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# Effects of magnesium and ATP on pre-steady-state phosphorylation kinetics of the $Na^+$,$K^+$-ATPase

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The aim of the present work was to elucidate the role played by ATP and $Mg^{2+}$ ions in the early steps of the $Na^+$,$K^+$-ATPase cycle. The approach was to follow pre-steady-state phosphorylation kinetics in $Na^+$-containing $K^+$-free solutions under variable ATP and $MgCl_2$ concentrations. The experiments were performed with a rapid mixing apparatus at 20 ± 2°C. The concentrations of free and complexes species of $Mg^{2+}$ and ATP were calculated on the basis of a dissociation constant of 0.091 ± 0.004 mM, estimated with Arsenazo III under identical conditions. A simplified scheme were ATP binds to the ENa enzyme, which is phosphorylated to MgEPNa and consequently dephosphorylated returning to the ENa form, was used. In the absence of ADP and phosphate four rate constants are relevant: $k_1$ and $k_{-1}$, the on and off rate constants for ATP binding; $k_2$, the transphosphorylation rate constant and $k_3$, the constant that governs the dephosphorylation rate. The values obtained were: $k_1 = 0.025 \pm 0.003\ \mu M^{-1}\,ms^{-1}$ for both free ATP and ATPMg; $k_{-1} = 0.038 \pm 0.004\ ms^{-1}$ for free ATP and $0.009 \pm 0.002\ ms^{-1}$ for ATPMg; $k_2 = 0.199 \pm 0.005\ ms^{-1}$; $k_3 = 0.0019 \pm 0.0002\ ms^{-1}$. The model that seems best to explain the data is one where (i) the role of true substrate can be played equally well by free ATP or ATPMg, and (ii) free $Mg^{2+}$, an essential activator acts by binding to a specific $Mg^{2+}$ site on the enzyme molecule.

## Introduction

Magnesium ion acts as an essential activator of the $Na^+$,$K^+$-ATPase enzyme [1]. This is the case for the overall cycle of ATP hydrolysis as well as for partial reactions that involve the formation of a phosphoenzyme. The optimal free $[Mg^{2+}]$ depends on the reaction being considered: in the micromolar range for ATPase activity [2], enzyme phosphorylation from ATP [3] and ATP–ADP exchange [4–6] and in the millimolar range for phosphatase activity [7] and phosphorylation by inorganic phosphate (Ref. 8; Campos and Beaugé, unpublished). In addition, when enzyme phosphorylation takes place in the presence of radioactive $Mg^{2+}$ analogues ($^{54}Mn^{2+}$ or $^{60}Co^{2+}$) an extremely stable $Mg^{2+}$ analogue-enzyme-phosphate complex is formed. This has been found both for ($Na^+$ + ATP)- [9,10] and for phosphate-dependent E-P formation (Ref. 10; Campos and Beaugé, unpublished). The MnEP complex from ATP is generated both in the $E_1$ and $E_2$ configuration [9]; in addition, $Mn^{2+}$ remains tightly bound as long as the enzyme stays phosphorylated [9]. Magnesium forms a complex with ATP, which at 20°C has a dissociation constant of 0.091 mM (this work); this means that for all practical purposes a mixture of free $Mg^{2+}$, free ATP and Mg-ATP complex will exist at the $[Mg^{2+}]$ and [ATP] normally used. This is the main reason why the relationships between these three species with $Na^+$,$K^+$-ATPase remained undefined and there is no agreement about what ATP form is the real substrate for the reaction [2,3,5,11–15]. On the basis of physical evidences a single $Mg^{2+}$ binding site has been recognized in the enzyme [16]. On the other hand, $Mg^{2+}$ affects conformational changes that are not associated with phosphorylation and is inhibitor of several total and partial reactions [8,17]; these effects could take place at more than one $Mg^{2+}$ site per enzyme molecule or a single site provided its affinity towards $Mg^{2+}$ changes during the reaction cycle [17–21]. Experiments with $Mg^{2+}$ analogues [9,10] indicate that during ATP phosphorylation their binding is: (i) with high affinity, (ii) very likely on a single site, and (iii) with a $Mg^{2+}$ analogue-inorganic phosphate stoichiometry of one to one. However, whether the $Mg^{2+}$ tightly bound to EP comes from the free $Mg^{2+}$ or the Mg-ATP complex remains an open question.

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This work was aimed to find an answer, or at least to shed light on, two points: (i) what ATP species is the real substrate, and (ii) which is the role of free $Mg^{2+}$ ions. To that purpose we followed pre-steady-state kinetics of the $Na^+$-dependent E-P formation from ATP. The idea was to determine the relative importance of the following alternative pathways:

$$E \longrightarrow MgE \longrightarrow MgEATP \longrightarrow EP \quad (i)$$

$$E \longrightarrow MgE \longrightarrow MgEATPMg \longrightarrow EP \quad (ia)$$

$$E \longrightarrow EATP \longrightarrow MgEATP \longrightarrow EP \quad (ii)$$

$$E \longrightarrow EATPMg \longrightarrow MgEATPMg \longrightarrow EP \quad (iia)$$

$$E \longrightarrow EATPMg \longrightarrow EP \quad (iii)$$

The distinction between (i)–(ia) and (ii)–(iia) is given by the order of ATP (or ATPMg) and $Mg^{2+}$ binding; that between (i) and (ia), or (ii) and (iia), is the ATP species (free ATP or ATPMg) that is the true substrate. In (iii) the true substrate is ATPMg while free $Mg^{2+}$ does not bind to the enzyme. The results are consistent with an scheme where both free ATP and Mg-ATP can act as substrates whereas $Mg^{2+}$ is an essential activator that gets into the enzyme independently of ATP; i.e. path (iii) is excluded. The order of ligand binding cannot be asserted from this work.

## Methods

Pig kidney $Na^+,K^+$-ATPase was partially purified according to Jørgensen [22]; the specific activity was 15–20 units/mg and remained stable for months when stored at −85°C (3–5 mg protein/ml) in 25 mM imidazole (pH (20°C) 7.5)/2 mM EDTA/10% sucrose. Immediately before use, and in order to remove any tightly bound $Mg^{2+}$, the enzyme was washed five times (1 : 10, v/v) and resuspended in a solution containing 100 mM NaCl/10 mM imidazole without sucrose and EDTA.

