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STEADY-STATE KINETIC STUDY OF MAGNESIUM AND ATP EFFECTS ON LIGAND AFFINITY AND CATALYTIC ACTIVITY OF SHEEP KIDNEY SODIUM, POTASSIUM-ADENOSINETRIPHOSPHATASE

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Summary

A non-linear least squares computer analysis of steady-state kinetic data of ATP hydrolysis catalyzed by sheep kidney (Na^+, K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) gives kinetic parameters of various enzyme intermediates.

Chemical models that fit the whole group of experimental data allow an overview of the effects of varying concentrations of Mg^{2+} and ATP on the ligand affinity and catalytic activity of the enzyme, and also make possible the following rationale in relation to the active transport of sodium and potassium across a cell membrane: In the presence of 60 mM of sodium and the absence of ATP, potassium binds favorably to the enzyme. This effective binding of potassium is consistent with extracellular binding of potassium prior to transport into a cell, because the extracellular medium consists of high sodium, low potassium and no ATP. Binding of potassium is decreased by free ATP and Mg · ATP complex. This "weakening" of potassium binding may be related to the low affinity sites of the enzyme for potassium inside a cell where ATP and Mg^{2+} are present. In the presence of 10 mM potassium, binding of sodium is insignificant, but is stimulated by Mg^{2+}, ATP, and Mg · ATP. This is consistent with the intracellular event of sodium binding prior to transport out of the cell. The breakdown of the Mg · ATP-containing enzyme complexes may cause release of potassium into cell and put the enzyme in a form that does not bind sodium favorably in the absence of ATP; hence, sodium is released to extracellular medium which contains no ATP.

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It was shown that a chemical model including Mg · ATP as a substrate is mathematically indistinguishable from one in which Mg · ATP is treated as a non-binding species.

Introduction

The membrane-bound (Na^+, K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) is responsible for the active transport of Na^+ and K^+ across biological membranes [1—5]. It has been found in erythrocytes that the hydrolysis of one molecule of ATP is usually associated with the efflux of about three Na^+ and the influx of about two K^+ [6—9]. Correlation of the properties of the (Na^+, K^+)-ATPase and the physiological transport phenomena has been supported mostly by the kinetic evidence obtained by fragmented membrane preparations and intact cells in vitro. In this context, attention has been focused on the effects of various ligands on the enzymatic hydrolysis of ATP. Previous kinetic data, using a crude preparation of beef brain (Na^+, K^+)-ATPase obtained in this laboratory, reflect the presumed in vivo stoichiometric relationship between ATP, sodium, and potassium; namely the hydrolysis of one ATP molecule most probably requires three sodiums and two potassiums at constant concentration of ATP (2.5 mM) and magnesium (2.5 mM) [10].

The present study was designed to approach the derivation of chemical kinetic models for the enzyme. (A portion of this study was presented at the 1975 Meeting of the Biophysical Society in Philadelphia, Biophys. J. (1975), 15, 244 Abstract). A purified sheep kidney (Na^+, K^+)-ATPase was used in order to minimize non-specific effects. From these studies, we obtained kinetic rate and dissociation constants associated with kinetically significant enzyme intermediates. The kinetic constants describe the effects of magnesium (another metal ion required for the hydrolytic activity) and ATP on the affinity of the enzyme for sodium and potassium, and on the initial rates of ATP hydrolysis under various ligand conditions. Also, best-fit steady-state kinetic models show that active enzyme complexes contain multiple numbers of Na^+ and K^+. The enzyme need not bind three sodiums and two potassiums simultaneously for ATP hydrolysis; active Mg · ATP-containing enzyme complexes that contain sodiums and potassiums in a ratio other than 3 to 2 may also decompose to yield products.

Two chemical models that are mathematically indistinguishable, but are in sharp contrast with respect to the involvement of the Mg · ATP complex as a substrate, are discussed. A rationale of the active transport of sodium and potassium across the intact cell membrane is also given in terms of the effects of magnesium and ATP on the kinetic dissociation constants of sodium- and potassium-enzyme complexes.

Methods

Equipment and materials. A flow diagram of the AutoAnalyzer manifold used for collecting kinetic data is shown in Fig. 1. The equipment consisted of a Proportioning Pump I, a colorimeter with a 15-mm tubular flow cell and a

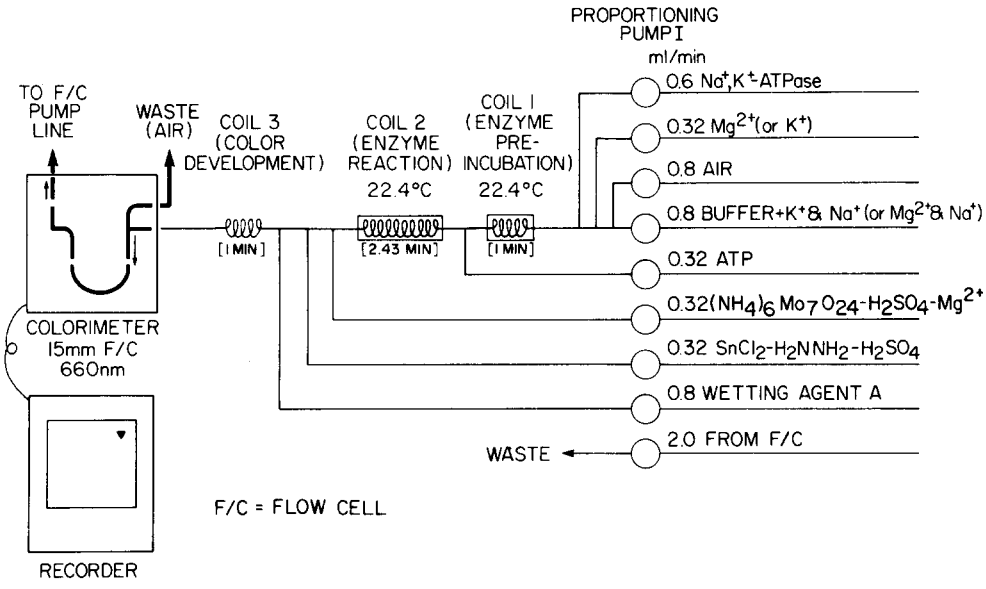


Fig. 1. Diagram of Technicon AutoAnalyzer manifold used for kinetic experiments. The numbers represent nominal pumping rates ml/min.

voltage stabilizer, and a single pen standard recorder. All modules, tubings, and fittings were products of Technicon Corp. The temperature of the water-jacketed preincubation coil 1 and reaction coil 2 was controlled with a Lauda Super K-2/R constant temperature circulator (Brinkmann Instruments). The temperature of the flowing solution in the coils was checked with a Telethermometer (Yellow Spring Instrument Company, Inc.) equipped with a microprobe.

All chemicals used in this work were Baker Analyzed reagents unless otherwise stated. The water used was glass-distilled deionized water. The concentration of ATP disodium salt (Sigma) stock solution in water was determined spectrophotometrically at 259 nm, using the extinction coefficient of 1.54×10^4 [11]. The solution was stored in a freezer before use. The concentration of $MgCl_2$ solution was determined by atomic absorption spectrophotometry (Beckmann DB-G), using a magnesium standard obtained from Fisher. Acid molybdate solution was prepared by dissolving, with stirring, 10 g of ammonium molybdate, 6 g of $MgCl_2 \cdot 6\,H_2O$ [12], and 50 ml of concentrated H_2SO_4 in 800 ml of water. The solution was then diluted to 1 l. Similarly, 1 l of stannous chloride/hydrazine sulfate solution consisting of 0.2 g of $SnCl_2 \cdot 2H_2O$, 2 g of $H_2NNH_2 \cdot H_2SO_4$ and 28 ml of concentrated H_2SO_4 was prepared [13]. Both solutions were stored at room temperature.

