membr. Biol.* 179, 263–273.
 40. Apell, H.-J., and Diller, A. (2002) Do H⁺ ions obscure electrogenic Na⁺ and K⁺ binding in the E₁ state of the Na,K-ATPase? *FEBS Lett.* 532, 198–202.
 41. Stein, W. D., Lieb, W. R., Karlish, S. J. D., and Eilam, Y. (1973) A model for active transport of sodium and potassium ions as mediated by a tetrameric enzyme, *Proc. Natl. Acad. Sci. U.S.A.* 70, 275–278.
 42. Repke, K. R. H., and Schön, R. (1973) Flip-flop model of (NaK)-ATPase function, *Acta Biol. Med. Ger.* 31, K19–K30.
 43. Askari, A. (1987) (Na⁺ + K⁺)-ATPase: on the number of the ATP sites of the functional unit, *J. Bioenerg. Biomembr.* 19, 359–374.
 44. Nørby, J. G., and Jensen, J. (1971) Binding of ATP to brain microsomal ATPase. Determination of the ATP-binding capacity and the dissociation constant of the enzyme-ATP complex as a function of K⁺ concentration, *Biochim. Biophys. Acta* 233, 104–116.
 45. Hegyvary, C., and Post, R. L. (1971) Binding of adenosine triphosphate to sodium and potassium ion-stimulated adenosine triphosphatase, *J. Biol. Chem.* 246, 5234–5240.
 46. Fedosova, N. U., Champeil, P., and Esmann, M. (2003) Rapid filtration analysis of nucleotide binding to Na,K-ATPase, *Biochemistry* 42, 3536–3543.
 47. Campos, M., and Beaugé, L. (1992) Effects of magnesium and ATP on pre-steady-state phosphorylation kinetics of the Na⁺,K⁺-ATPase, *Biochim. Biophys. Acta* 1105, 51–60.
 48. Cornelius, F. (1999) Rate determination in phosphorylation of shark rectal Na,K-ATPase by ATP: Temperature sensitivity and effects of ADP, *Biophys. J.* 77, 934–942.
 49. Fedosova, N. U., Champeil, P., and Esmann, M. (2002) Nucleotide binding to Na,K-ATPase: The role of electrostatic interactions, *Biochemistry* 41, 1267–1273.

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