

Mechanism of Mg^{2+} Binding in the Na^+, K^+ -ATPase

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ABSTRACT The Mg^{2+} dependence of the kinetics of the phosphorylation and conformational changes of Na^+, K^+ -ATPase was investigated via the stopped-flow technique using the fluorescent label RH421. The enzyme was preequilibrated in buffer containing 130 mM NaCl to stabilize the $\text{E1}(\text{Na}^+)_3$ state. On mixing with ATP, a fluorescence increase was observed. Two exponential functions were necessary to fit the data. Both phases displayed an increase in their observed rate constants with increasing Mg^{2+} to saturating values of $195 (\pm 6) \text{ s}^{-1}$ and $54 (\pm 8) \text{ s}^{-1}$ for the fast and slow phases, respectively. The fast phase was attributed to enzyme conversion into the E2MgP state. The slow phase was attributed to relaxation of the dephosphorylation/rephosphorylation (by ATP) equilibrium and the buildup of some enzyme in the E2Mg state. Taking into account competition from free ATP, the dissociation constant (K_d) of Mg^{2+} interaction with the $\text{E1ATP}(\text{Na}^+)_3$ state was estimated as $0.069 (\pm 0.010) \text{ mM}$. This is virtually identical to the estimated value of the K_d of Mg^{2+} -ATP interaction in solution. Within the enzyme-ATP- Mg^{2+} complex, the actual K_d for Mg^{2+} binding can be attributed primarily to complexation by ATP itself, with no apparent contribution from coordination by residues of the enzyme environment in the E1 conformation.

INTRODUCTION

An important role of Mg^{2+} in biology is as a cofactor of ATP. The Mg^{2+} ion is complexed by the negatively charged oxygens of its phosphate groups. The Mg^{2+} is thus thought to help shield the negative charges of the phosphates, allowing reaction with the electron pairs of attacking groups and facilitating phosphoryl transfer (1). One of the most important enzymes in which this is the case is the Na^+, K^+ -ATPase, which is responsible for maintaining electrochemical potential gradients for Na^+ and K^+ across the plasma membrane.

To our knowledge, no crystal structure of the Na^+, K^+ -ATPase in the E1 state with bound Mg^{2+} and ATP has yet been reported. Nevertheless, based on a published crystal structure of the related enzyme sarcoplasmic reticulum Ca^{2+} -ATPase (2), and using computer modeling, Patchornik et al. (3) suggested that, like the phosphates of ATP, the aspartate residues D710, D443, and D714 contribute to Mg^{2+} coordination. Although this may be correct, it is difficult from crystal structural data to make conclusions about the relative strengths of interactions. The aim of this article is to provide reliable experimental data on the strength of binding of Mg^{2+} ions to the Na^+, K^+ -ATPase.

A difficulty in studying Mg^{2+} interaction with the Na^+, K^+ -ATPase under physiological conditions, i.e., in the presence of ATP and Na^+ ions, is that it immediately induces phosphorylation, so that Mg^{2+} binding cannot be separated from the phosphorylation reaction. This precludes equilibrium binding studies. Here we have, therefore, applied a pre-steady-state kinetic technique (stopped-flow

spectrofluorimetry) utilizing the voltage-sensitive fluorescent probe RH421. Together with data recently obtained under the same experimental conditions for Mg^{2+} binding to ATP (4), this allows us to analyze the question of the relative contributions of the enzyme and ATP in binding Mg^{2+} in the enzyme-ATP- Mg^{2+} complex. In the case of the E1 conformation of the enzyme, it will be shown here that, although the enzyme environment is definitely important for the catalysis of phosphoryl transfer from ATP, coordination by ATP dominates the dissociation constant (K_d) for Mg^{2+} binding.

MATERIALS AND METHODS

Enzyme and reagents

Na^+, K^+ -ATPase-containing membrane fragments from shark rectal glands were purified essentially as described by Skou and Esmann (5). The specific ATPase activity at 37°C and pH 7.4 was measured according to the methods of Ottolenghi (6). The activity of the preparation used was $1679 \mu\text{mol ATP hydrolyzed h}^{-1} (\text{mg of protein})^{-1}$ and the protein concentration was 4.82 mg mL^{-1} . The protein concentration was determined according to the Peterson modification (7) of the Lowry method (8) using bovine serum albumin as a standard. For the calculation of the molar protein concentration, a molecular mass for an $\alpha\beta$ unit of the Na^+, K^+ -ATPase of $147,000 \text{ g mol}^{-1}$ (9) was used. The origins of the various reagents used were imidazole ($\geq 99\%$, Sigma, Castle Hill, Australia), NaCl (suprapure, Merck, Kilsyth, Australia), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (analytical grade, Merck), EDTA (99% , Sigma), ATP disodium $3\text{H}_2\text{O}$ ($\geq 99\%$, Sigma) and HCl (0.1 N Titrisol solution, Merck).

Stopped-flow spectrofluorimetry

Stopped-flow experiments were carried out using an SF-61 stopped-flow spectrofluorimeter from Hi-Tech Scientific (Salisbury, United Kingdom) as described previously (10). To improve the signal/noise ratio, typically 20–30 experimental traces were averaged before the k_{obs} values were

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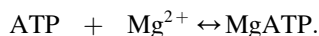
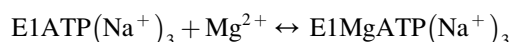
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evaluated. This was done by fitting a sum of two exponential functions to the averaged experimental trace. The kinetics of the Na^+, K^+ -ATPase conformational changes and ion translocation reactions were investigated at 24°C in the stopped-flow apparatus by mixing Na^+, K^+ -ATPase (labeled with RH421) with an equal volume of a 2-mM ATP solution. Both solutions were prepared in a buffer containing 30 mM imidazole, 130 mM NaCl, and varying concentrations of MgCl_2 . No change in the Na^+ or Mg^{2+} concentration occurred on mixing.