The (Na^+, K^+)-ATPase prepared from outer medulla of frozen sheep kidney was prepared by a previously described procedure [14] with some modifications and was kindly supplied to us by Dr. Lois K. Lane of this laboratory. The purified enzyme fraction consisted of only two polypeptides and associated phospholipids. The enzyme in reaction coil 2 (Fig. 1) was $1 \cdot 10^4$—$3 \cdot 10^4$-fold dilution of an ammonium sulfate-precipitated fraction (dialyzed for 3 days

to remove NH_4^+) suspended in a medium consisting of 25 mM imidazole and 1 mM Tris/EDTA. The protein concentrations was 10 mg/ml, determined by the method of Lowry et al. [15]. The specific activity of this enzyme, determined by the automated method, was 1.8 μmol $P_i \cdot mg^{-1} \cdot min^{-1}$ at pH 7.1, 22.4°C and in 100 mM imidazole buffer consisting of 0.9 mM ATP, 2.0 mM magnesium, 60 mM sodium and 2.0 mM potassium. The enzymatic activity of 0.01 mg/ml solution of this enzyme in the imidazole buffer was preserved for a few days when stored at 4°C. The specific activity of the ammonium sulfate-precipitated enzyme was 3.1 μmol $Pi \cdot mg^{-1} \cdot min^{-1}$, as determined by the Fiske-SubbaRow method [16] at pH 7.1, 22°C and in 30 mM histidine buffer consisting of 5 mM ATP, 5 mM magnesium, 100 mM Sodium and 10 mM potassium (Lane, L.K., personal communication).

Measurement of ATP hydrolysis. The automated method used for the phosphate assay is a modified Martin and Doty procedure [12,13]. All concentrations of reagents refer to the adjusted concentrations in reaction coil 2 (Fig. 1) unless otherwise stated. The enzyme was preincubated with necessary reagents for about 1 min (i.e. the time required for the solution to flow through coil 1) before the addition of ATP to initiate the reaction. The enzymatic hydrolysis of ATP was allowed to proceed for 2.43 min in reaction coil 2, and was then stopped immediately by acid from the ammonium molybdate line after the solution had flowed through the reaction coil. The phosphate ion formed was complexed with the acid ammonium molybdate solution in the color development coil 3, and was assayed at 660 nm as a blue complex formed after the addition of $SnCl_2/H_2NNH_2$ solution. An optimum quantity of magnesium (6 g in 1 l of ammonium molybdate solution) [12] and adequate length of the color development coil 3 were used to minimize the effect of ATP on the phosphate assay.

Kinetic experiments. The standard phosphate (Fisher) assay and the effect of ATP on the phosphate assay are shown in Fig. 2, which shows that the linear relationship of absorbance of the blue complex to the phosphate concentration holds at different levels of ATP.

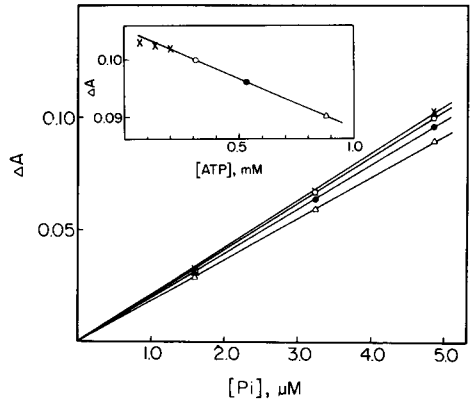


Fig. 2. Effect of ATP on standard phosphate assay. ATP concentrations are: x, 0—0.198 mM; ○, 0.307 mM; ●, 0.527 mM; △, 0.878 mM. Other conditions are: 22.4°C, 100 mM imidazole buffer, pH 7.1, 60 mM sodium, 10 mM potassium and 2.0 mM magnesium. Inset, demonstration of linear suppression of absorbance of 4.87 μM P_i by ATP. Similar effects of ATP on P_i assay were observed under various conditions.

The responses of ATP hydrolysis to increasing enzyme concentration was determined after correction for small absorbance changes observed at each enzyme concentration in the absence of ATP. The required linear relationship was observed over the range of enzyme concentrations (1—3 mg/l).

The time course of ATP hydrolysis was followed by separate measurement of the extent of the reaction in 1.25 and 2.43 min using two coils of different lengths. These experiments were carried out at temperatures ranging from 15 to 37°C (Fig. 3). Adequate initial velocity at 2.43 min could be obtained below 24°C. An Arrhenius plot of these data shows no break point in the temperature range from 15 to 24°C.

All kinetic experiments were carried out at pH 7.1, 22.4°C, and in 100 mM imidazole buffer. Concentration ranges employed for various ligands are: ATP, 0.066—0.878 mM; Mg^{2+}, 0.02—7.4 mM; Na^+, 2.5—60 mM; K^+, 0.125—10 mM. At constant sodium (60 mM) the effects of seven concentrations of magnesium (0.02, 0.05, 0.08, 0.12, 0.56, 1.9, and 4.5 mM) and six concentrations of ATP (0.066, 0.132, 0.197, 0.307, 0.527, and 0.878 mM) on the initial rates of ATP hydrolysis were studied at eight concentrations of potassium (0.125, 0.25,

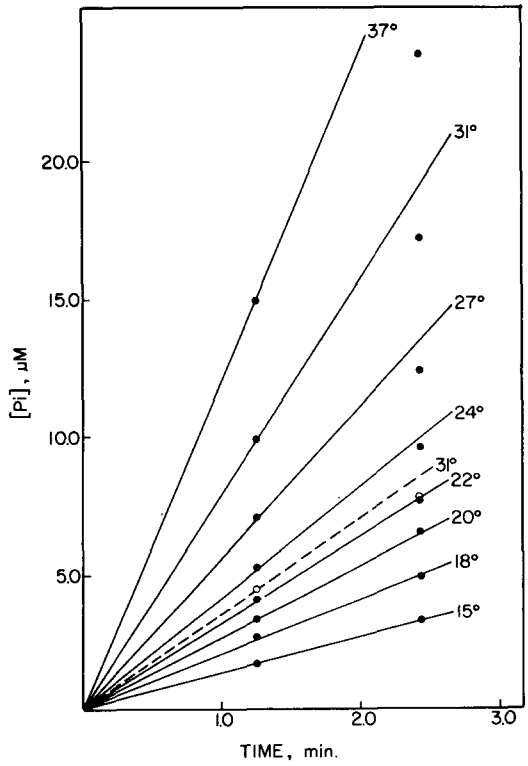


Fig. 3. Effect of temperature on initial production of phosphate (P_i) in ($Na^+ + K^+$)-ATPase-catalyzed hydrolysis of ATP. Lines were drawn from the origin through the values obtained at 1.25 min. Deviation of the values obtained at 2.43 min were apparent at temperatures above 24°C. The broken line (- - - o - - -) is a representative run usingabout 2-fold diluted enzyme solution, showing that deviation from initial velocity is independent of enzyme concentration. Reaction conditions are: 100 mM imidazole buffer, pH approx. 7, 60 mM sodium, 10 mM potassium, 2 mM magnesium, 0.878 mM ATP and (Na^+, K^+)-ATPase approx. 2 mg/l. Similar results were observed using 0.066 mM ATP.

0.50, 1.0, 2.0, 3.5, 6.0, and 10 mM). At constant potassium (10 mM) the effects of eight concentrations of magnesium (0.02, 0.05, 0.08, 0.19, 0.56, 1.9, 4.5, and 7.4 mM) and six concentrations of ATP (0.066, 0.132, 0.197, 0.307, 0.527, and 0.878 mM) were studied at seven concentrations of sodium (2.5, 5.5, 10.5, 20.5, 30.5, 46.0 and 60.0 mM). In the procedure, a series of six ATP solutions of various concentrations was used for each level of Mg^{2+}, Na^+ and K^+ which were infused from separate lines. Data that were treated as the true effect on ATP hydrolysis of various levels of each ligand, Mg^{2+}, for example, were obtained in the following manner: background (no added Mg^{2+}) ATP hydrolysis in the presence of enzyme at desired levels of Na^+ and K^+ was first recorded which was then subtracted from the amount of ATP hydrolysis at various levels of Mg^{2+} under the same condition. Typical experimental runs are shown in Fig. 4. Initial velocities obtained from different enzyme solutions were normalized to 3 mg/l enzyme activity by comparing data obtained under identical ligand concentration. Data were also corrected for the additional sodium from Na_2ATP and for the effect of ATP on phosphate assay. The maximum hydrolysis of ATP in any experiment was 4%.

The total ionic strength of the reaction medium was 120—140 mM when the sodium concentration was held at 60 mM. At 10 mM potassium, when less than 60 mM sodium was used, choline chloride was added to maintain the total concentration of sodium and choline chloride at 60 mM. Choline chloride up to 100 mM did not interfere with the automated assay of phosphate ion.

Analysis of kinetic data. Because of the high reproducibility of the automated method in mixing reagents of various concentrations and in the recorder reading of absorbance, no attempt was made to duplicate each of a large number of observed initial velocities. Ligand concentrations were considered accurately known.