ATPase activity was assayed as in Ref. 23 following the release of $[^{32}P]P_i$ from $[\gamma\text{-}^{32}P]ATP$ labelled according to Glynn and Chappell [24] with modifications [25]. Protein was determined by the method of Lowry et al. [26].

Pre-steady-state phosphorylations were carried out with an Intermekron DSM 3-SF Rapid Mixing apparatus (Uppsala, Sweden) [27] where the tangential mixers were substituted with Berger Ball Mixers [28] kindly provided by Dr. J. Froehlich. Two syringes and one chamber, three syringes and two chambers or four syringes and three chambers were used; the volume in each syringe was 1 ml. Syringe 1 (which always contained the enzyme) and syringe 2 were connected to chamber 1; the output of that chamber went into the stopping solution or was mixed in chamber 2 with the content of syringe 3; the mixture from chamber 2 went into the stopping solution or was mixed, in chamber 3, with the content of syringe 4 before the reaction was halted. The equilibration time of $Na^+,K^+$-ATPase and the ligands present in syringe 1 was 2 min. Temperature was 20–22°C. The stopping solution comprised ice-cold 12% perchloric acid/1 mM ATP/10 mM inorganic phosphate. The denatured protein was collected on Whatman GF/F filters, washed with 5% trichloroacetic acid/10 mM inorganic phosphate and counted in a liquid scintillation counter. Imidazole (70 mM and pH 7.4 at 20°C) was always present. Other experimental details and solutions are described in the text and figure and table legends. The steady-state levels of phosphoenzyme were estimated in each condition in which rates of phosphorylation were measured. To this end, the final reaction mixture was introduced into an empty tube where the stopping solution was added after 0.5 to 3 s.

The number of phosphorylating sites in the enzyme was determined on the basis of the E-P formed after 10 min incubation at 37°C in the presence of 1 mM $[^{32}P]$phosphate and 1 mM ouabain (total volume of 0.1 ml). The stopping solution consisted of 1.0 ml of 12% perchloric acid/50 mM inorganic phosphate/10% polyphosphate/10 mM pyrophosphate; the collection of the denatured protein and radioactivity counting were performed as indicated above. Enzyme heat denatured prior to phosphorylation was used as blank. The phosphorylating sites ranged from 1.7 to 2.1 nmol/mg protein. These estimation were performed in each experiment, and the values used in the kinetic calculations.

All solutions were made with de-ionized bidistilled water. NaCl and KCl were of spectrometric grade; the other chemicals were of reagent grade. Ouabain and imidazole were from Sigma, Chemical Co., USA. Vanadium-free ATP was from Boehringer Mannheim, USA. Inorganic $[^{32}P]$phosphate was purchased from the Comisión Nacional de Energía Atómica of Argentina.

Radioactivity assays were performed in a Beckman liquid scintillation counter using a toluene based scintillator; counting times were long enough to obtain standard errors of about one percent. Curve fitting was performed with the Scop non-linear regression computer program (National Biomedical Simulation Resource, Durham, NC, USA).

## Results

Magnesium ions form a complex with ATP. Using the Arsenazo III indicator [29] we obtained, under the conditions of our experiments, a ATPMg dissociation constant of $0.091 \pm 0.004$ mM (not shown). This $K_d$ confirms that, at the $MgCl_2$ and ATP concentrations

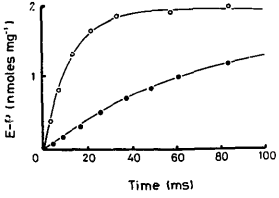



Fig. 1. Pre-steady-state phosphorylation from ATP of $Na^+,K^+$-ATPase in the ENa form. This experiment was designed to test for symmetry between $MgCl_2$ and ATP. Aliquots of 50 $\mu$g enzyme, equilibrated in syringe 1 with 100 mM NaCl and 100 $\mu$M (open symbols) or 10 $\mu$M (filled symbols) $MgCl_2$, were mixed with a final concentration of 10 $\mu$M (open symbols) or 100 $\mu$M (filled symbols) of [$\gamma$-$^{32}$P]ATP coming from syringe 2; the phosphorylation reaction was stopped at different times with an acid solution. Note: (i) the same free $Mg^{2+}$ was present during equilibration and phosphorylation reactions; (ii) 100 mM NaCl and 70 mM imidazole (pH 7.4 at 20°C) were present all throughout. (iii) the volume in each syringe was 1 ml. Temperature was 20±2°C. The lines through the points are the best fit to the equation resulting from the oversimplified reaction E ⇌ EP; this fit gave a value of 0.080±0.003 $ms^{-1}$ (open symbols) and 0.011±0.001 $ms^{-1}$ (filled symbols) for the forward rate constant ($k_f$). For more details see text and Methods.

used here, three species coexist: free $Mg^{2+}$, free ATP and ATPMg.

In the general case of an essential activator and a substrate that form a complex, there are several possible mechanisms for activation [30]. The simplest is one in which the true substrate is the activator-substrate complex while the enzyme does not bind either of the free ligands. This case can be easily checked, for it has the property of symmetry between ligands; i.e. the same activity or rate is obtained when the ratio activator/substrate is, let us say, $A$ or $1/A$. We tested this hypothesis in experiments like that described in Fig. 1 and summarized in Table I. Two syringes and one mixing chamber were used; the enzyme, pre-equilibrated with 100 mM NaCl and variable [$MgCl_2$], was mixed with also variable concentrations of [$\gamma$-$^{32}$P]ATP coming from syringe 2 and the phosphorylation reaction stopped at different times. An important feature, which will become relevant in latter analysis, is that the data points could be acceptably fitted to an equation resulting from the simple relationship E ⇌ EP. In this scheme we have: (i) the overall pseudo-first order rate constant for EP formation ($k_f$), which includes several steps, minimally the reactions comprising ATP binding and phosphorylation [3,31,32], (ii) the steady-state values of EP (which were experimentally determined), and (iii) the rate constant for EP dissociation ($k_3$ given below), estimated from the rate of EP breakdown.

The pseudo-first order rate constants, depicted in rows 2 and 3 of Table I, were 0.079 ± 0.005 $ms^{-1}$ for 10 $\mu$M ATP/100 $\mu$M $MgCl_2$ and 0.012 ± 0.002 $ms^{-1}$ for 100 $\mu$M ATP/10 $\mu$M $MgCl_2$. As in both instances the [ATPMg] was the same, ATPMg cannot be the true substrate and the only ligand interacting with the enzyme (reaction (iii) in Introduction).