Data fitting

Nonlinear least-squares fitting of equations describing the Mg^{2+} dependence of the fluorescence amplitudes to the data was performed using Origin 6.0 (Microcal Software, Northampton, MA). Fitting of equations describing the Mg^{2+} dependence of the k_{obs} values to the data were performed using Mathematica (version 6.0.2, Wolfram Research, Champaign, IL) via a globally convergent variation of the Newton-Raphson methods to find the roots of Eqs. 1 and 2.

Immediately after mixing with ATP, but before any phosphorylation, the following equilibria would be expected to exist in solution:



K_E and K_{ATP} are the association constants for each equilibrium. We assume that because the enzyme is saturated by ATP and Na^+ there is no enzyme in the $\text{E1}(\text{Na}^+)_3$, E1ATP , or E1 states, and therefore, binding of Mg^{2+} to these states has been ignored. We also assume that under conditions of excess ATP and Mg^{2+} over enzyme, the concentrations of $\text{E1MgATP}(\text{Na}^+)_3$ and $\text{E1ATP}(\text{Na}^+)_3$ can be neglected when calculating the total ATP concentration and that the concentration of $\text{E1MgATP}(\text{Na}^+)_3$ can also be neglected when calculating the total Mg^{2+} concentration. Under these conditions, taking into account mass balance for ATP, the free ATP concentration is related to the total Mg^{2+} concentration ($[\text{Mg}^{2+}]_{\text{tot}}$) and the total ATP concentration ($[\text{ATP}]_{\text{tot}}$) by

$$[\text{ATP}] + \frac{K_{\text{ATP}}[\text{ATP}][\text{Mg}^{2+}]}{1 + K_{\text{ATP}}[\text{ATP}]} - [\text{ATP}]_{\text{tot}} = 0. \quad (1)$$

Solving for the roots of Eq. 1 allows $[\text{ATP}]$ to be calculated for any values of $[\text{Mg}^{2+}]_{\text{tot}}$ and $[\text{ATP}]_{\text{tot}}$. Considering mass balance for Mg^{2+} , the free Mg^{2+} concentration is related to the $[\text{Mg}^{2+}]_{\text{tot}}$ and the total enzyme concentration ($[\text{E}]_{\text{tot}}$) by

$$[\text{Mg}^{2+}] + K_{\text{ATP}}[\text{ATP}][\text{Mg}^{2+}] + \frac{K_E[\text{E}]_{\text{tot}}[\text{Mg}^{2+}]}{1 + K_E[\text{Mg}^{2+}]} - [\text{Mg}^{2+}]_{\text{tot}} = 0. \quad (2)$$

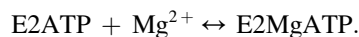
Once Eq. 1 has been solved for $[\text{ATP}]$, this can be used to solve for the roots of Eq. 2 to find $[\text{Mg}^{2+}]$ for any values of $[\text{Mg}^{2+}]_{\text{tot}}$ and $[\text{E}]_{\text{tot}}$. Taking into account mass balance for the enzyme, from the expression for the association constant of Mg^{2+} with the enzyme, the concentration of $\text{E1MgATP}(\text{Na}^+)_3$, the species which must be produced to allow phosphorylation to occur, is

$$[\text{E1MgATP}(\text{Na}^+)_3] = \frac{K_E[\text{E}]_{\text{tot}}[\text{Mg}^{2+}]}{1 + K_E[\text{Mg}^{2+}]} \quad (3)$$

The degree of saturation (S_{E1}) of the Mg^{2+} sites on the E1 conformation of the enzyme is given by

$$S_{\text{E1}} = \frac{[\text{E1MgATP}(\text{Na}^+)_3]}{[\text{E}]_{\text{tot}}} \quad (4)$$

After the enzyme has been mixed with ATP and cycling has begun, Mg^{2+} could also bind to the E2 conformation of the enzyme according to the equilibrium



K_F represents the association constant for this equilibrium. Taking an approach similar to that taken for the E1 conformation, one can find the free Mg^{2+} concentration when the enzyme is in the E2 conformation by solving an expression analogous to Eq. 2, i.e.,

$$[\text{Mg}^{2+}] + K_{\text{ATP}}[\text{ATP}][\text{Mg}^{2+}] + \frac{K_F[\text{E}]_{\text{tot}}[\text{Mg}^{2+}]}{1 + K_F[\text{Mg}^{2+}]} - [\text{Mg}^{2+}]_{\text{tot}} = 0. \quad (5)$$

Equation 1 can still be used to calculate the free ATP concentration. The degree of saturation (S_{E2}) of the Mg^{2+} sites on the enzyme is given by

$$S_{\text{E2}} = \frac{K_F[\text{Mg}^{2+}]}{1 + K_F[\text{Mg}^{2+}]} \quad (6)$$

RESULTS

Rates of ATP-induced stopped-flow fluorescence traces

On mixing Na^+, K^+ -ATPase-containing membrane fragments labeled with RH421 with Na_2ATP (as described under [Materials and Methods](#)), an increase in fluorescence occurred (see [Fig. 1](#)). Two exponential time functions were required to fit the data. The faster phase constituted on average 80% of the overall amplitude and the slower phase 20%. As the MgCl_2 concentration of the enzyme suspension and the ATP solution was increased, the overall kinetics of the fluorescence change became faster until a saturating limit was reached with an observed rate constant, k_{obs} , of ~ 190 – 200 s^{-1} for the faster phase (see [Fig. 2](#)) and ~ 45 – 50 s^{-1} for the slower phase (see [Fig. 3](#)). These data agree qualitatively with previous measurements using the same source of enzyme (11). The k_{obs} values measured agree quantitatively with similar measurements on enzyme from rabbit and pig kidney (10,12). The fact that the kinetics of both phases saturate at constant values implies that the overall rate-determining step for each process is a first-order reaction (i.e., phosphorylation or an enzyme conformational change). The slower kinetics observed under nonsaturating Mg^{2+} concentrations implies that both first-order reactions are preceded by Mg^{2+} binding.