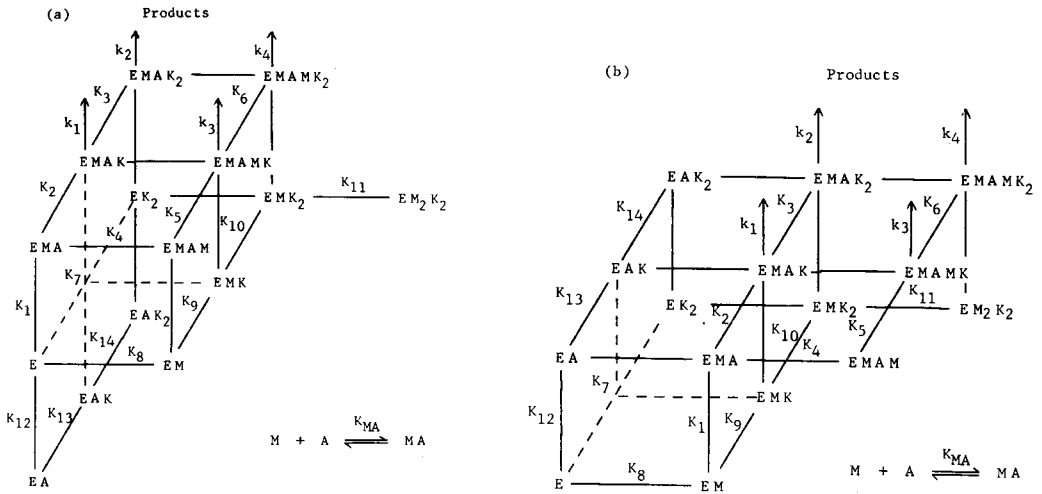
A non-linear least squares computer program (BMD07R) [17], which is applicable to the estimation of up to 100 parameters, was used for the data analysis. This program minimizes the error mean square (s^2) for the defined function v by means of stepwise Gauss-Newton iteration on $\theta_1, ..., \theta_p$.

$$v = f(C_1, ..., C_t; \theta_1, ..., \theta_p)$$

$$s^2 = \frac{1}{n-p} \sum_{i=1}^{\eta} ([v_i - f(C_{i1}, ..., C_{it}; \theta_1, ..., \theta_p)]^2 \cdot w_i)$$

In these equations, n is the total number of observations; v_i, observed initial velocities (i.e. dependent variables); $C_{i1}, ..., C_{it}$, concentration or product of concentrations of various ligands (i.e. independent variables); and $\theta_1, ..., \theta_p$, parameters (i.e. coefficients of $C_{i1}, ..., C_{it}$) to be estimated. Equal weights of unity were used in this work.

The algebraic equation used for data analysis is in the form of Eqs. 1 and 2. These equations are derived, respectively, from Schemes 1(a) and 2(a) in which rapid equilibrium is assumed among all enzyme complexes and the products are formed in rate-determining steps.



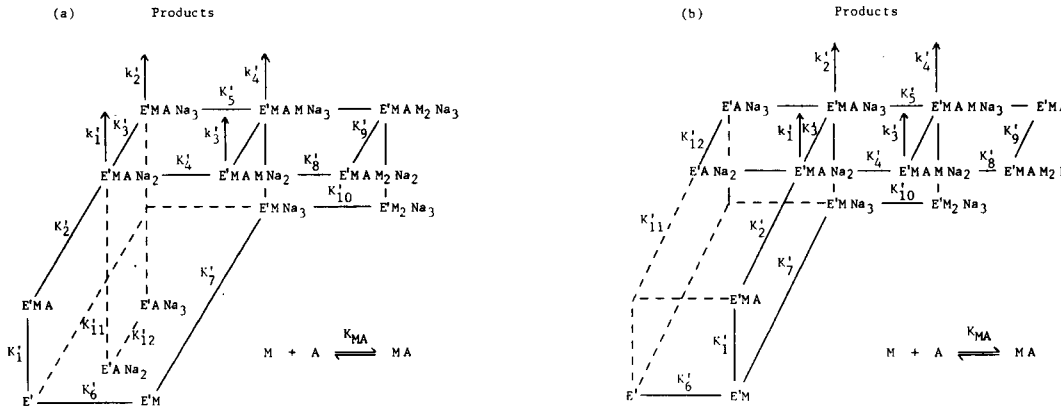
Schemes 1(a) and 1(b). Chemical kinetic models that best fit the data obtained for varying concentrations of ATP (A), magnesium (M) and potassium (K) at 60 mM sodium. K_1—K_{14} are kinetic dissociation constants and k_1—k_4 are rate constants for decomposition of MA-containing enzyme complexes to inorganic phosphate.

$$\frac{v_{obs}}{[E]_0} = k_{obs} = \frac{\frac{k_1}{K_1K_2}[MA][K] + \frac{k_2}{K_1K_2K_3}[MA][K]^2 + \frac{k_3}{K_1K_4K_5}[MA][M][K] + \frac{k_4}{K_1K_4K_5K_6}[MA][M][K]^2}{1 + \frac{1}{K_1}[MA] + \frac{1}{K_1K_2}[MA][K] + \frac{1}{K_1K_2K_3}[MA][K]^2 + \frac{1}{K_1K_4}[MA][M] + \frac{1}{K_1K_4K_5}[MA][M][K]}$$

$$+ \frac{1}{K_1K_4K_5K_6}[MA][M][K]^2 + \frac{1}{K_7}[K]^2 + \frac{1}{K_8}[M] + \frac{1}{K_8K_9}[M][K] + \frac{1}{K_8K_9K_{10}}[M][K]^2$$

$$+ \frac{1}{K_8K_9K_{10}K_{11}}[M]^2[K]^2 + \frac{1}{K_{12}}[A] + \frac{1}{K_{12}K_{13}}[A][K] + \frac{1}{K_{12}K_{13}K_{14}}[A][K]^2$$

$$[MA] = \frac{[A]_0 + [M]_0 + K_{MA} - \sqrt{([A]_0 + [M]_0 + K_{MA})^2 - 4[A]_0[M]_0}}{2.0}; [M] = [M]_0 - [MA]; [A] = [A]_0 - [MA] \qquad (1)$$



Schemes 2(a) and 2(b). Chemical kinetic models that best fit the data obtained for varying concentrations of ATP (A), magnesium (M) and sodium (Na) at 10 mM potassium. K'_1—K'_{12} are kinetic dissociation constants and k'_1—k'_4 are rate constants for decomposition of MA-containing enzyme complexes to inorganic phosphate.

$$\frac{v_{obs}}{[E']_0} = k_{obs} = \frac{\frac{k'_1}{K'_1K'_2}[MA][Na]^2 + \frac{k'_2}{K'_1K'_2K'_3}[MA][Na]^3 + \frac{k'_4}{K'_1K'_1K'_3K'_5}[MA][M][Na]^3}{1 + \frac{1}{K'_1}[MA] + \frac{1}{K'_1K'_2}[MA][Na]^2 + \frac{1}{K'_1K'_2K'_3}[MA][Na]^3 + \frac{1}{K'_1K'_2K'_4}[MA][M][Na]^2}$$

$$+ \frac{1}{K'_1K'_2K'_3K'_5}[MA][M][Na]^3 + \frac{1}{K'_6}[M] + \frac{1}{K'_6K'_7}[M][Na]^3 + \frac{1}{K'_1K'_2K'_4K'_8}[MA][M]^2[Na]^2$$

$$+ \frac{1}{K'_1K'_2K'_4K'_8K'_9}[MA][M]^2[Na]^3 + \frac{1}{K'_6K'_7K'_{10}}[M]^2[Na]^3 + \frac{1}{K'_{11}}[A][Na]^2 + \frac{1}{K'_{11}K'_{12}}[A][Na]^3$$

$$[MA] = \frac{[A]_0 + [M]_0 + K_{MA} - \sqrt{([A]_0 + [M]_0 + K_{MA})^2 - 4[A]_0[M]_0}}{2.0}; [M] = [M]_0 - [MA]; [A] = [A]_0 - [MA] \qquad (2)$$

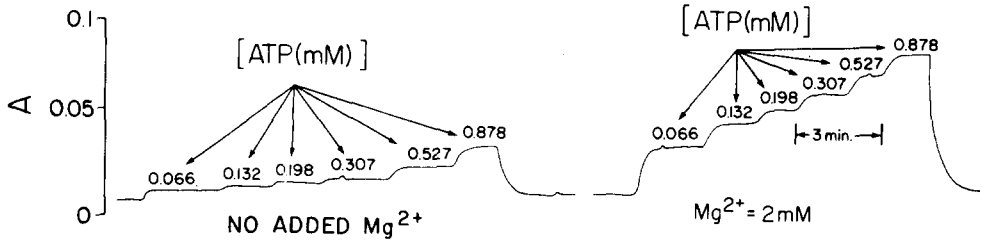


Fig. 4. Recorder traces of typical kinetic runs in the presence and absence of magnesium. Reaction conditions are: 24.4°C, 100 mM imidazole buffer, pH 7.1, 60 mM sodium, 10 mM potassium and approx. 1 mg/l (Na^+, K^+)-ATPase. ATP and magnesium concentrations are shown in the figure.

