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KINETIC STUDIES OF MEMBRANE (Na^+-K^+-Mg^{2+})-ATPase*

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SUMMARY

Kinetic studies on a microsomal (Na^+-K^+-Mg^{2+})-ATPase (ATP phosphohydrolase EC 3.6.1.3) from rat brain are reported. The results indicate that MgATP is the real substrate of the reaction and ATP is a weak competitive inhibitor. Kinetic studies in which MgATP is varied at several Na^+ and K^+ concentrations reveal that the order of addition of cations and substrate to the enzyme is random. Product inhibition experiments suggest that the release of products is ordered, with P_i being released before ADP. A double reciprocal plot of $1/v$ vs $1/[S]$ at temperatures ranging from 7–37° produced a linear Arrhenius plot with an energy of activation of 23 150 cal/mole. Studies of the effect of pH on the kinetic constants show that two ionizing groups with pK values of 7.1 and 7.6 control the catalysis by (Na^+-K^+-Mg^{2+})-ATPase.

INTRODUCTION

In 1957, SKOU[1] demonstrated the presence of a monovalent cation-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) in crab nerve which catalyzes the hydrolysis of ATP to ADP and P_i. Na^+, K^+ and Mg^{2+} at critical concentrations are required for maximal activity. Since that time the enzyme has been demonstrated in a variety of tissues and cells. It is now widely accepted that the enzyme is an integral part of the cation transport system located in the plasma membrane[2]. Studies in several laboratories[3–5] have shown that the reaction mechanism involves the formation of a phosphorylated enzyme and the intermediate appears to be a protein bound γ-glutamyl phosphate[6]. The work reported in this paper utilized kinetic studies to determine whether the addition of substrate and cations to the enzyme and the release of products is ordered or random. The effects of temperature and pH on the kinetic parameters, V and K_m, are also reported.

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MATERIALS AND METHODS

Preparation of the enzyme

Five female albino rats (Sprague–Dawley) were decapitated, their brains removed and cortexes isolated and frozen in liquid nitrogen or a dry ice–acetone mixture. A 20% w/w homogenate of the cortexes was made with cold distilled water using a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 31 000 × *g* for 30 min at 2° in a Sorvall refrigerated centrifuge. The supernatant was discarded. The pellet was suspended to 10 ml with water and fractionated in a sucrose density gradient[7] ranging from 1.05 to 1.20 g/ml; 3 ml homogenate was carefully layered on the 27-ml gradient. The tubes were placed in an SW-25 swinging bucket-head and centrifuged at 2° for 120 min in a Spinco Model L centrifuge at 25 000 rev./min. Each 30-ml gradient tube was punctured at the bottom and 3-ml fractions were collected. Fractions 5, 6 and 7, which contained most of the $(Na^+\text{-}K^+\text{-}Mg^{2+})$-ATPase activity, were combined, dialyzed for 20 h against 4 changes of 1 l water, freeze-dried and stored at −20° *in vacuo*. In other cases the combined Fractions 5–7 were diluted 3-fold with water and the solution was centrifuged at 31 000 × *g* for 30 min. Either the resultant pellet or the freeze-dried material was treated by the sodium iodide method of NAKAO[8]. 25 ml of the NaI solution which contained 2 M NaI, 2.5 mM EDTA, 5 mM cysteine–HCl, and 2 mM ATP, adjusted to a final pH of 8.0, was added to an amount of enzyme equivalent to 50 mg of protein and mixed with the aid of a homogenizer. This mixture was allowed to stand on ice for 30 min, then diluted 1/2.5 with water and centrifuged at 20 000 × *g* for 30 min. The pellet was washed twice with water, freeze-dried and stored *in vacuo*. For most experiments, the dried sodium iodide enzyme preparation was suspended in water to a final protein concentration of 0.5 mg/ml. The enzyme prepared by the above procedure had a specific activity at 37° of 80–90 μmoles P_i formed/h per mg protein. The Mg^{2+}-ATPase activity of the enzyme preparation was usually 0–5% of the $(Na^+\text{-}K^+\text{-}Mg^{2+})$-ATPase activity.

Assay of the enzyme

Preliminary studies showed that the enzyme preparation contained adenylate kinase activity. This was first indicated in studies performed to demonstrate the stoichiometry of the reaction. During the first few minutes the amount of P_i formed was equal to the ADP formed. After about 5 min the P_i continued to increase but the ADP concentration decreased slowly. The enzyme-catalyzed production of ATP from ADP was shown by incubating the enzyme with ADP and coupling the ATP formed with an ATP requiring enzyme, formyltetrahydrofolate synthetase[9]. This was done under conditions where the $(Na^+\text{-}K^+\text{-}Mg^{2+})$-ATPase activity was inhibited by ouabain. Enzyme, 0.1 mg; ADP, 1.0 mM; ouabain, 1.6 mM; $MgCl_2$, 12 mM; (±)-tetrahydrofolate, 2 mM; sodium formate, 40 mM; 2-mercaptoethanol, 200 mM; triethanolamine HCl, pH 8.0, 100 mM; and formyltetrahydrofolate synthetase, 20 μg; were incubated at 37° for 10 min. The reaction was stopped with 2.0 ml of 0.36 M HCl. The amount of 5,10-methenyltetrahydrofolate formed was calculated from the absorption at 350 mμ using an ε_M of 24 900. The specific activity of adenylate kinase under these conditions expressed as μmoles ATP formed/min per mg protein was 1.6% of the specific activity of the $(Na^+\text{-}K^+\text{-}Mg^{2+})$-ATPase. Since the presence of this amount of adenylate kinase does interfere with $(Na^+\text{-}K^+\text{-}Mg^{2+})$-ATPase assays if the time of assay is over 5 min,

we used an assay time of 2 min. Many studies of the enzyme described in the literature involve assay times of 10–60 min and may be subject to error caused by the adenylate kinase activity.