A control experiment in which 5 mM EDTA and no MgCl_2 were included in the buffer resulted in almost complete disappearance of the fluorescence change (see [Fig. 1](#)). In previous studies (10), it was shown that if the Na^+, K^+ -ATPase membrane fragments were preequilibrated with 30 mM vanadate, subsequent mixing with ATP caused no fluorescence change. These control experiments indicate that the observed fluorescence changes are due to the hydrolytic action of the Na^+, K^+ -ATPase.

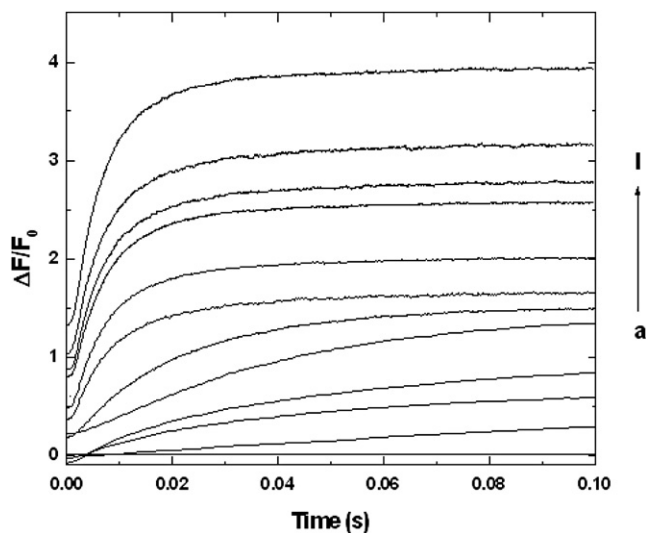


FIGURE 1 Stopped-flow fluorescence transients of Na⁺,K⁺-ATPase non-covalently labeled with RH421 (100 nM after mixing). Na⁺,K⁺-ATPase (270 nM after mixing) was rapidly mixed with an equal volume of Na₂ATP solution (1 mM after mixing). Each solution was in buffer containing 130 mM NaCl, 30 mM imidazole, and either 5 mM EDTA or varying concentrations of MgCl₂ (pH 7.4, 24°C). The traces have been labeled in order of increasing fluorescence intensity change or, equivalently, increasing final fluorescence level, i.e., curve *a* has the lowest and curve *l* the highest final fluorescence intensity. Curve *a* is a control experiment in the presence of 5 mM EDTA and no added MgCl₂. Curves *b*–*l* correspond to the MgCl₂ concentrations 0.0025 mM, 0.01 mM, 0.15 mM, 0.2 mM, 0.4 mM, 1.0 mM, 2.0 mM, 2.5 mM, 3.5 mM, 4.5 mM, and 5.0 mM, respectively. The excitation and emission wavelengths were 577 nm and ≥665 nm (RG665 glass cutoff filter), respectively.

Based on previous studies (11,13,14), it is known that RH421 responds to the formation of the E2P state with an increase in fluorescence. The fast phase of the fluorescence transients observed can therefore confidently be attributed to the reaction $E1MgATP(Na^+)_3 \rightarrow E2MgP + 3Na^+ + ADP$. The maximum k_{obs} for the fast phase (195 s⁻¹) agrees with k_{obs} values determined for enzyme phosphorylation using the quenched-flow technique and radioactively labeled ATP, which yield values of the order of 200 s⁻¹ (10,15,16). Phosphorylation can thus be considered as the rate-determining step for the reaction sequence responsible for the fast fluorescence phase under these experimental conditions. At lower temperatures, i.e., ≤15°C, a difference in the kinetics observed via stopped-flow using RH421 and quenched flow is apparent (11), indicating that as the temperature decreases, the $E1MgP \rightarrow E2MgP$ transition makes an increasing contribution to the overall rate determination.

Based on additional stopped-flow measurements and theoretical simulations (12), the slow phase has previously been attributed to relaxation of the dephosphorylation/rephosphorylation equilibrium. The meaning of this requires some explanation. In the absence of K⁺ ions, as in the experiments performed here, dephosphorylation of E2MgP is slow. Measured rate constants are in the range 2–7 s⁻¹

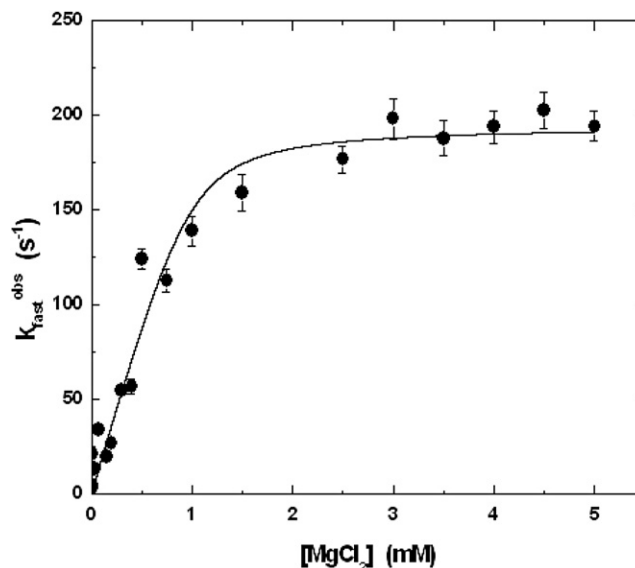


FIGURE 2 Dependence of the observed rate constant of the fast phase (k_{obs}^{fast}) of the RH421 fluorescence change on the concentration of MgCl₂ (after mixing). The solid line is a nonlinear least-squares fit of the data to Eq. 8 and Eqs. 1–4. The fit parameters were $k_1 = 195 (\pm 6) \text{ s}^{-1}$ and $K_E = 1.45 (\pm 0.22) \times 10^4 \text{ M}^{-1}$. K_E corresponds to a K_d of 0.069 (± 0.010) mM.