A fixed dissociation constant for the Mg·ATP complex, $K_{Mg \cdot ATP}$, was used in each computation. Five values of this constant were examined: 0.005, 0.01, 0.05, 0.1, 0.5 mM. Inclusion of this constant as an adjustable parameter necessitates the use of a set of partial differential equations (of the dependent variable with respect to each of the parameters to be estimated) in a complicated form; computation using these equations would increase tremendously the amount of computer time required.

Initial estimates of parameters required by the computer program were obtained from primary (graphical) plots of pertinent data. For example, rough estimates of dissociation constants associated with enzyme species containing two potassiums in Scheme 1(a) were obtained from a family of sigmoidal curves of initial velocity vs. potassium concentration. At 1.9 mM magnesium and 0.878 mM ATP, the curve gave a value for $K_5 \cdot K_6$. At the above magnesium level various constants were obtained at various ATP concentrations and extrapolation of these constants to zero ATP concentration gave a value for $K_9 \cdot K_{10}$. Based on these two values, reasonable initial estimates of K_5, K_6, K_9 and K_{10} could be obtained. The initial estimate for the maximal velocity was usually a value 1.5—2 times as high as the observed maximum velocity in the appropriate region of ligand concentrations. The upper and lower constraints of the parameter values required for computation differ two orders of magnitude from the initial estimates for the parameters.

The error mean square and asymptotic standard deviation of parameters are two criteria used for judging the best fit of data. The uniqueness of the resultant minima and parameter values was determined by using different initial estimates of parameters and parameter constraints in the computation.

Protonated species of ATP at pH 7.1, association of sodium and potassium with ATP, which is usually weak [18] and association of magnesium with imidazole buffer and with a very small amount of EDTA (from enzyme preparation) were ignored in the data analysis for simplicity.

Results

In the assay of phosphate ion by the automated procedure (Fig. 1), the effect of ATP on the phosphate color development (Fig. 2) was, unlike previously reported [12], significant and was proportional to the ATP concentration if greater than 0.2 mM. A 10% reduction in absorbance was observed in the presence of 0.9 mM ATP, the highest concentration of ATP used in this work. Fig. 3 shows that the adequate initial velocity could not be maintained

for 2.43 min at a temperature higher than 24°C in the imidazole buffer (100 mM, pH 7.1). For this reason, kinetic experiments of this study were carried out at 22.4°C.

All parameters in Eqs. 1 and 2 converged to a minimum when fixed values for the Mg·ATP complex dissociation constant ($K_{Mg\cdot ATP}$ = 0.005, 0.01, 0.05, 0.1, and 0.5 mM) were used in the iteration. The dependence of parameter values on $K_{Mg\cdot ATP}$ is worth noting. The results show that a change in $K_{Mg\cdot ATP}$ from 0.05 to 0.1 mM results only in a small change in all parameters. However, an increase in $K_{Mg\cdot ATP}$ from 0.1 to 0.5 mM or a decrease from 0.05 to 0.01 mM (and 0.005 mM) causes a significant increase in the values of a subset of the parameters. Thus, the minimal or close to minimal values of the whole set of parameters were obtained when $K_{Mg\cdot ATP}$ is in the range from 0.05 to 0.1 mM. The associated error mean squares are smaller with $K_{Mg\cdot ATP}$ = 0.05 mM than 0.1 mM: $1.18\cdot10^{-3}$ vs. $1.22\cdot10^{-3}$ for Eq. 1 and $1.27\cdot10^{-3}$ vs. $1.51\cdot10^{-3}$ for Eq. 2 (the size of quantities measured is less than 2.0 as shown in Figs. 5 and 6).

The resultant best fit parameters of Eqs. 1 and 2 using 0.05 mM for $K_{Mg\cdot ATP}$ are tabulated in Table I with their asymptotic standard deviations. In the case of Eq. 2, the same best-fit values of the parameters were obtained when the initial estimates of the first eleven (arbitrarily chosen) parameters are increased and the remaining parameters decreased by two orders of magnitudes, and also when the upper and lower constraints of parameters were changed to $+10^{20}$ and -10^{20}. The best-fit parameters of Eq. 1 were based solely on error mean square and asymptotic standard deviation. Various sets of parameters with unreasonably high standard deviations (which render a parameter value negative) were obtained when different initial estimates of parameters, varied as stated above, were used for Eq. 1.

The kinetic rate and dissociation constants reported below are defined by the chemical Schems 1(a), 1(b), 2(a), and 2(b) and their numerical values are derived from the mean values of the parameters in Table I.

At 60 mM sodium, the shapes of the sigmoidal curves of initial velocities vs. $[K^+]$ (at various Mg^{2+} and ATP concentrations, not shown) do not justify the need to fit the experimental data to a model that includes enzyme species containing more than two potassiums. Neither do the shapes of initial velocities vs. $[Na^+]$ curves justify a model with enzyme species containing more than three sodiums.

Effects of Mg^{2+} and ATP on (Na^+, K^+)-ATPase at constant sodium (60 mM)

Fig. 5 shows plots of the observed rate constants ($k_{obs} = v_{obs}/[E]_0$) versus magnesium concentrations. The solid lines in this figure are calculated with best-fit values of parameters of Eq. 1. The initial velocity of ATP hydrolysis at 60 mM sodium can be extrapolated to zero at zero potassium concentration (plots not shown), therefore ATP hydrolysis due to Na-ATPase is considered insignificant.

Schemes 1(a) and 1(b) show the chemical models that best fit the whole set of data. In these schemes missing enzyme (E) complexes such as E·K and $E\cdot Mg\cdot ATP\cdot Mg_2\cdot K_2$ were first included in computation, but they were rejected because the coefficients of the corresponding ligand concentrations $[K^+]$ and