There are other pertinent data in Table I: (a) a 50% drop in [ATPMg]/[free ATP] ratio or [free $Mg^{2+}$] coexists with a similar drop in the rate of phosphorylation, although [ATPMg] increases 5-fold (rows 1 and 3); (b) an 8-fold increase in [ATPMg] with a 40% reduction in [free $Mg^{2+}$] and [ATPMg]/[free ATP] ratio does no affect the phosphorylation rate (rows 2 and 4). These results cannot differentiate between the remaining alternatives: (i) ATPMg is the only substrate; free ATP acts as competitive inhibitor or free ATP is ineffective but there is a stimulation by $Mg^{2+}$ ions; (ii) free or total ATP is/are the substrate/s while $Mg^{2+}$ acts on specific sites on the enzyme. The experiments that follow we designed to look into these possibilities.

In order to obtain more meaningful quantitative information, we looked into a minimal kinetic model that could satisfactory describe our pre-steady-state

TABLE I

*Overall rate constant for phosphorylation from ATP ($k_f$) of $Na^+,K^+$-ATPase in the presence of different concentrations of ATP and $MgCl_2$*

The general experimental procedure is explained in the legend to Fig. 1 and in the text. The concentrations of free $Mg^{2+}$, free ATP and the ATPMg complex were estimated on the basis of a ATPMg dissociation constant of 0.091 mM. The overall rate constants for phosphorylation were calculated fitting the data points to a growing single-exponential function. The total number of phosphorylating sites were taken as the amount of [$^{32}$P]phosphate incorporated after incubating the enzyme in media containing 1 mM [$^{32}$P]phosphate and 1 mM ouabain. See text for more details. All values are means ± S.D.

| ATP ($\mu$M) | $MgCl_2$ ($\mu$M) | $Mg^{2+}$ ($\mu$M) | free ATP ($\mu$M) | ATPMg ($\mu$M) | ATPMg / free ATP ratio | $k_f$ ($ms^{-1}$) |
|---|---|---|---|---|---|---|
| 10 | 10 | 9.1 | 9.1 | 0.9 | 0.10 | 0.023 ± 0.002 |
| 10 | 100 | 94.9 | 4.9 | 5.1 | 1.02 | 0.079 ± 0.005 |
| 100 | 10 | 4.9 | 94.9 | 5.1 | 0.05 | 0.012 ± 0.002 |
| 100 | 100 | 60.4 | 60.4 | 39.6 | 0.66 | 0.085 ± 0.006 |

phosphorylation. We decided to use a model simpler than that put forward by Froehlich et al. [33] and Hobbs et al. [34], such as:

$$E + ATP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}ATP \xrightarrow{k_2} EP \xrightarrow{k_3} E \qquad (1)$$

where

$k_1$ is the on rate constant for ATP binding,

$k_{-1}$ is the off rate constant for ATP binding,

$k_2$ is a rate constant that includes all steps involved in EP formation (possible conformational changes prior to phosphorylation, etc.),

$k_3$ is the rate constant for EP breakdown.

We included only EP (total acid-stable EP), which is what we measured, without distinguishing between $E_1P$ and $E_2P$. The rationale for using $k_3$ is that under these conditions most of the phosphoenzyme is rapidly converted into $E_2P$ [35]. The $E_2$–$E_1$ transition was ignored, for it is relatively fast and not rate-limiting in the absence of $K^+$ ions (see Ref.36). The constants for the reverse reactions, $k_{-2}$ and $k_{-3}$, were also ignored since the expected ADP and phosphate concentrations following ATP hydrolysis are too low to reverse those parts of the cycle [5,21]. Eqn. 1 was used in data fitting when free ATP, ATPMg or both were considered substrates. When ATPMg (or free ATP) was taken as true substrate and free ATP (or ATPMg) as a competitive inhibitor, the interactions between enzyme and all ATP species were included.

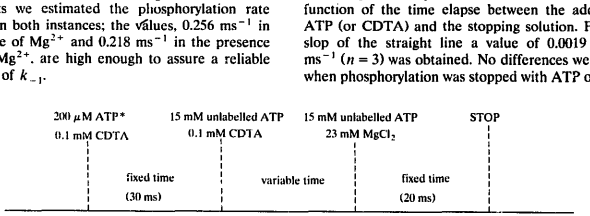
The resulting simultaneous differential equations, both for simulation and fitting of the data, were solved numerically with the Scop program. In order to reduce the number of unknown rate constants, $k_{-1}$ and $k_3$ were independently estimated.

Two different $k_{-1}$ were determined. One for free ATP and one for ATPMg. The experimental design depended on which one was considered, although both protocols had the enzyme preincubated with ATP in the absence or presence of $MgCl_2$. In introductory experiments we estimated the phosphorylation rate constants in both instances; the values, 0.256 $ms^{-1}$ in the absence of $Mg^{2+}$ and 0.218 $ms^{-1}$ in the presence of 1 mM $Mg^{2+}$, are high enough to assure a reliable estimation of $k_{-1}$.

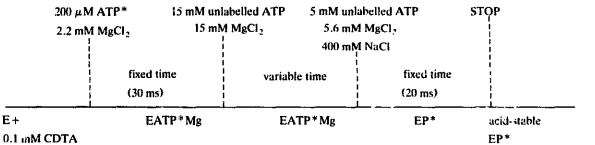
In the case of free ATP the protocol shown in Scheme I was used. Four syringes and three mixing chamber were required. In the first experiments 100 mM NaCl and 70 mM imidazole were present all throughout. We later found that in $Mg^{2+}$-containing $Na^+$-free solutions ($k_{-1}$ for ATPMg) imidazole activated some phosphorylation from ATP; this was low and amounted to no more than 4% in steady state. However, in view of that result we repeated the estimations of $k_{-1}$ using Tris-HCl instead. The values for $k_{-1}$ were practically the same, but the results in Tris are reported here. The experimental details are given in the legend to Fig. 2. The equation for this approach is a linear decay of the logarithm of the acid-stable [EP] as a function of the variable time elapsed between the addition of unlabelled ATP and excess $MgCl_2$. The absolute value of slope gives $k_{-1}$. The figure was $0.038 \pm 0.004$ $ms^{-1}$ ($n = 3$). In the absence of $Mg^{2+}$ and with CDTA no ATP hydrolysis is observed. Under these conditions $k_{-1}$ can also be estimated using three syringes and two mixing chambers (the enzyme and [$\gamma$-$^{32}$P]ATP are pre-equilibrated in the presence of 0.1 mM CDTA). We performed some of these experiments and the values of $k_{-1}$ were identical to those seen with four syringes ($0.035 \pm 0.003$ $ms^{-1}$).