(17–20). After dephosphorylation, because excess ATP is present, the enzyme can undergo a conformational transition back to the E1 state and be rephosphorylated by ATP, i.e., $E2 \rightarrow E1(Na^+)_3 + ATP \rightarrow E2P + 3Na^+ + ADP$ (Mg²⁺ ions have been omitted here for simplicity). The rate-

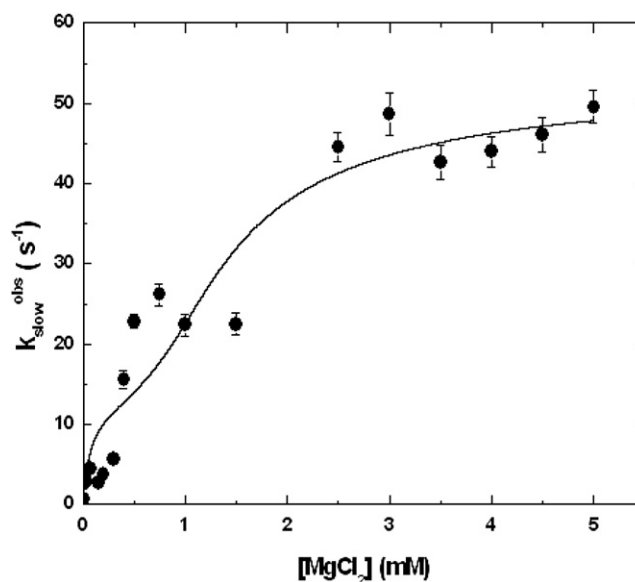


FIGURE 3 Dependence of the observed rate constant of the slow phase (k_{obs}^{slow}) of the RH421 fluorescence change on the concentration of MgCl₂ (after mixing). The solid line is a nonlinear least-squares fit of the data to Eq. 12 and Eqs. 1–6. The fit parameters were $k_4^{min} = 13(\pm 4) \text{ s}^{-1}$, $k_4^{max} = 74(\pm 10) \text{ s}^{-1}$, and $K_F = 1.3 (\pm 0.8) \times 10^3 \text{ M}^{-1}$. K_F corresponds to a K_d of 0.8 (± 0.5) mM.

determining step in this rephosphorylation pathway is the conformational transition, $E2 \rightarrow E1(\text{Na}^+)_3$, which based on previous measurements would be expected to have a rate constant in the range $65\text{--}90\text{ s}^{-1}$ (21–23) at saturating Na^+ , Mg^{2+} , and ATP concentrations. Based on the Van Slyke approximation (24), the reciprocal of the overall expected rate constant for the entire pathway from E2 to E2P via ATP rephosphorylation is given by the sum of the reciprocals of the rate constants of the separate reaction steps, $E2 \rightarrow E1(\text{Na}^+)_3$ and $E1(\text{Na}^+)_3 + \text{ATP} \rightarrow \text{E2P} + 3\text{Na}^+ + \text{ADP}$. Taking values of $65\text{--}90\text{ s}^{-1}$ for the first reaction, and the value of 195 s^{-1} measured here for the fast phase for the second reaction, yields an overall expected rate constant in the range $49\text{--}62\text{ s}^{-1}$.

Enzyme dephosphorylation and rephosphorylation via ATP represent a coupled equilibrium that must relax subsequent to the initial phosphorylation of the enzyme by ATP. The k_{obs} value for the relaxation of any first or pseudo-first-order process is given by the sum of the forward and backward rate constants. Therefore, adding the rate constants given above for dephosphorylation and rephosphorylation yields an expected k_{obs} of $51\text{--}69\text{ s}^{-1}$. The k_{obs} of $45\text{--}50\text{ s}^{-1}$ measured here for the slow phase at a saturating MgCl_2 concentration is only slightly below this range. This small difference may perhaps be accounted for by the different enzyme sources (i.e., animal species) employed in the previous studies.

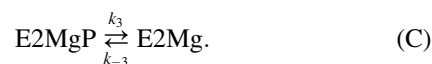
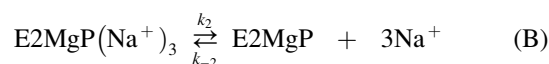
Further evidence supporting the assignment of the slow phase to the relaxation of the dephosphorylation/rephosphorylation equilibrium comes from measurements in which 7 mM KCl was included in the buffer before mixing with ATP (19). Under these conditions, an increase in fluorescence was still observed, but the fluorescent transient was then monoexponential. This can be explained by an acceleration of the dephosphorylation reaction by K^+ ions. The rate constant for K^+ -stimulated dephosphorylation has been estimated based on stopped-flow measurements (19,25) to have a value of $\sim 312\text{ s}^{-1}$. Adding this value to the rate constant for rephosphorylation given above ($49\text{--}62\text{ s}^{-1}$), then yields an expected k_{obs} for relaxation of the dephosphorylation/rephosphorylation equilibrium of $361\text{--}374\text{ s}^{-1}$. This is far greater than the k_{obs} found here for the fast phase due to initial ATP phosphorylation of 195 s^{-1} . Therefore, under K^+ -saturating conditions, the dephosphorylation/rephosphorylation equilibrium would be expected to relax instantaneously on the timescale of the initial phosphorylation, which is consistent with the experimental observation of only a single phase under these conditions.

It has been suggested previously (11) that the slow phase could be due to a slower conformational change after formation of the E2P species, i.e., $\text{E2P} \rightarrow \text{E2}^*\text{P}$, where the $\text{E2}^*\text{P}$ phosphoenzyme form is a K^+ -insensitive form in contrast to the K^+ -sensitive E2P form. However, it has since been found that the model we present here is capable of explaining the

ATP concentration dependence of both the k_{obs} values and the amplitudes of the transients (21).