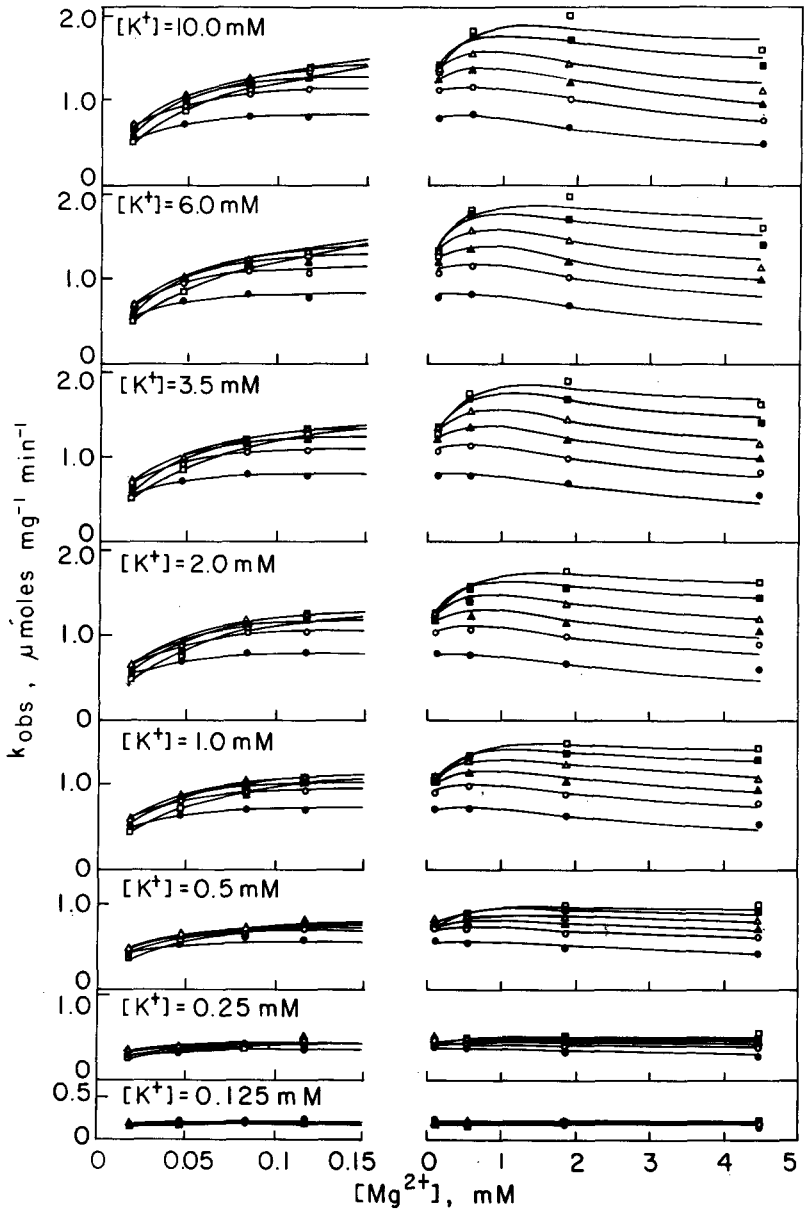


Fig. 5. Plots of observed rate constants (k_{obs}) versus magnesium concentrations demonstrating the fit of calculated curves to all experimental points at eight levels of potassium (as shown) and six levels of ATP: ●, 0.066 mM; ○, 0.132 mM; ▲, 0.198 mM; △, 0.307 mM; ■, 0.527 mM; and □, 0.878 mM. Sodium concentration was fixed at 60.1—61.8 mM (range due to additional Na^+ from Na_2ATP). Curves were calculated with the best-fit parameters (Eq. 1) in Table I. Other conditions are: 22.4°C, 100 mM imidazole buffer, pH 7.1, and 3 mg/l (Na^+, K^+)-ATPase.

$[Mg{\cdot}ATP][Mg]^2[K^+]^2$, when included in Eq. 1, drift to very small values in the process of interation; these small values indicate very large dissociation constants associated with these complexes, hence their presence can be considered insignificant.

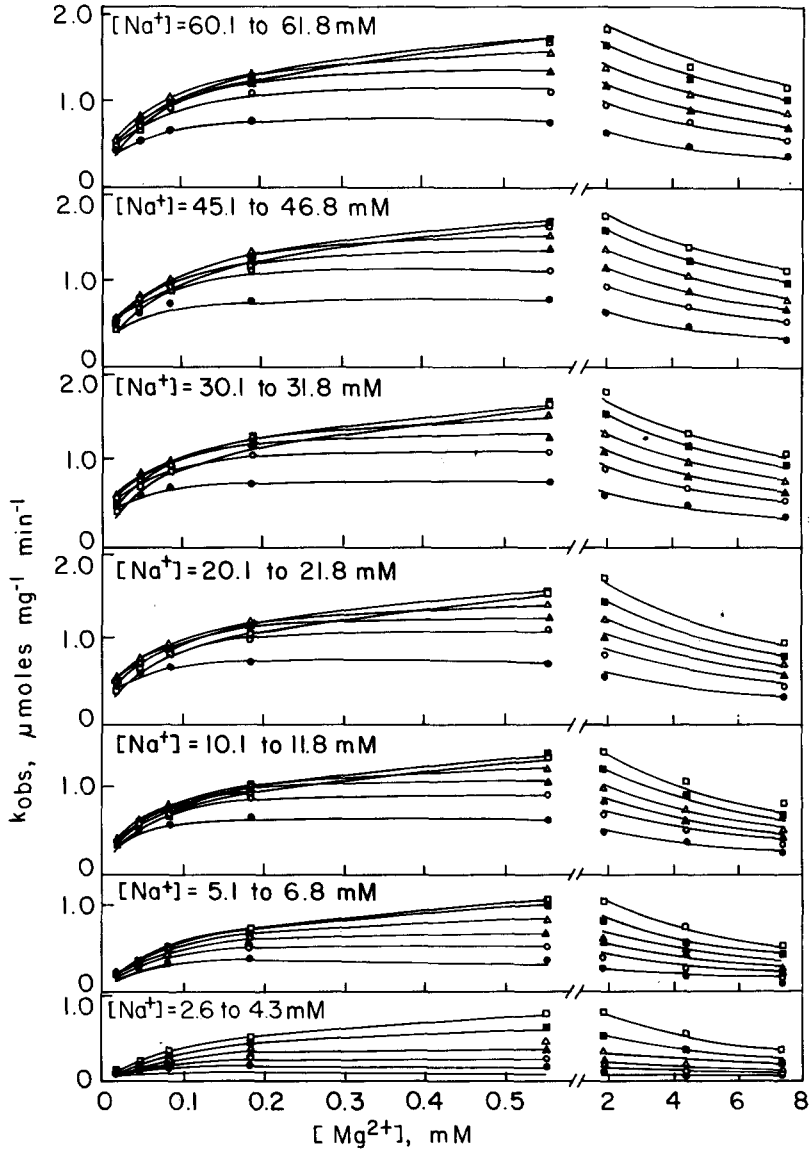


Fig. 6. Plots of observed rate constants (k_{obs}) versus magnesium concentrations demonstrating the fit of calculated curves to all experimental points at seven levels of sodium (as shown, range due to additional Na^+ from Na_2ATP) and six levels of ATP: ●, 0.066 mM; ○, 0.132 mM; ▲, 0.198 mM; △, 0.307 mM; ■, 0.527 mM; and □, 0.878 mM. Potassium concentration was fixed at 10 mM. Curves were calculated with the best-fit parameters (Eq. 2) in Table I. Other conditions are: 22.4°C, 100 mM imidazole buffer, pH 7.1 and 3 mg/l (Na^+, K^+)-ATPase.

Scheme 1(a) shows that at 60 mM sodium the affinity of the enzyme for Mg·ATP complex is higher than for free ATP by nearly 60-fold ($K_1 = 3.6 \cdot 10^{-4}$ and $K_{12} = 2.0 \cdot 10^{-2}$ mM) in the absence of potassium and by 20-fold ($K_{17} = 1.3 \cdot 10^{-2}$ mM for $E \cdot K_2 \rightleftharpoons E \cdot Mg \cdot ATP \cdot K_2$ and $K_{22} = 2.7 \cdot 10^{-1}$ mM for $E \cdot K_2 \rightleftharpoons E \cdot K_2 \cdot ATP$) in the presence of high potassium. Magnesium present at the magnesium site (E·Mg) decreases the affinity of the enzyme for Mg·ATP complex,

TABLE I

NUMERICAL VALUES OF PARAMETERS OF EQUATIONS 1 AND 2

All parameters in each equation were obtained simultaneously using the same set of data. A fixed value of Mg·ATP dissociation constant, 0.05 mM, was used in computation. Units of kinetic rate constants (k_1—k_4 and k'_1—k'_4) are in μmol·mg^{-1}·min^{-1}; units of kinetic dissociation constants are in mM except K_7 (mM^2), K'_2 (mM^2), K'_7 (mM^3) and K'_{11} (mM^3).