For ATPMg the protocol shown in Scheme II was used. As before, four syringes and three mixing chamber were employed. Tris-HCl (70 mM) was included in all solutions but 400 mM NaCl (100 mM final concentration) was only in syringe 4. The constant $k_{-1}$ was gotten from the linear decay of the logarithm of the acid-stable [EP] as a function of the variable time elapsed between the addition of unlabelled ATP and NaCl; that value was $0.009 \pm 0.002$ $ms^{-1}$ ($n = 3$) (see Fig. 2).

For $k_3$, the experimental design, using three syringes and two mixing chambers, shown in Scheme III was used. The details are given in the legend to Fig. 3, that illustrates one of these experiments. The figure is a semilogarithmic plot of the acid stable [EP] as a function of the time elapse between the addition of ATP (or CDTA) and the stopping solution. From the slop of the straight line a value of $0.0019 \pm 0.0002$ $ms^{-1}$ ($n = 3$) was obtained. No differences were found when phosphorylation was stopped with ATP or CDTA




Scheme I




Scheme II

(see Fig. 3) nor was $k_3$ affected when 1000 $\mu$M instead of 10 $\mu$M free $Mg^{2+}$ were present (not shown).

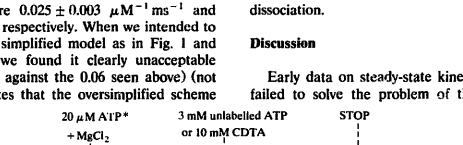
Once the two $k_{-1}$ and $k_3$ had been estimated, the fit of the experimental data to Eqn. 1 contained three unknowns; these were reduced to two, $k_1$ and $k_2$, for the total number of phosphorylating sites was estimated separately on the basis of the E-P formation from [$^{32}$P]phosphate in the presence of ouabain (see Methods).

The next group of experiments was aimed to determine the remaining unknown constants ($k_1$ and $k_2$), varying total [ATP] while ionized [$Mg^{2+}$] remained fixed at about 1000 $\mu$M. Eight ATP concentrations were chosen: 0.75 $\mu$M, 1.5 $\mu$M, 3 $\mu$M, 5 $\mu$M, 10 $\mu$M, 25 $\mu$M, 50 $\mu$M and 100 $\mu$M; under these conditions most of the nucleotide existed as ATPMg complex. Introductory experiments had indicated that with 100 $\mu$M ATP and 1000 $\mu$M free $Mg^{2+}$ the overall phosphorylation rate was saturated. Therefore, the time-dependent EP formations with the eight ATP concentrations were simultaneously fitted to Eqn. 1 assuming that $k_1$ and $k_2$ were constant; that gave two fitting parameters for about 80 data points. For the sake of clarity, only five of the resulting curves are illustrated in Fig. 4. The fit was very good (standard error of fitting of 0.06); this is important considering that [ATP] varied over more than 100-fold. The values of $k_1$ and $k_2$ were $0.025 \pm 0.003$ $\mu M^{-1} ms^{-1}$ and $0.196 \pm 0.007$ $ms^{-1}$, respectively. When we intended to fit these data to a simplified model as in Fig. 1 and Table I ($E \rightleftharpoons EP$) we found it clearly unacceptable (fitting error of 0.6 against the 0.06 seen above) (not shown); this indicates that the oversimplified scheme cannot account for the kinetics under these conditions.

If $Mg^{2+}$ ions stimulate the rate of phosphorylation by an action different from, or in addition to, the formation of the ATPMg complex, that should be reflected in the value of $k_2$ of Eqn. 1. That was investigated measuring the pre-steady-state rate of EP formation at constant total [ATP] (10 $\mu$M) in enzyme equilibrated with variable [$Mg^{2+}$] (1.9 $\mu$M, 4.7 $\mu$M, 9.1 $\mu$M, 13 $\mu$M, 23 $\mu$M, 47 $\mu$M, 190 $\mu$M and 990 $\mu$M). Again, the multiple data sets were simultaneously fitted to the corresponding equation. The value of $k_3$ was the same of before, whereas that of $k_1$ was the mean 0.025 $\mu M^{-1} ms^{-1}$ obtained above. The total number of phosphorylating sites was determined as indicated. The assumption made (see Discussion for alternatives) was that both free ATP and ATPMg served as substrate. The two values chosen for $k_{-1}$, 0.034 $ms^{-1}$ and 0.006 $ms^{-1}$, had no influenced on the computed $k_2$. The data points as well as the fitted curves of five different [$Mg^{2+}$] are illustrated in Fig. 5. On the other hand, Fig. 6 is a plot of the values of $k_2$ as a function of the logarithm of the $Mg^{2+}$ concentration. The line through the points in Fig. 6 represents the best fit to an hyperbolic function with a $K_m$ of $38 \pm 3$ $\mu$M [$Mg^{2+}$] and a $k_{2max}$ of $0.199 \pm 0.005$ $ms^{-1}$. Under these conditions, the kinetic $K_m$ is a complex function of several constants, including that for $Mg^{2+}$ dissociation.