Model simulations of the stopped-flow fluorescence transients

To gain a deeper understanding of the Mg^{2+} dependence of the observed kinetic behavior, we have carried out simulations of the fluorescence transients based on the assignment of the fast and slow kinetic phases described above. Accordingly, the sequence of reaction steps used was



Mathematical details of the simulations are given in the Appendix. Remember that k_{-3} represents the rate constant for rephosphorylation of the enzyme by continuing around the enzymatic cycle via E1 and undergoing phosphorylation by ATP; it is not the rate constant for “back-door” phosphorylation by inorganic phosphate. Reaction B describes the Na^+ -binding equilibrium to the E2P state. Reaction A is required for the initial turnover of the enzyme after mixing with ATP. For subsequent turnovers, this reaction is incorporated in the reverse direction of reaction C.

Simulations based on this model (see Fig. 4) show that a biphasic fluorescence increase is expected as long as the fluorescence of dye associated with enzyme in the E2Mg state is greater than that of dye associated with the initial $\text{E1MgATP}(\text{Na}^+)_3$ state. This agrees with the results of previous stopped-flow experiments showing that the fluorescence of RH421 decreases when the enzyme undergoes the transition from the E2 state to the $\text{E1}(\text{Na}^+)_3$ state (10,12,26). In fact, as shown previously (12), a biphasic fluorescence increase would also be expected for a mechanism involving only reactions A and C. This would require the fluorescence level of the E2Mg state to be greater than that of the E2MgP state. However, since evidence exists (14,20,27) that fluorescence changes of RH421 associated with the Na^+, K^+ -ATPase arise predominantly from steps involving ion binding to or release from the enzyme, we favor the complete reaction scheme A–C.

Based on the simulations, the observed two-phase fluorescence increase can be understood as follows. Initially, the enzyme is in the low fluorescent state, $\text{E1MgATP}(\text{Na}^+)_3$. The fast phase of the fluorescence increase on mixing with ATP can be explained by phosphorylation of the enzyme and its conversion into the higher fluorescent state E2MgP. The slow phase of the fluorescence increase is attributed to the conversion of some of the enzyme into the other high fluorescent state, E2Mg.

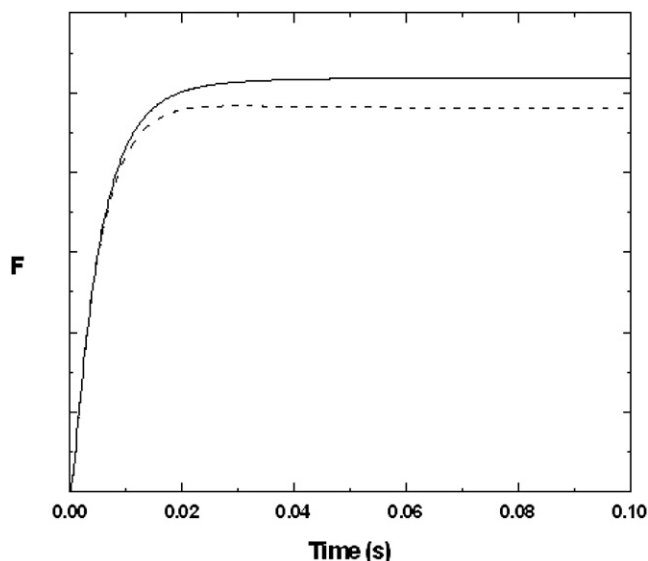


FIGURE 4 Computer simulations of RH421 stopped-flow kinetic transients for experiments in which Na⁺,K⁺-ATPase membrane fragments are mixed with ATP in the presence of Mg²⁺. The simulations are based on reaction scheme A–C and have been calculated according to Eqs. A1–A5 (see Appendix). The values of the rate constants chosen for the simulations are $k_1 = 237 \text{ s}^{-1}$, $k_2 = k_{-2} = 1000 \text{ s}^{-1}$, $k_3 = 5 \text{ s}^{-1}$, and $k_{-3} = 63 \text{ s}^{-1}$. The solid line represents a simulation in which the fluorescence levels of the E2Mg and E2MgP states are assumed to be equal ($f_{\text{E2Mg}} = f_{\text{E2MgP}} = 1$) and the fluorescence level of the E1MgATP(Na⁺)₃ state is defined as zero ($f_{\text{E1}} = 0$). This simulation shows a biphasic fluorescence increase, as experimentally observed (cf. Fig. 1, curve I). The dashed line represents a simulation in which the fluorescence level of the E2Mg state is assumed to be less than that of the E2MgP state and equal to that of the E1MgATP(Na⁺)₃ state, i.e., $f_{\text{E2MgP}} = 1$, $f_{\text{E2Mg}} = f_{\text{E1}} = 0$. This simulation reaches a maximum in fluorescence intensity 0.3 s after mixing (not easily observable by eye), after which there is a slight drop in fluorescence. The time course of the dashed curve does not correspond to the experimentally observed behavior.

Fitting of the fast phase of the stopped-flow kinetic data

Because phosphorylation of the enzyme by ATP also requires a Mg²⁺ ion to bind as a cofactor, under conditions of excess Na⁺, ATP, and Mg²⁺ over enzyme, the initial formation of the E2MgP state can be considered as a pseudo-first-order reaction that requires complete saturation of the Mg²⁺ binding sites to relax with its maximum rate constant. Under these conditions, it can be shown (10) that if one neglects competition between enzyme and unbound ATP for Mg²⁺, the expected dependence of the observed rate constant for the fast phase ($k_{\text{fast}}^{\text{obs}}$) on the Mg²⁺ concentration is given by

$$k_{\text{fast}}^{\text{obs}} = k_1 \times \frac{[\text{Mg}^{2+}]}{K_d + [\text{Mg}^{2+}]}, \quad (7)$$

where k_1 is the overall rate constant for phosphorylation of the enzyme and its conversion into the E2P state and K_d is the apparent dissociation constant of Mg²⁺ and enzyme in the E1ATP state. The term $[\text{Mg}^{2+}]/(K_d + [\text{Mg}^{2+}])$ represents

the fraction of Mg²⁺ sites of E1ATP occupied by Mg²⁺. Equation 7 predicts a hyperbolic dependence of $k_{\text{fast}}^{\text{obs}}$ on the Mg²⁺ concentration. However, in fact the situation is more complicated, because if the dissociation constants of ATP and enzyme for Mg²⁺ are of a similar order of magnitude, excess unbound ATP would compete effectively with the enzyme for the available Mg²⁺. This effect needs to be taken into account if one wishes to derive accurate values of k_1 and K_d .