Equation 1		Equation 2	
k_1/K_1K_2	$(5.52 \pm 2.03) \times 10^6$	$k'_1/K'_1K'_2$	$(7.39 \pm 0.57) \times 10^2$
$k_2/K_1K_2K_3$	$(1.59 \pm 0.32) \times 10^8$	$k'_2/K'_1K'_2K'_3$	$(2.43 \pm 1.14) \times 10$
$k_3/K_1K_4K_5$	$(3.24 \pm 0.36) \times 10^7$	$k'_4/K'_1K'_2K'_3K'_5$	$(2.31 \pm 0.65) \times 10^3$
$k_4/K_1K_4K_5K_6$	$(5.97 \pm 0.0\ ) \times 10^8$	$1/K'_1$	2.64 ± 0.98
$1/K_1$	$(2.74 \pm 0.74) \times 10^3$	$1/K'_1K'_2$	$(4.94 \pm 0.50) \times 10^{-1}$
$1/K_1K_2$	$(5.76 \pm 0.98) \times 10^3$	$1/K'_1K'_2K'_3$	$(1.13 \pm 0.57) \times 10^{-2}$
$1/K_1K_2K_3$	$(1.17 \pm 0.14) \times 10^4$	$1/K'_1K'_2K'_4$	2.09 ± 0.69
$1/K_1K_4$	$(7.74 \pm 1.03) \times 10^4$	$1/K'_1K'_2K'_3K'_5$	$(6.58 \pm 1.85) \times 10^{-1}$
$1/K_1K_4K_5$	$(5.30 \pm 0.88) \times 10^4$	$1/K'_6$	$(1.97 \pm 0.53) \times 10$
$1/K_1K_4K_5K_6$	$(1.96 \pm 0.03) \times 10^5$	$1/K'_6K'_7$	$(7.17 \pm 1.98) \times 10^{-2}$
$1/K_7$	$(1.54 \pm 0.27) \times 10^2$	$1/K'_1K'_2K'_4K'_8$	1.63 ± 0.45
$1/K_8$	$(8.67 \pm 5.63) \times 10^2$	$1/K'_1K'_2K'_4K'_8K'_9$	$(3.54 \pm 1.08) \times 10^{-2}$
$1/K_8K_9$	$(3.67 \pm 1.61) \times 10^3$	$1/K'_6K'_7K'_{10}$	$(2.15 \pm 0.63) \times 10^{-2}$
$1/K_8K_9K_{10}$	$(1.85 \pm 0.07) \times 10^4$	$1/K'_{11}$	$(3.08 \pm 0.51) \times 10^{-2}$
$1/K_8K_9K_{10}K_{11}$	$(6.53 \pm 0.25) \times 10^3$	$1/K'_{11}K'_{12}$	$(3.82 \pm 2.62) \times 10^{-4}$
$1/K_{12}$	$(4.97 \pm 3.14) \times 10$		
$1/K_{12}K_{13}$	$(9.07 \pm 8.95) \times 10$		
$1/K_{12}K_{13}K_{14}$	$(5.69 \pm 0.57) \times 10^2$		

both in the presence and absence of potassium by about 30—7-fold (K_1 and $K_{18} = 1.1 \cdot 10^{-2}$ mM for E·Mg ⇌ E·Mg·ATP·Mg; K_{17} and $K_{20} = 9.4 \cdot 10^{-1}$ mM for E·Mg·K_2 ⇌ E·Mg·ATP·Mg·K_2). Despite this decrease in affinity for the Mg·ATP complex that is required for activity, the overall hydrolytic activity of the enzyme was compensated for by higher decomposition rates of the resultant Mg·ATP·Mg-containing complexes, especially at low potassium concentrations: E·Mg·ATP·Mg·K_2 decomposes at nearly the same rate as E·Mg·ATP·K_2 ($k_4 = 2.1$ and $k_2 = 1.9$ μmol/mg per min), whereas E·Mg·ATP·Mg·K decomposes about three times as fast as E·Mg·ATP·K ($k_3 = 2.1$ and $k_1 = 6.7 \cdot 10^{-1}$ μmol/mg per min). It is noteworthy that enzyme complexes containing one and two potassiums can decompose to yield products.

In the presence of 60 mM sodium, the effects of magnesium and ATP on the affinity of the enzyme for potassium are significant. The results show that when the medium is free of magnesium and ATP, a condition obtained by extrapolation to zero magnesium and ATP concentrations, the binding of the first potassium increases the affinity for the second potassium to such an extent that the presence of E·K becomes insignificant. This cooperative binding of potassium is not so effective when the enzyme is bound with magnesium, Mg·ATP, or free ATP. In these cases enzyme complexes containing only one potassium are also present. The first and the second dissociation constants with respect to potassium are nearly equal when the enzyme contains Mg^{2+} or Mg·ATP ($K_9 = 2.4 \cdot 10^{-1}$ and $K_{10} = 2.0 \cdot 10^{-1}$ mM; $K_2 = 4.8 \cdot 10^{-1}$ and $K_3 = 4.9 \cdot 10^{-1}$ mM); the second dissociation constant is smaller than the first one when the enzyme contains Mg·ATP·Mg or ATP ($K_5 = 1.46$ and $K_6 = 2.7 \cdot 10^{-1}$ mM; $K_{13} = 5.5 \cdot 10^{-1}$ and $K_{14} = 1.6 \cdot 10^{-1}$ mM).

In the absence of ATP, two potassiums bind strongly to the enzyme both with and without the magnesium site occupied ($K_7 = 6.5 \cdot 10^{-3}$ mM^2 and $K_9 \cdot K_{10} = 4.8 \cdot 10^{-2}$ mM^2); but the affinity of the enzyme for two potassiums was reduced by free ATP ($K_{13} \cdot K_{14} = 4.8 \cdot 10^{-2}$ mM^2) and by Mg·ATP complex ($K_2 \cdot K_3 = 2.4 \cdot 10^{-1}$ and $K_5 \cdot K_6 = 3.9 \cdot 10^{-1}$ mM^2) by 8—40-fold. On the other hand, potassium reduces the affinity of the enzyme for Mg·ATP by 36-fold (K_1 and K_{17}); the reduction in affinity for Mg·ATP is 9-fold when the magnesium site is occupied (K_{18} and K_{20}). Potassium also reduces the affinity of the enzyme for free ATP by 14-fold (K_{12} and K_{22}) and reduces the affinity of the enzyme for free magnesium by 7-fold in the absence of Mg·ATP ($K_8 = 1.2 \cdot 10^{-3}$ and $K_{21} = 8.3 \cdot 10^{-3}$ mM for $E \cdot K_2 \rightleftharpoons E \cdot Mg \cdot K_2$) by about 3-fold in the presence of Mg·ATP ($K_4 = 3.5 \cdot 10^{-2}$ and $K_{16} = 1.2 \cdot 10^{-1}$ mM for $E \cdot Mg \cdot ATP \cdot K_2 \rightleftharpoons E \cdot Mg \cdot ATP \cdot Mg \cdot K_2$).

While magnesium is required for the enzymatic activity, it inhibits the activity when present in large excess over ATP at high sodium and potassium concentration by forming an inhibitory complex $E \cdot Mg \cdot ATP \cdot Mg_2 \cdot K_2$ ($K_{11} =$ 2.8 mM). Free ATP, when present in excess over magnesium, inhibits the enzymatic activity through its competition with Mg·ATP for the enzyme (K_{12} and K_{22}).

Mathematically, substitution of $[Mg][ATP]/K_{Mg \cdot ATP}$ for [Mg·ATP] in Eq. 1 results in an equation in which the coefficients of all the terms containing [Mg]-[ATP] are the original parameters multiplied by $1/K_{Mg \cdot ATP}$. For example, $(1/K_1K_2)$[Mg·ATP][K] becomes $(1/K_1K_2K_{Mg \cdot ATP})$[Mg][ATP][K]. Thus, this new mathematical equation is numerically identical with the original one and can be derived from the alternative Scheme 1(b). Scheme 1(b) is in sharp contrast to Scheme 1(a) with respect to the involvement of Mg·ATP as a substrate: the Mg·ATP complex is a substrate in Scheme 1(a), but is a non-binding species in Scheme 1(b). This alternative model offers information about the affinities of the magnesium-containing enzyme for ATP ($K_1 = 1.6 \cdot 10^{-2}$ mM; $K_{17} = 3.2 \cdot 10^{-2}$ mM for $E \cdot Mg \cdot K \rightleftharpoons E \cdot Mg \cdot ATP \cdot K$; $K_{18} = 7.8 \cdot 10^{-2}$ mM for $E \cdot Mg \cdot K_2 \rightleftharpoons E \cdot Mg \cdot ATP \cdot K_2$) and also of the ATP-containing enzyme for magnesium ($K_{19} = 9 \cdot 10^{-4}$ mM for $E \cdot ATP \rightleftharpoons E \cdot Mg \cdot ATP$; $K_{20} = 8 \cdot 10^{-4}$ mM for $E \cdot ATP \cdot K \rightleftharpoons E \cdot Mg \cdot ATP \cdot K$; and $K_{21} = 2.4 \cdot 10^{-3}$ mM for $E \cdot ATP \cdot K_2 \rightleftharpoons E \cdot Mg \cdot ATP \cdot K_2$). All other numerical values of the kinetic rate and dissociation constants and their associated enzyme species remain the same as in Scheme 1(a).

Effects of Mg^{2+} and ATP on (Na^+, K^+)-ATPase at constant potassium (10 mM)

Fig. 6 shows plots of the observed rate constants ($k_{obs} = v_{obs}/[E]_0$) versus magnesium concentrations. The solid lines in this figure are calculated with best-fit values of parameters of Eq. 2. The initial velocity of ATP hydrolysis at 10 mM potassium can be extrapolated to zero at zero concentration of sodium (plots not shown), therefore ATP hydrolysis due to potassium phosphatase is considered insignificant.