## Discussion

Early data on steady-state kinetics [2–4,11–15] has failed to solve the problem of the relative roles of




Scheme III

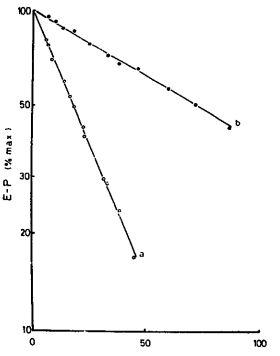



Fig. 2. Experiments designed to estimate the value of the off rate constant ($k_{-1}$) for free ATP binding to the NaE and for ATPMg binding to the E form of $Na^+,K^+$-ATPase. (a) Free ATP (open circles): Aliquots of 50 $\mu$g enzyme equilibrated with 100 mM NaCl, buffer and 0.1 mM CDTA (syringe 1) were mixed, in chamber 1, with 200 $\mu$M [$\gamma$-$^{32}$P]ATP and 0.1 mM CDTA (syringe 2); after 20 ms this suspension was mixed, in chamber 2, with the contents of syringe 3 (15 mM unlabelled ATP and 0.1 mM CDTA); after variable times the effluent of chamber 2 was mixed, in chamber 3, with 5 mM unlabelled ATP and 22.8 mM $MgCl_2$ (final free $[Mg^{2+}]$ about 1 mM). The phosphorylation reaction was stopped at a fixed time of 20 ms. The volume of each syringe was 1 ml and all contained 100 mM NaCl and 70 mM Tris-HCl (pH 7.4 at 20°C). Temperature was 20 ± 2°C. Note that the semilogarithmic plot of acid-stable phosphoenzyme as a function of the variable time between addition of unlabelled ATP and $MgCl_2$ gives a straight line with a negative slope. Three different experiments gave a value for $k_{-1}$ of 0.038 ± 0.004 $ms^{-1}$. (b) ATPMg (filled circles): Aliquots of 50 $\mu$g enzyme equilibrated with buffer and no sodium (syringe 1) were mixed, in chamber 1, with 200 $\mu$M [$\gamma$-$^{32}$P]ATP and 2.2 mM $MgCl_2$ (syringe 2); after 30 ms this suspension was mixed, in chamber 2, with the contents of syringe 3 (15 mM unlabelled ATP and 15 mM $MgCl_2$); after variable times the effluent of chamber 2 was mixed, in chamber 3, with 5 mM unlabelled ATP, 5.6 mM $MgCl_2$ (final free $[Mg^{2+}]$ of about 1 mM) and 400 mM NaCl (final concentration 100 mM). The phosphorylation reaction was stopped at a fixed time of 20 ms. The volume of each syringe was 1 ml and all contained 70 mM Tris-HCl (pH 7.4 at 20°C). Temperature was 20 ± 2°C. NaCl was present only during the phosphorylation reaction. Note that the semilogarithmic plot of acid-stable phosphoenzyme as a function of the variable time between addition of unlabelled ATP and NaCl gives a straight line with a negative slope. Three different experiments gave a value for $k_{-1}$ of 0.009 ± 0.002 $ms^{-1}$. See text for more details.

$Mg^{2+}$ and ATP in the $Na^+,K^+$-ATPase. Specially illustrative is the thorough work of Skou [15]. He found stimulation of the $Na^+,K^+$-ATPase activity by low $[Mg^{2+}]$ relative to that of total ATP followed by a sharp inhibition at higher $[Mg^{2+}]$; inhibition was also

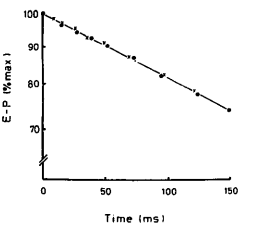



Fig. 3. Independent determination of the rate constant for the breakdown, in $Na^+$-containing $K^+$-free media, of EP formed from ATP ($k_3$). Three syringes and two mixing chambers were used. Aliquots of 50 $\mu$g enzyme, equilibrated in syringe 1 with 10 $\mu$M or 1000 $\mu$M $MgCl_2$ were mixed in chamber 1 with the contents of syringe 2 (20 $\mu$M [$\gamma$-$^{32}$P]ATP and 10 $\mu$M or 1000 $\mu$M free Mg; after about 100 ms the phosphorylation reaction was halted by mixing the suspension in chamber 2 with final concentrations of 1 mM ATP (filled circles) or 3 mM CDTA (crosses) coming from syringe 3. The dephosphorylation reaction was stopped at variable times up to about 150 ms. The volume of each syringe was 1 ml and all contained 100 mM NaCl and 70 mM imidazole (pH 7.4 at 20°C). Temperature was 20 ± 2°C. The EP levels at $t = 0$ dephosphorylation were obtained including the acid stopping solution in syringe 3. The figure is a semilogarithmic plot of the acid-stable EP as a function of the time elapse between the addition of ATP (or CDTA) and the stopping solution. From the slope of the straight line a value of 0.0019 ± 0.0002 $ms^{-1}$ ($n = 3$) was estimated. No difference was found when phosphorylation was stopped with ATP or CDTA; moreover the same $k_3$ was obtained in the presence of 10 $\mu$M or 1000 $\mu$M free $[Mg^{2+}]$. See text and Methods for other details.

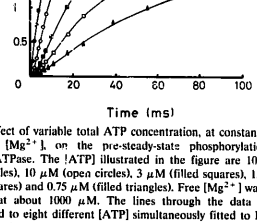



Fig. 4. Effect of variable total ATP concentration, at constant 1000 $\mu$M free $[Mg^{2+}]$, on the pre-steady-state phosphorylation of $Na^+,K^+$-ATPase. The [ATP] illustrated in the figure are 100 $\mu$M (filled circles), 10 $\mu$M (open circles), 3 $\mu$M (filled squares), 1.5 $\mu$M (open squares) and 0.75 $\mu$M (filled triangles). Free $[Mg^{2+}]$ was kept constant at about 1000 $\mu$M. The lines through the data points correspond to eight different [ATP] simultaneously fitted to Eqn. 1 using the values of $k_{-1}$ (for ATPMg) and $k_3$ obtained from experiments like those illustrated in Figs. 2 and 3. The estimates of the fitting parameters were 0.025 ± 0.003 $\mu M^{-1} ms^{-1}$ for $k_1$ and 0.196 ± 0.007 $ms^{-1}$ for $k_2$. Note: In addition to the ligands indicated above all solutions contained 100 mM NaCl and 70 mM imidazole (pH 7.4 at 20°C). For more details see text.