Equation 7 can be written in a more model-independent form as

$$k_{\text{fast}}^{\text{obs}} = k_1 S_{\text{E1}}, \quad (8)$$

where S_{E1} represents the degree of saturation of the Mg²⁺ sites on the E1ATP(Na⁺)₃ species. Competition between enzyme and free ATP for Mg²⁺ can be taken into account as described under Materials and Methods. Equations 1–4 allow one to calculate a value of S_{E1} for any given values of the binding constants of enzyme and ATP for Mg²⁺ and, in combination with Eq. 8, to fit this model to the experimental $k_{\text{fast}}^{\text{obs}}$ data. Following this procedure leads to values of k_1 and K_d of $195 (\pm 6) \text{ s}^{-1}$ and $0.069 (\pm 0.010) \text{ mM}$, respectively. The value of k_1 agrees with measurements on enzyme from other sources under comparable conditions (10,12,21,22). The value of K_d is indistinguishable from the dissociation constant of ATP alone for Mg²⁺ ($0.071 (\pm 0.003) \text{ mM}$) under the same buffer conditions (4).

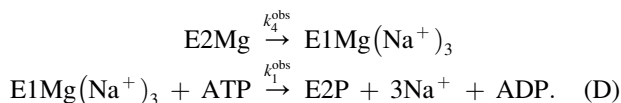
The salt concentrations are important here, because Mg²⁺ can also interact with the Na⁺ transport sites with an intrinsic K_d of $\sim 0.8 \text{ mM}$ (28,29) and ATP can be complexed by Na⁺ ions, both of which could make the apparent K_d dependent on the NaCl concentration. In the absence of any other added ions, Grisham and Mildvan (30) reported that Mg²⁺ binding to the enzyme in the absence of ATP and of added salt occurred with an apparent K_d of 0.15 mM , whereas in a buffer containing 100 mM NaCl and 10 mM KCl , i.e., comparable to our conditions of 130 mM NaCl , the apparent K_d was 1.0 mM . This value can't be directly compared to the K_d determined here, though, because it is due to direct binding of Mg²⁺ to the enzyme, whereas we are measuring the binding of Mg²⁺ to an enzyme-ATP complex.

Fitting of the slow phase of the stopped-flow kinetic data

In the case of the slow phase, attributed to the relaxation of reaction C above, under saturating conditions the observed rate constant should be given by the sum of the forward and backward rate constants:

$$k_{\text{slow}}^{\text{obs}} = k_3^{\text{obs}} + k_{-3}^{\text{obs}}. \quad (9)$$

The “obs” superscripts are used to indicate that under non-saturating conditions, these rate constants of reaction C could depend on the Mg²⁺ concentration due to coupling to other reactions. In fact, the backward reaction of reaction C involves at least two steps:



ATP and Na^+ binding reactions have not been explicitly included here, because we assume that these reactions are in fast equilibrium and that both ATP and Na^+ are present at saturating concentrations. Furthermore, we have neglected the reverse reaction of the conformational change of unphosphorylated enzyme ($\text{E1Mg}(\text{Na}^+)_3 \rightarrow \text{E2Mg}$), because in the absence of K^+ and the presence of high concentrations of both Na^+ and ATP, the E1/E2 equilibrium would be expected to lie greatly in favor of E1. According to the Van Slyke approximation (24) for sequential irreversible reactions, the overall observed rate constant for this reaction sequence can be approximated by

$$\frac{1}{k_{-3}^{\text{obs}}} = \frac{1}{k_4^{\text{obs}}} + \frac{1}{k_1^{\text{obs}}}, \quad (10)$$

where k_1^{obs} is the same as the experimentally determined $k_{\text{fast}}^{\text{obs}}$. Therefore, after substituting from Eq. 8, rearranging and substituting the resulting expression for k_{-3}^{obs} into Eq. 9 gives

$$k_{\text{slow}}^{\text{obs}} = k_3^{\text{obs}} + \frac{k_4^{\text{obs}} k_1 S_{\text{E1}}}{k_1 S + k_4^{\text{obs}}}. \quad (11)$$

This equation predicts an approximately hyperbolic increase in $k_{\text{slow}}^{\text{obs}}$ with increasing Mg^{2+} as experimentally observed (see Fig. 3) with a saturating value given by $k_3^{\text{obs}} + (k_4^{\text{obs}} k_1 / (k_1 + k_4^{\text{obs}}))$. The Mg^{2+} dependence of $k_{\text{fast}}^{\text{obs}}$ could, thus, be explained by Mg^{2+} -stimulation of the phosphorylation reaction, as is the case for the fast phase. In the first instance, therefore, Eqs. 11 and 1-4 were fitted to the experimental $k_{\text{slow}}^{\text{obs}}$ data with the assumption that k_3^{obs} and k_4^{obs} are both independent of the Mg^{2+} concentration. An adequate fit to the data could, however, only be achieved if K_{E} , the binding constant of Mg^{2+} to $\text{E1ATP}(\text{Na}^+)_3$, was also allowed to vary. The best-fit value of K_{E} was a factor of 10 lower than that already determined from the analysis of the fast phase (i.e., K_{d} was a factor of 10 higher). Because this situation would be inconsistent, the possibility that k_4^{obs} is also Mg^{2+} -dependent needs to be considered.