Schemes 2(a) and 2(b) show the chemical models that best fit the data. In these schemes, missing enzyme complexes, $E' \cdot Na_x$ ($x = 1$—3) and many others were first included, but they were rejected as a result of computations as described above.

Scheme 2(a) shows that at 10 mM potassium the enzyme binds the Mg·ATP

complex with a low affinity ($K'_1 = 3.8 \cdot 10^{-1}$ mM) in the absence of sodium, but the complex of the enzyme with free ATP may not exist under the same condition. Direct comparison of the affinities of the enzyme for Mg·ATP complex and for free ATP is not possible throughout the entire range of sodium concentrations studied.

Unlike potassium which inhibits the ATP and Mg·ATP binding, sodium increases the binding of these two ligands, and vice versa. While the presence of E′·ATP is not significant in the absence of sodium, the enzyme binds free ATP in the presence of sodium as indicated by the presence of E′·ATP·Na_2 and E′·ATP·Na_3 in the scheme. In the presence of equimolar excess of magnesium over ATP, sodium increases the affinity of the enzyme for Mg·ATP complex by 4-fold (K'_1 and $K'_{13} = 1.1 \cdot 10^{-1}$ mM).

In the absence of both magnesium and ATP, the model implies that the enzyme does not bind sodium; no enzyme-sodium complexes (E′·Na_x, $x = 1$—3) are present. However, when the magnesium site is occupied (E′·Mg), the binding of three sodiums occurs. The binding of the first sodium greatly enhances the binding of the second and the third sodiums ($K'_7 = 2.7 \cdot 10^{-2}$ mM^3). The enhancement of the binding of the third sodium is modulated by Mg·ATP complex ($K'_3 = 4.4 \cdot 10$, $K'_9 = 4.6 \cdot 10$ mM) throughout the entire range of magnesium concentrations employed and by free ATP ($K'_{12} = 7.5 \cdot 10$ mM) in the absence of magnesium.

Different effects of magnesium on the decomposition rates of Mg·ATP-containing enzyme complexes are noted at different concentrations of sodium. At high concentrations of sodium where the enzyme complexes may contain three sodiums, magnesium activates the decomposition of Mg·ATP-containing enzyme complexes ($k'_2 = 1.50$ and $k'_4 = 2.45$ μmol/mg per min). However, at the midrange of sodium concentrations, where most enzyme complexes may contain only two sodiums, magnesium drastically stabilizes these Mg·ATP-containing enzyme complexes: ($k'_1 = 1.05$ and $k'_3 = 0$ μmol/mg per min).

At 10 mM potassium and various concentrations of sodium, the inhibitory properties of magnesium and ATP are the same as those noted in the presence of 60 mM sodium and various concentrations of potassium (Scheme 1(a)).

Substitution of [Mg][ATP]/$K_{Mg \cdot ATP}$ for [Mg·ATP] in Eq. 2, as described above in the case of Eq. 1, gives an alternative chemical model as shown in Scheme 2(b). Information about the affinities of the magnesium-containing enzyme species for ATP ($K'_1 = 3.8 \cdot 10^{-1}$ mM and $K'_{13} = 3.2 \cdot 10^{-1}$ mM for E·Mg·$Na_3 \rightleftharpoons$ E·Mg·ATP·Na_3) and the ATP-containing enzyme species for magnesium ($K'_{14} = 3.2 \cdot 10^{-3}$ mM for E′·ATP·$Na_2 \rightleftharpoons$ E·Mg·ATP·Na_2 and $K'_{15} = 1.8 \cdot 10^{-3}$ mM for E′·ATP·$Na_3 \rightleftharpoons$ E′·Mg·ATP·Na_3) are available. Again, all other numerical values of kinetic rate and dissociation constants and their associated enzyme species remain the same as in Scheme 2(a).

In Schemes 1(a), 1(b), 2(a) and 2(b), it is not clear to what extent sodium (at 60 mM) or potassium (at 10 mM) is bound to each significant enzyme complex at various concentrations of other ligands. However, it is obvious in both schemes that many of these complexes, such as those containing Mg·ATP or Mg·ATP·Mg, may contain both sodium and potassium, although they are not shown as such in the schemes.

Discussion

Since the work of Michaelis and Menten [19] it has been known that the affinity of an enzyme for its substrate can be studied by measuring the initial velocities of the enzymatic reaction at varying substrate concentrations. Such study may provide additional information about the maximal rate of the enzymatic reaction. One can extend this method to involve enzymes which need more than one substrate or ligand for their activity and expect to gain insights into the affinities of the enzyme for each ligand by measuring initial velocities over a wide range of concentration of these ligands. As a result, catalytic activity and ligand affinity of the enzyme at various concentrations of ligands can be recognized. The chemical Schemes 1 and 2 that fit the whole set of experimental data presented in Figs. 5 and 6 are based on this premise.

The hydrolysis of ATP by (Na^+, K^+)-ATPase requires magnesium, sodium and potassium. Since each observation of the initial velocity is the result of the overall equilibrium, it is necessary to include, as we have done, all ligand concentrations and the corresponding initial velocities in the same mathematical equation for estimation of parameters. The non-linear least-squares method was used to calculate the best-fit parameters directly from the observed values, not only for its rapidity in generating numerical values of parameters, but also for its known advantages over conventional graphical or linear least-squares methods involving transformation of the observed values [20,21]. Understandably, the standard deviations of some parameters in Table I are high. The reason for this may be that an insufficient amount of data associated with these parameters was used in the computation, as such effect is known in non-linear regression method [21,22]. The results of the effect of $K_{Mg \cdot ATP}$ values on parameters obtained indicated that the appropriate $K_{Mg \cdot ATP}$ values fall in the region of 0.05—0.1 mM, which coincide with the literature values [18,23]. Since the associated error mean squares are smaller with $K_{Mg \cdot ATP}$ = 0.05 mM than 0.1 mM, we will use kinetic constants associated with 0.05 mM $K_{Mg \cdot ATP}$ for discussion. Numerical values of kinetic dissociation constants associated with the antagonism between potassium and ATP [24—27], mutual enhancement of binding between sodium and ATP [27—29], and inhibitory properties of free magnesium and free ATP in large excess [30—34] that have been well recognized in the literature are reported in Results.

Under the experimental conditions, magnesium or ATP in low quantities exist as Mg·ATP complex when the other ligand is in excess, and in Schemes 1(a) and 2(a) enzyme complexes E''·Mg·ATP (E'' denotes any enzyme complexes capable of binding ATP, Mg^{2+} and Mg·ATP) are formed from the combination of various enzyme complexes with Mg·ATP. The low value of the dissociation constant for E $\rightleftharpoons$ E·Mg·ATP (K_1 = 0.36 μM in Scheme 1(a)), is similar to the dissociation constants for E''·ATP at 0—2°C in the presence of EDTA and without any added metal cations, which usually range from 0.06 to 0.22 μM [35]. In both schemes, the presence of E''·ATP or E''·Mg is possible because insufficient amounts of free magnesium or ATP are available for converting either complex to the active E''·Mg·ATP form.

It is possible that the enzyme may acquire both magnesium and ATP separately to form E''·Mg·ATP for its hydrolytic activity. The alternative chemical

models, Schemes 1(b) and 2(b), aptly demonstrate this possibility. Here, active Mg·ATP-containing enzyme complexes are formed from separate addition of magnesium and ATP to the enzyme. Since the conversion of Schemes 1(a) and 2(a) to 1(b) and 2(b) is a result of the simple mathematical substitution of $[Mg][ATP]/K_{Mg\cdot ATP}$ for [Mg·ATP] in Eqs. 1 and 2, Schemes 1(a) and 1(b) and also 2(a) and 2(b) are numerically identical. These alternative models offer information about the affinities of the magnesium-containing enzyme complexes for free ATP ($E''\cdot Mg + ATP \rightleftharpoons E''\cdot Mg\cdot ATP$) and also those of the ATP-containing enzyme species for magnesium ($E''\cdot ATP + Mg \rightleftharpoons E''\cdot ATP\cdot Mg$). All other rate and dissociation constants and the associated enzyme complexes remain identical with those in Schemes 1(a) and 2(a).