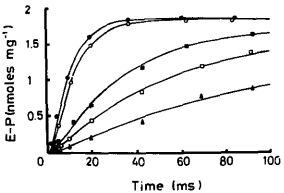



Fig. 5. Effect of variable free $Mg^{2+}$ concentrations, at constant 10 $\mu$M total [ATP], on the pre-steady state phosphorylation of $Na^+,K^+$-ATPase equilibrated with variable ionized $Mg^{2+}$ concentrations. The $[Mg^{2+}]$ illustrated in the figure are 193 $\mu$M (filled circles), 47 $\mu$M (open circles), 9 $\mu$M (filled squares), 4.5 $\mu$M (open squares) and 1.8 $\mu$M (filled triangles). The lines through the points correspond to two to three different experiments, consisting of eight different $[Mg^{2+}]$ simultaneously fitted to Eqn. 1. For that fitting, the values of $k_{-1}$, $k_3$ and $k_1$, obtained from experiments like those illustrated in Figs. 2, 3 and 4 were used; therefore, the only fitting parameter was $k_2$. All solutions contained 100 mM NaCl and 70 mM imidazole (pH 7.4 at 20°C) and the variable $[Mg^{2+}]$. For more details see text.

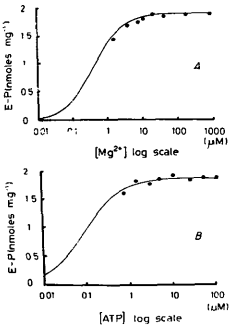



Fig. 7. Semilogarithmic plot of predicted (solid lines) and measured (filled circles) steady-state [EP] as a function of free $[Mg^{2+}]$ at constant 10 $\mu$M [ATP] (A) and of total [ATP] at constant 1000 $\mu$M free $[Mg^{2+}]$ (B). The predicted values were calculated, for a wide range of $[Mg^{2+}]$ and [ATP], from Eqn. 1 using the rate constants of Table II. Their plots were then fitted to a Michaelian function. The resulting $K_m$ values were $0.433 \pm 0.002$ $\mu$M for $Mg^{2+}$ and $0.094 \pm 0.002$ $\mu$M for total ATP. Note that the measured $[EP]_{ss}$ values fall close to the solid lines in both instances.

produced by increasing the [free ATP]/[ATPMg] ratio. This occurred at all [ATP] investigated. Looking into all possible roles of free ATP, ATPMg and free $Mg^{2+}$ he concluded that, with the steady-state strategy, there was no answer to the problem. In a recent abstract, Covarrubias and De Weer [14] went through a meticulous statistical analysis and arrived at a similar conclusion. In addition [14], they followed the simultaneous effects of $Mg^{2+}$ on two reactions at steady state: ATPase and ATP–ADP exchange. With that approach their data favours the separate binding of ATP and $Mg^{2+}$ ions. However, being an abstract one cannot know if a possible inhibition of ATP–ADP exchange by ADPMg [5] was considered. The use of pre-steady-state

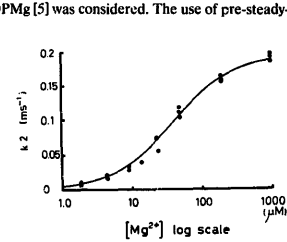
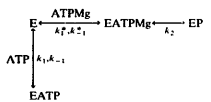



Fig. 6. Effects of $Mg^{2+}$ concentration on the value of the $k_2$ rate constant obtained from experiments like those illustrated in Fig. 5. The data points, plotted in semilogarithmic scale, were fitted to a Michaelian equation. The resulting values were a $k_{2max}$ of $0.199 \pm 0.005$ $ms^{-1}$ and $K_m$ for $Mg^{2+}$ of $38 \pm 3$ $\mu$M.

kinetics had already allowed some progress. Thus, the work of Mardh and Post [3] was the first to establish that: (i) a random binding of free ATP and $Mg^{2+}$ was feasible, and (ii) a preferential route may exist, because the release of ATP from the enzyme was four times faster than that of ATPMg.

The scheme used here is internally consistent and can fit the results very well, but its choice was still arbitrary; this is particularly true in Figs. 5 and 6. The question is if there are alternatives that can also account for the data. Before looking into that it seems important to see how reliable are the estimation of $k_{-1}$ and $k_3$. The 'off' rate constant ($k_{-1}$) for free ATP of $0.038 \pm 0.004$ $ms^{-1}$ agrees very well with 0.033 $ms^{-1}$ and 0.040 $ms^{-1}$ reported in pig kidney [3] and eel [33,34] $Na^+,K^+$-ATPase, respectively. The 'off' rate constant for ATPMg binding ($0.009 \pm 0.002$ $ms^{-1}$) is four times lower than that for free ATP. This coincides with the observation of Mardh and Post [3] who found that in enzyme preincubated with ATP and $Mg^{2+}$, $k_{-1}$ was reduced to about one fourth (0.009 $ms^{-1}$). It should be pointed out that, because $k_{-2}$ and $k_{-3}$ are zero (phosphorylation is irreversible) the system behaves as a sink; hence, the fitting of all the data points should be relatively insensitive to the value of $k_{-1}$. In fact, we found that the computed $k_1$ and $k_2$ were the same whether we took a $k_{-1}$ equal to 0.038 $ms^{-1}$ or



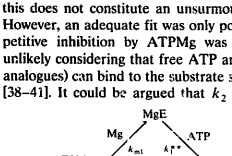

Scheme IV

0.009 $ms^{-1}$ (not shown). For the overall dephosphorylation rate constant ($k_3$), our estimate of 0.0019 $ms^{-1}$ agrees with the available 0.0028 $ms^{-1}$ [3] and 0.002 $ms^{-1}$ [33,34].

The lack of symmetry between [ATP] and [$MgCl_2$] (Fig. 1 and Table I) requires other effects of ATP and/or $MgCl_2$ than just forming the ATPMg complex. Let us consider them:

(a) $Mg^{2+}$ acts only by forming the ATPMg complex, which is the real substrate, without affecting $k_2$ in Eqn. 1; free ATP inhibits by competing with ATPMg (Scheme IV). This can explain Fig. 1 and Table I as well as Fig. 4, where most of the nucleotide is bound to $Mg^{2+}$. Therefore, the value of $k_1$ ($k_1^*$ here) would still be of the order of 0.025 $\mu M^{-1} ms^{-1}$ and that of $k_2$ around 0.2 $ms^{-1}$. However, this model gives inadequate fittings to the experimental points of Fig. 5: If we take each [$MgCl_2$] isolated from the others, an acceptable fitting can be obtained provided the combined values of the 'on' ($k_1$, $k_1^*$) and 'off' ($k_{-1}$, $k_{-1}^*$) rate constants result in a kinetic $K_m$ for free ATP much higher than that for ATPMg; in the case of 10 $\mu M$ $MgCl_2$ the difference is about 50-fold. Moreover, there is no way to get a simultaneous fit of all [$MgCl_2$] data (not shown).