A Mg^{2+} dependence of k_4^{obs} could come about if Mg^{2+} binding to E2 stimulates the $\text{E2} \rightarrow \text{E1}$ transition via an allosteric effect. This is already known to be the case for ATP (31). To consider such an effect, Eq. 11 must be modified to

$$k_{\text{slow}}^{\text{obs}} = k_3^{\text{obs}} + \frac{[k_4^{\text{min}} + (k_4^{\text{max}} - k_4^{\text{min}})S_{\text{E2}}]k_1 S_{\text{E1}}}{k_1 S_{\text{E1}} + [k_4^{\text{min}} + (k_4^{\text{max}} - k_4^{\text{min}})S_{\text{E2}}]}, \quad (12)$$

where k_4^{min} and k_4^{max} represent the minimum and maximum values of k_4 when the E2 state of the enzyme has no bound Mg^{2+} ions and when it is saturated by Mg^{2+} , respectively.

S_{E1} and S_{E2} represent the degrees of saturation of the E1 and E2 states by Mg^{2+} . S_{E1} can be calculated by taking into account competition from the excess unbound ATP, according to Eqs. 1–6. Analogous equations can be written for the E2 state to allow the calculation of S_{E2} .

A fit of Eq. 12 to the experimental data is shown in Fig. 3. The fit was carried out using a fixed value of K_{ATP} , taken from calorimetric measurements (4), and fixed values of k_1 and K_{E} , based on the values determined from the analysis of the fast phase (see the previous section). The only parameters allowed to vary were k_3^{obs} , k_4^{min} , k_4^{max} and K_{F} (the binding constant of Mg^{2+} to the E2ATP conformation of the protein). It was found that the value of k_3^{obs} , the rate constant for dephosphorylation, was indistinguishable from zero and could in fact be removed from Eq. 12 with no change to the fit. This is interesting, because previous direct measurements of the rate constant for dephosphorylation in the absence of K^+ but in the presence of Mg^{2+} have yielded values in the range $2\text{--}7 \text{ s}^{-1}$ (17–19). Therefore, this result suggests that Mg^{2+} may also have an allosteric effect in accelerating the rate of dephosphorylation. However, this possibility should be investigated by more direct measurements than those reported here. An allosteric effect of Mg^{2+} on k_3 hasn't been included in Eq. 12. This could be done, but with the number of fitted parameters already included in the equation, a significantly better fit to the experimental data would not be obtained. The values of the other parameters obtained from the fit were $k_4^{\text{min}} = 13(\pm 4)\text{ s}^{-1}$, $k_4^{\text{max}} = 74(\pm 10)\text{ s}^{-1}$ and $K_{\text{F}} = 1.3 (\pm 0.8) \times 10^3 \text{ M}^{-1}$. The value of K_{F} corresponds to a dissociation constant of Mg^{2+} with the E2ATP conformation of the protein of $0.8 (\pm 0.5) \text{ mM}$. In comparison with the $\text{E1ATP}(\text{Na}^+)_3$ conformation, this represents an order of magnitude weaker binding of Mg^{2+} . It is interesting that this parallels a much weaker binding of ATP itself to the E2 conformation relative to the E1 conformation (26).

The value of k_4^{max} derived from the fitting of 74 s^{-1} is consistent with the values in the range $65\text{--}90 \text{ s}^{-1}$ (21–23) previously found for the $\text{E2} \rightarrow \text{E1}(\text{Na}^+)_3$ transition in the presence of saturating concentrations of Mg^{2+} and ATP for Na^+, K^+ -ATPase from pig and rabbit kidney. The lower value of k_4^{min} , the rate constant for the same transition but in the absence of Mg^{2+} , of 13 s^{-1} is in accordance with the idea that Mg^{2+} has an allosteric effect similar to ATP in accelerating this reaction. One needs to keep in mind, however, that although the fit shown in Fig. 3 is consistent with the data obtained for the fast phase, there are many more rate constants and equilibrium constants affecting the relaxation of the slow phase. This makes it difficult to give precise estimates of the parameters in the fitting model. For this reason, as in the case of the dephosphorylation reaction, the effect of Mg^{2+} on the $\text{E2} \rightarrow \text{E1}(\text{Na}^+)_3$ transition should be investigated by more direct means, e.g., a direct analysis of the effect of Mg^{2+} on the time course of the $\text{E2} \rightarrow \text{E1}(\text{Na}^+)_3$ reaction alone.

DISCUSSION

The kinetics of Na⁺- and Mg²⁺-dependent reactions of the Na⁺,K⁺-ATPase have been investigated via the stopped-flow technique by mixing enzyme-containing membrane fragments fluorescently labeled with the probe RH421 with ATP at varying Mg²⁺ concentrations. As in the case of previous stopped-flow studies using the same technique (10–12,21,22), two kinetic phases were observed, both associated with a fluorescence increase. The faster phase is attributed to the phosphorylation of the enzyme and its conversion to the E2MgP state. The slower phase is attributed to a subsequent relaxation of the dephosphorylation/rephosphorylation (via ATP) equilibrium and build-up of some enzyme in the E2Mg state.

It was found that both phases showed similar roughly hyperbolic increases in their observed rate constants to saturating values, but the k_{obs} of the fast phase saturated at a lower Mg²⁺ concentration than that of the slow phase. The Mg²⁺ concentration dependence of the fast phase can be explained by Mg²⁺ stimulation of the enzyme's phosphorylation by acting as a cofactor of ATP. This phase involves the direct phosphorylation of enzyme in the E1Mg(Na⁺)₃ state by ATP. The slow phase also involves phosphorylation by ATP, but after the enzyme has proceeded once around its enzymatic cycle, i.e., after dephosphorylation and a conformational change back to the initial E1Mg(Na⁺)₃ state. Since the slow phase also involves enzyme phosphorylation, the Mg²⁺ concentration dependence of its k_{obs} can also partly be explained by Mg²⁺ stimulation of ATP phosphorylation. However, the fact that the slow phase saturates at a higher Mg²⁺ concentration than the fast phase implies that this is not the only effect. The concentration dependence of the slow phase could be explained by an additional stimulation by Mg²⁺ of the E2ATP → E1ATP(Na⁺)₃ reaction.