When magnesium (or ATP) is present in excess over ATP (or magnesium), Schemes 1(a) and 2(a) show that the inhibition by the ligand in excess involves the inhibitory binding of magnesium (or ATP) to the pertinent enzyme complexes, whereas Schemes 1(b) and 2(b) show that such inhibition by excess magnesium (or ATP) is, in addition to the inhibitory binding (Scheme 2(b)), due to the reduction in the amount of the active ligand through the formation of the non-binding Mg·ATP complex. Thus, the long-standing question of whether or not the Mg·ATP complex is a true substrate cannot be resolved based on the steady-state kinetic data.

The chief attraction of the resultant chemical models, Schemes 1(a), 1(b), 2(a), and 2(b), is in their usefulness in demonstrating the relationship of responses of the enzyme to various ligands. Notably, information can be gained from extrapolation to zero concentration of certain ligands. For example, Schemes 1(a) and 1(b) show that one can expect to increase binding of two potassiums to the enzyme in the presence of sodium by decreasing ATP concentration because K_7 and $K_9\cdot K_{10}$ are smaller than $K_2\cdot K_3$ and $K_5\cdot K_6$. Also, Schemes 2(a) and 2(b) show that more sodium can be expected to be released from the enzyme in the presence of potassium if both magnesium and ATP are lowered, presumably because dissociation constants associated with $E\cdot Na_x$ ($x = 1,2,3$) are so high that concentrations of the sodium-containing enzyme complexes are insignificant.

It is important to try to correlate the present kinetic results with the results obtained from experiments designed to study transport of sodium and potassium in intact cells. The chemical models proposed in this work make possible the following rationale: Schemes 1(a) and 1(b) show that in the presence of sodium and the absence of ATP, potassium binds favorably to the enzyme ($E \rightleftharpoons E\cdot K_2$). The enzyme exposed to this medium may mimic the external portion of the (Na^+, K^+)-ATPase bound to the intact cell membrane because the extracellular medium consists of high sodium and low potassium and no ATP. Thus, the effective binding of K^+ under the steady-state kinetic conditions may describe the extracellular event of potassium binding to the membrane prior to transport into the cell. The binding of potassium is modulated by free ATP and Mg·ATP ($E\cdot ATP \rightleftharpoons E\cdot ATP\cdot K_2$ and $E\cdot Mg\cdot ATP \rightleftharpoons E\cdot Mg\cdot ATP\cdot K_2$). The "weakening" of binding may be related to the internal sites of weak affinity of the enzyme for potassium inside the cell where ATP and magnesium are present. The breakdown of Mg·ATP-containing enzyme complexes may effect the release of potassium into the cell.

On the other hand, Schemes 2(a) and 2(b) show that in the presence of potassium, binding of sodium is insignificant in the absence of both ATP and magnesium, and is stimulated by magnesium, ATP and Mg·ATP ($E' \cdot Mg \rightleftharpoons E' \cdot Mg \cdot Na_3$; $E' \cdot ATP \rightleftharpoons E' \cdot ATP \cdot Na_2$; $E' \cdot Mg \cdot ATP \rightleftharpoons E' \cdot Mg \cdot ATP \cdot Na_2$). This is presumably the intracellular event occurring on the internal side of the membrane where both magnesium and ATP are present. The breakdown of the Mg·ATP-containing complexes may produce an enzyme conformation in a form that does not bind sodium in the absence of ATP; hence, sodium is released to the extracellular medium which contains no ATP. The enzyme in this state is presumably the same enzyme conformation which binds two potassiums favorably in the absence of ATP as noted in Schemes 1(a) and 1(b).

The discussion thus far has been based on rapid equilibrium models, which describe simultaneous presence of sodium and potassium in the hydrolytically active enzyme complexes. Such models describe how ligands at various concentrations may affect initial rates of ATP hydrolysis catalyzed by (Na^+, K^+)-ATPase in solution, but do not account for all properties of the enzyme under different conditions. An alternative reaction pathway of ATP hydrolysis includes phosphorylation of the enzyme by ATP in the presence of sodium and magnesium, subsequent change of conformation of the phosphorylated enzyme and its decomposition by potassium to give phosphate ion (P_i). Reaction mechanisms based on this simple scheme are supported mainly by isolation and characterization of phosphoenzyme intermediates [36,37] and also by transient kinetic data obtained by rapid-mixing technique [38—40].

However, it is relevant to emphasize that phosphorylation experiments including rapid-mixing technique usually require large amounts of enzyme in contrast to the much smaller ratio of enzyme to ligands concentrations (less than 1/1000) required for steady-state kinetic conditions [41]. We have not been able to fit our large group of steady-state data to a reaction model that includes the phosphorylated enzyme intermediates wherein interactions of sodium and potassium with the enzyme occur in a consecutive manner. Phosphoenzyme intermediates are not necessary for the present steady-state kinetic schemes, because inclusion of any number of these intermediates on the reaction path, such as the following sequence suggested by Skou [42], does not affect the kinetic constants. When the phosphoenzyme intermediates are included, only the meaning of the rate constants (k_1—k_4 and k_1'—k_4') will change; they will consist of individual rate constants that are associated with these intermediates and are experimentally undeterminable under the steady-state condition [43].

$$\underset{n\mathrm{Na}}{\overset{m\mathrm{K}}{\mathrm{E}}} \cdot Mg_2 \cdot ATP \rightleftarrows \underset{n\mathrm{Na}}{\overset{m\mathrm{K}}{\mathrm{E}}} \sim P \rightarrow E + P_i + nNa^+ + mK^+$$

On the other hand, the present steady-state kinetic study certainly does not vitiate the requirement of a phosphoenzyme intermediate state.

Thus, in terms of Schemes 1(a), 1(b), 2(a), and 2(b), our data appear to be consistent with the transport model of simultaneous interactions of inner and outer ligands with the membrane-bound (Na^+, K^+)-ATPase [1,8,9,44—47] and not with the consecutive model which consists of different conformers of the enzyme for consecutive binding of sodium and potassium [1,48—51]. The

reaction sequence involving phosphorylated enzyme intermediates E_1-P and E_2-P as mentioned above is often linked to the latter transport model.

It is not known as to what extent sodium is bound to each of the significant enzyme complexes in Schemes 1(a) and 1(b) and to what extent potassium is bound to each complex in Schemes 2(a) and 2(b), because sodium and potassium concentrations were not used simultaneously in the data analysis in developing Scheme 1(a) and Scheme 2(a). However, the present results are consistent with the interesting possibility that there may be various enzyme complexes that decompose to yield products. These complexes may contain one and two potassiums and two and three sodiums, depending upon the concentrations of potassium and sodium, similar to the dependence of the coupling ratio between potassium influx and sodium efflux on the internal concentration in squid axons [52]. Because of the procedures of data analysis, previous results derived from intact red cells [8] and a fragmented beef brain enzyme [10] suggest that there is only one hydrolytically active enzyme complex most probably containing three sodiums and two potassiums regardless of sodium and potassium concentrations, and the sodium binding sites may be equivalent and independent of the two non-equivalent potassium binding sites [10]. Although the present results provide no information regarding interdependence of sodium and potassium binding sites, the cooperative nature of the binding of sodium and potassium suggests that all binding sites are probably non-equivalent.

Finally, inviewing steady-state kinetic studies, it is important to place certain limitations on the meaning of the data. One feature of the present study is the avoidance of classification of internal and external sites of the fragmented membrane-bound (Na^+, K^+)-ATPase. This is because the whole set of kinetic constants is derived from the same kinetic equation, which states only the dependence of the change in initial velocities of ATP hydrolysis on different concentrations of various ligands. Appropriate interpretation of the results from this study is in terms of kinetic rate and dissociation constants defined by the chemical models. Each of the enzyme complexes in these models represents all possible complexes with the same stoichiometry; it gives no information about its structure. Furthermore, as mentioned earlier, the steady-state initial velocity data in this paper neither prove nor disprove the involvement of Mg·ATP complex as a true substrate and that "phosphorylated enzyme" is a necessary reaction intermediate in the course of ATP hydrolysis. Although the results are in accord with the simultaneous model rather than with the consecutive model, the data cannot be used to disprove the consecutive model under non-steady-state conditions. Notwithstanding these limitations, however, the present kinetic results based on the simultaneous models, Scheme 1 and Scheme 2, do provide some insights of the role that magnesium and ATP may play inside the cell in association with the transport of Na^+ and K^+. Quantitative analysis of the kinetic data in terms of the subunits, which the (Na^+, K^+)-ATPase may be composed of [53], awaits further study.

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