(b) Free ATP is the real substrate and $Mg^{2+}$ increases $k_2$ in Eqn. 1 (Scheme V). In this case $k_1$ and $k_1^{**}$ should be around 0.25 $\mu M^{-1} ms^{-1}$ (that is 10-times the formerly computed $k_1$) to account for the data of Fig. 4. Although those values are more than 20-times higher than the published estimates [33,34,37], this does not constitute an unsurmountable difficulty. However, an adequate fit was only possible when competitive inhibition by ATPMg was ignored. This is unlikely considering that free ATP and ATPMg (or its analogues) can bind to the substrate site of the enzyme [38–41]. It could be argued that $k_2$ is actually higher




Scheme V

than estimated, and by proper adjustments a rise in $k_1$ would not be required. We tried it, but figures as unrealistic for $k_2$ as 100 $ms^{-1}$ did not work even when competition by ATPMg was disregarded; this was also the case with 100 $\mu M$ ATP, indicating it is not due to a rate limiting step at low [ATP] (not shown).

(c) Following the suggestion of one of the reviewers, we considered the possibility that the three Mg-enzyme-ATP species (MgEATP, EATPMg and MgEATPMg) can lead to EP formation. The potential alternative pathways, taking the forward reactions only, are:

(i) $E \xrightarrow{ATPMg} EATPMg \xrightarrow{k_2^{**}} EP$

(ii) $E \xrightarrow{ATPMg} EATPMg \xrightarrow{Mg} MgEATPMg \xrightarrow{k_2^*} EP$

(iii) $E \xrightarrow{ATP} EATP \xrightarrow{Mg} MgEATP \xrightarrow{k_2} EP$

(iv) $E \xrightarrow{ATP} EATP \xrightarrow{Mg} EATPMg \xrightarrow{Mg} MgEATPMg \xrightarrow{k_2^*} EP$

(v) $E \xrightarrow{Mg} MgE \xrightarrow{ATP} MgEATP \xrightarrow{Mg} MgEATPMg \xrightarrow{k_2^*} EP$

(vi) $E \xrightarrow{Mg} MgE \xrightarrow{ATPMg} MgEATPMg \xrightarrow{k_2^*} EP$

The interconversions MgEATP ⇌ MgEATPMg, EATP ⇌ MgEATP and EATP ⇌ EATPMg are also included. In fitting the data we took eight experimental conditions simultaneously. This was done first with those obtained at saturating concentration of [$Mg^{2+}$] (data points from Fig. 4), where most ATP was as ATPMg and the enzyme was taken as a MgE complex. The reduced scheme produced the following values: $k_1^{**} = 0.028 \pm 0.005$ $\mu M^{-1} ms^{-1}$; $k_1^{***} = 0.020 \pm 0.004$ $\mu M^{-1} ms^{-1}$; $k_2 = 0.30 \pm 0.05$ $ms^{-1}$; $k_2^* = 0.21 \pm 0.03$ $ms^{-1}$; i.e. the 'on' rate of binding to MgENa is the same for ATP and ATPMg; likewise, there is no difference between the rates of E-P formation from MgEATP and MgEATPMg. The overall scheme was used to fit the data at constant 10 $\mu M$ [ATP] and variable [$Mg^{2+}$] (the same as in Fig. 5). In this case we used the values obtained before for $k_1^{**}$, $k_1^{***}$, $k_2$ and $k_2^*$. When the system was left free to choose the remaining parameters a good fit (error function of 0.040) resulted in a value for $k_2^{**}$ equal to zero; those for Mg binding varied from 0.014 to 0.04 $\mu M^{-1} ms^{-1}$. On the other hand, if we kept $k_2^{**} = k_2 = k_2^*$ the fitting was poor (error function around 0.2) and the other parameters had unrealistic values (for instance there was no binding of $Mg^{2+}$ to EATP and EATPMg). In summary, the analysis indicates that MgEATP and MgEATPMg form E-P whereas EATPMg does not.

A scheme that can account for all the data presented in this work is one where free ATP and ATPMg are equally well suited to act as substrate. As mentioned before, several studies showed that free ATP and ATPMg can bind tightly to the substrate site of the $Na^+,K^+$-ATPase [3,38–42]. On the other hand, the simultaneous fit to Eqn. 1 in Fig. 5 can be attained with a unique $k_1$ (0.025 $\mu M^{-1} ms^{-1}$) despite the fact that the [free ATP]/[ATPMg] ratio varies from 49 to 0.47 (100-fold); this conforms with an scheme where $k_1$ is the same for both ATP species. But, increasing $[Mg^{2+}]$ stimulates the transphosphorylation rate. If this is not due to the formation of a ATPMg complex, $Mg^{2+}$ must act on a specific binding site on the enzyme. Separates sites for $Mg^{2+}$ and ATP have already been proposed [16,43]; we extended that view by considering that both free ATP and ATPMg can attach to the ATP site independently of what happens at the $Mg^{2+}$ binding site (paths (i), (ia), (ii) or (iia) in Introduction). The kinetic $K_m$ for $Mg^{2+}$ in Fig. 6 is 38 $\mu M$; this might appear incompatible with the equilibrium $K_d$ of 1000 $\mu M$ reported on the basis of $Mn^{2+}$ displacement from the enzyme [16]. Nonetheless, there may be a way out of this discrepancy. The original $K_d$ for $Mn^{2+}$ was 0.74 $\mu M$ [16]. In a latter publication [44] using a purer $Na^+,K^+$-ATPase, the $K_d$ for $Mn^{2+}$ under identical conditions was 0.21 $\mu M$; the affinity for $Mg^{2+}$ was not determined, but if it did follow that of $Mn^{2+}$ it should have been about 280 $\mu M$. It is not inconceivable that the $K_m$ of 38 $\mu M$ results from a combination of its kinetic estimation and a different enzyme preparation. Accepting that ATP (free or as ATPMg) and $Mg^{2+}$ bind to different sites on the enzyme the question remains as to what is the mechanism for the $Mg^{2+}$ effect. The original proposal [4,45] that high $[Mg^{2+}]$ was needed for the $E_1P$–$E_2P$ transition seems ruled out by the work of Klodos and Skou [46] who showed that the results at low $Mg^{2+}$ were actually due to EDTA. Kanazawa et al. [47] suggested the existence of two EATP complexes: one where ATP is loosely bound and does not phosphorylate and another where ATP is tightly bound and can phosphorylate. The role of $Mg^{2+}$ was precisely to transform the first into the second or to form the second directly. In this system $Mg^{2+}$ could accelerate the overall transphosphorylation reaction ($k_2$ in this work). Finally, it may be that $k_2$ is constant and independent of $[Mg^{2+}]$; in this case the results of Fig. 6 would express the rate-limiting step of $Mg^{2+}$ binding at non saturating $Mg^{2+}$ concentrations.