From crystal structures (32,33) of the related P-type ATPase, the sarcoplasmic reticulum Ca²⁺-ATPase, it is known that Mg²⁺ binds to the enzyme in a complex with a nucleotide (i.e., ATP under physiological conditions but its inert analog AMPPCP in crystals). To gain an insight into how Mg²⁺ is held in place within the enzyme, it is interesting to compare the Mg²⁺ K_d determined here with the K_d of Mg²⁺ with ATP in free solution. We have recently determined this using isothermal titration calorimetry, under the same buffer conditions as used here for the stopped-flow measurements, to be 0.071 (± 0.003) mM (4). This is indistinguishable from the value of K_d determined here in the presence of the enzyme, i.e., 0.069 (± 0.010) mM. This implies that although Mg²⁺ carries out an important function in the enzyme's mechanism in allowing phosphoryl transfer, the enzyme environment of the E1 state has no effect on the strength of complexation of Mg²⁺ by ATP. The enzyme does have to provide space within its structure so that Mg²⁺ can access ATP, but it is the ATP, not the enzyme, that dominates in holding the Mg²⁺ ion in place. This could explain why no crystal structure of an E1Mg²⁺ state of a P-type

ATPase has ever been determined despite success with many other states around the enzymatic cycle. Faller (34) recently reported a weak dissociation constant of Mg²⁺ to the E2 form of the Na⁺,K⁺-ATPase of 2.5 mM. He attributed Mg²⁺ binding alone to an “induced-fit” mechanism, because, as shown in crystal structures of the Ca²⁺-ATPase, the protein residues that interact with the Mg²⁺ ions are more than twice as far apart in the Ca₂E1 state (2) as in the E2 P_i state (35). Therefore, in an Na₃ E1 state, one might expect an even weaker binding of Mg²⁺ to the enzyme in the absence of ATP, i.e., $K_d > 2.5$ mM, but definitely much greater than the K_d determined here for the E1ATP complex of 0.069 (± 0.010) mM. Schneeberger and Apell (29) reported an apparent K_d of Mg²⁺ to the E1 state of ~30 mM in the presence of 50 mM NaCl, but this was attributed to Mg²⁺ binding to the Na⁺ transport sites, not the site within the enzyme where it coordinates with ATP. Aspartate residues of the protein may be in relatively close proximity to the Mg²⁺ required for phosphorylation, as suggested by Patchornik et al. (3), but it seems that their importance for the strength of binding of Mg²⁺ is far outweighed by the phosphates of ATP. It needs to be pointed out, however, that the above argument does not rule out significant contributions of the enzyme toward the strength of Mg²⁺ binding in other conformations of the enzyme cycle. It also needs to be made clear that the results described here only refer to the Mg²⁺ ion necessary for phosphorylation, which acts as a cofactor of ATP. In crystals containing Mg²⁺, ADP and the phosphate analog AlF₄[−] (36), which represents an E1 ~PADP transition state, a second Mg²⁺ ion was found between the α- and β-phosphates of ADP. This second Mg²⁺ was, however, not present in the complex of the enzyme with AMPPCP, which is the more relevant state for this study.

APPENDIX: SIMULATIONS

Computer simulations of the time course of fluorescence changes experimentally observed via stopped flow were performed using the program Berkeley Madonna 8.0 (University of California, Berkeley) via the variable-step-size Rosenbrock integration method. Based on reaction scheme A–C, the differential rate equations describing the changes in the concentrations of all the enzyme intermediates are

$$\frac{d[\text{E1MgATP}(\text{Na}^+)_3]}{dt} = -k_1[\text{E1MgATP}(\text{Na}^+)_3] \quad (\text{A1})$$

$$\begin{aligned} \frac{d[\text{E2MgP}(\text{Na}^+)_3]}{dt} &= k_1[\text{E1MgATP}(\text{Na}^+)_3] \\ &\quad - k_2[\text{E2MgP}(\text{Na}^+)_3] \\ &\quad + k_{-2}[\text{E2MgP}] \end{aligned} \quad (\text{A2})$$

$$\begin{aligned} \frac{d[\text{E2MgP}]}{dt} &= k_2[\text{E2MgP}(\text{Na}^+)_3] - k_{-2}[\text{E2MgP}] \\ &\quad - k_3[\text{E2MgP}] + k_{-3}[\text{E2Mg}] \end{aligned} \quad (\text{A3})$$

$$\frac{d[\text{E2Mg}]}{dt} = k_3[\text{E2MgP}] - k_{-3}[\text{E2Mg}]. \quad (\text{A4})$$

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 fluorescence increases after mixing with ATP and the enzyme starts in the $\text{E1MgATP}(\text{Na}^+)_3$ state, we have defined the fluorescence level of this state to be zero. Because the major changes in fluorescence are thought to involve the binding or release of ions (14,20,27), the fluorescence level of the $\text{E1MgP}(\text{Na}^+)_3$ has also been taken to be zero. F is then given by

$$F = f_{\text{E2MgP}}[\text{E2MgP}] + f_{\text{E2Mg}}[\text{E2Mg}]. \quad (\text{A5})$$

Numerical integration of Eqs. A1–A4 and calculation of F using Eq. A5 yields a biexponential fluorescence transient with both phases associated with a fluorescence increase (as experimentally observed) using any values of f_{E2MgP} and f_{E2Mg} greater than zero. Based on the experimental results obtained, at saturating Mg^{2+} concentrations, the values of k_1 , k_3 , and k_{-3} used were 237 s^{-1} , 5 s^{-1} , and 63 s^{-1} , respectively. The values of k_2 and k_{-2} for Na^+ release and binding to the E2MgP state were both chosen to have values of 1000 s^{-1} (37).

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