A summary of all the constants obtained with Eqn. 1 is in Table II. Among other things, they allow us to estimate the $K_m$ values for ATP ($k_{-1}/k_1$). The figures are 1.52 $\mu M$ for free ATP and 0.36 $\mu M$ for ATPMg. This represents two major discrepancies with binding data: (1) For free ATP, the $K_m$ is 7–13-fold higher than the equilibrium dialysis values of 0.22 $\mu M$ in kidney [39] and 0.12 $\mu M$ in ox brain [38] enzyme; however it is close to the 2.4 $\mu M$ calculated from pre-steady-state data in eel $Na^+,K^+$-ATPase [33, 34]. There is no clear explanation for the disparity; perhaps the lower temperature used in binding experiments (0–2°C) increases the affinity for the nucleotide. (2) For ATPMg our estimated $K_m$ (0.36 $\mu M$) is four times lower than for free ATP. This is the opposite to what has been reported in binding experiments with $\beta$-$\gamma$-imido ATP [40,41] where the presence of $Mg^{2+}$ decreases the affinity of the enzyme for the analogue. We can offer no explanation for the discrepancy; unless the ATPMg and Mg-analogue complexes are not equivalent. Our estimates result from a reduction of $k_{-1}$ at constant $k_1$. In experiments similar to ours, Mardh and Post [3] also reported a 4-fold reduction in the 'off' rate constant for ATP binding in the presence of $Mg^{2+}$. A relevant question is what one is actually measuring in experiments like those of Fig. 2 in the presence of high $[Mg^{2+}]$. That this is the 'off' rate of ATPMg binding is just an assumption. Equally possible is that they reflect the effects of $Mg^{2+}$ binding to the enzyme on the rate of ATP release, a difference in dissociation from $E_1$ and $E_1Na$ forms, or a mixture of both. Finally, an affinity of the enzyme which is higher for ATPMg than for free ATP might represent the existence of a preferential kinetic route where that of ATPMg is favoured. However, this is not necessarily the case because $k_{-1}$ is the only constant affected and the system behaves as a sink ($k_{-2}$ and $k_{-3}$ are zero). In accordance with this view, the computed $k_1$ was the same for free ATP and ATPMg; in addition, simulations were not appreciably different when $k_{-1}$ was taken as 0.038 $ms^{-1}$ or 0.009 $ms^{-1}$ (not shown).

Regarding $k_1$, a value of $2.5 \cdot 10^7$ $M^{-1}s^{-1}$, is only twice as large as other appraisals in eel [33,34] and pig

TABLE II

*Summary of the rate constants for on ($k_1$) and off ($k_{-1}$) ATP binding, phosphorylation ($k_2$) and dephosphorylation ($k_3$) of $Na^+,K^+$-ATPase in $Na^+$-containing $K^+$-free media*

All values are means ± S.D.

| Rate constant | Value and unit | | Effect of $Mg^{2+}$ |
|---|---|---|---|
| $k_1$ | $0.025 \pm 0.001$<br>$2.5 \cdot 10^7$ | $\mu M^{-1} ms^{-1}$<br>$M^{-1} s^{-1}$ | None up to 1 mM |
| $k_{-1}$ | | | |
| free ATP | $0.038 \pm 0.004$ | $ms^{-1}$ | [a] |
| ATPMg | $0.009 \pm 0.002$ | $ms^{-1}$ | [b] |
| $k_{2max}$ | $0.199 \pm 0.005$ | $ms^{-1}$ | Stimulates ($K_m$ 38 ± 3 $\mu M$) |
| $k_3$ | $0.0019 \pm 0.0002$ | $ms^{-1}$ | None up to 1 mM |

[a] 1 mM $Mg^{2+}$ was added at the time phosphorylation was started.
[b] 1 mM $Mg^{2+}$ was present during pre-incubation with [$\gamma$-$^{32}$P]ATP.

kidney enzymes [37] but much smaller than the $10^9$ $M^{-1}s^{-1}$ expected for a diffusion controlled process [48].

From the rate constants in Table II we computed EP in steady state ($[EP]_{ss}$) as a function of a wide range of $Mg^{2+}$ and ATP concentrations. Their plot could be fitted to Michaelian functions for both ligands, giving a $K_m(ss)$ of $0.433 \pm 0.002$ $\mu$M for $Mg^{2+}$ and $0.094 \pm 0.002$ $\mu$M for total ATP; these figures are lower than the 38 $\mu$M for $Mg^{2+}$ (Fig. 6) and the 1.52 $\mu$M or 0.36 $\mu$M for ATP obtained from the $k_{-1}/k_1$ ratio. From these estimates the solid lines in Figs. 7A and 7B were drawn. The measured $[EP]_{ss}$ (filled symbols in the figures) fall close to the predicted curves. This is very important, for it means that the $Na^+$-ATPase activity, which is the product of $[EP] \times k_3$, should have the same apparent affinitics for these ligands. At 37°C, the reported $K_m$ of that activity for ATP is below 0.5 $\mu$M [17,49], while that for $Mg^{2+}$ is around 12 $\mu$M [2]. On the other hand, at 0°C and at steady state, the $K_m$ for $MnCl_2$ in the formation of a MnEP complex with 5 $\mu$M ATP is 0.78 $\mu$M [9].

Lastly, if the real substrate for the reaction is total ATP, free and Mg-bound, it is likely that the $Mg^{2+}$ that binds tightly to the phosphoenzyme, forming the MgEP complex, is the same that attaches to the enzyme before phosphorylation takes place.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