$(Na^+\text{-}K^+\text{-}Mg^{2+})$-ATPase activity was determined by measuring either the amount of P_i or ADP produced. The reaction vessel contained in a final volume of 1.0 ml: Tris–HCl, pH 8.0, 50 mM; NaCl, 75 mM; KCl, 15 mM; $MgCl_2$, 15 mM; and Tris–ATP, 3 mM. The media for determining the Mg^{2+}-ATPase activity was the same except for the omission of NaCl and KCl. After incubation at 37° for 5 min the reaction was started by the addition of an enzyme suspension containing 50–100 μg of protein. The reaction vessels were incubated in a continuous-shaking water bath at 37° for 2 min. The reaction was stopped by the addition of 1 ml of cold 10% TCA. The precipitated protein was removed by centrifugation. One ml of the supernatant solution was removed and assayed for P_i using a modified Fiske–SubbaRow method[10]. Velocities are expressed as μmoles P_i produced/min. Two different types of control tubes were included with each assay: one contained the appropriate amount of ATP and all other components except the enzyme; the other contained all components except ATP. In experiments involving P_i as an inhibitor it was necessary to measure activity by determining the amount of ADP produced. At the end of the 2-min incubation time the reaction vessels were placed in a dry ice–acetone bath. Freezing was complete within 8 sec. The frozen solutions were brought to 4° and centrifuged at 31 000 × *g* for 10 min. The supernatants were assayed for ADP content by the lactate dehydrogenase–phosphoenolpyruvate kinase method[11]. An aliquot of the solution was added to a cuvette which contained in a final volume of 0.5 ml: triethanolamine–HCl, pH 8.0, 100 mM; $MgCl_2$, 10 mM; 2-mercaptoethanol, 142 mM; phosphoenolpyruvate 1.5 mM; NADH, 0.2 mM; 20 μg of pyruvate kinase (Sigma) and 30 μg of lactic dehydrogenase (Sigma). The oxidation of NADH was measured at 340 mμ using an ε_M of 6200.

By measuring both the P_i and ADP formation and ATP utilization, it was found that one P_i and one ADP was formed for every ATP utilized over a 2-min period. The velocities of product formation were constant over this time period. Concentrations of ADP solutions were determined by the lactate dehydrogenase–phosphoenolpyruvate kinase method described above. ATP concentration was measured using formyltetrahydrofolate synthetase[9]. Protein determinations were done by a modified phenol-reagent method[12].

RESULTS

The effect of Mg^{2+} and ATP on the initial velocity

Since Mg^{2+} and ATP in solution form a MgATP complex, it is reasonable to assume that MgATP is the substrate which complexes with $(Na^+ + K^+ + Mg^{2+})$-ATPase. However, it has been shown[13] that in some ATP-requiring enzyme reactions, the divalent metal is bound to the enzyme before ATP binds. These two mechanisms of cation activation can be differentiated on the basis of kinetic studies[14]. The velocity of the reaction is determined at varying concentrations of the divalent ion and ATP and the data expressed in terms of free concentrations of Mg^{2+} and ATP. If it is found that the apparent K_m for ATP_{free} at one concentration of $Mg^{2+}{}_{free}$ is equal to the apparent K_m for $Mg^{2+}{}_{free}$ at the same concentration of ATP_{free} then the mechanism

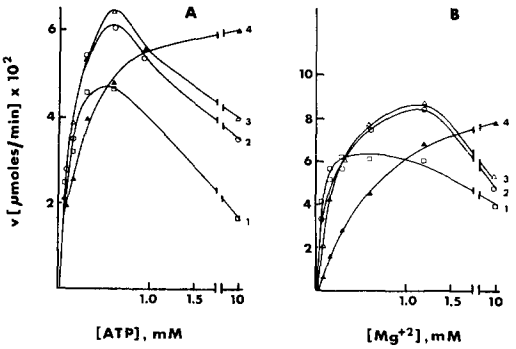


Fig. 1. The effect of varying Mg_{total} and ATP_{total} on the reaction velocity. The conditions and components are as described under METHODS except that Mg^{2+} and ATP concentrations are varied as shown. A. ATP varied at 4 concentrations of Mg^{2+} (mM), 1: 0.25, 2: 0.50, 3: 0.75, 4: 4.0. B. Mg^{2+} varied at 4 concentrations of ATP (mM), 1: 0.25, 2: 0.50, 3: 0.75, 4: 4.0.

of the reaction involves the addition of MgATP to the enzyme[14]. Plots of the reciprocal of the velocity against the reciprocal of Mg^{2+}_{free} concentration at several ATP_{free} concentrations and vice versa should show "competitive activation", *i.e.* increasing concentrations of Mg^{2+}_{free} result in a linear decrease in the K_m for ATP_{free} and likewise the K_m for Mg^{2+}_{free} decreases linearly with an increase in the concentration of ATP_{free} while the maximum velocity remains unchanged. ATP_{free} and Mg^{2+}_{free} may act as inhibitors when present in excess of the concentration of MgATP.

The effect of Mg^{2+} concentration on the initial velocity of the reaction at several ATP concentrations and the effect of ATP concentration at several Mg^{2+} concentrations are shown in Fig. 1. The data show that when either of these components is present in a large excess over the other inhibition occurs. The data in Fig. 1 were expressed in terms of the concentrations of Mg^{2+}_{free}, ATP_{free} and MgATP by using the association constant of 20 000 M^{-1} for the formation of MgATP[15]. Using an association constant of 15 M^{-1} (see ref. 15) for the formation of Na_2ATP and K_2ATP, it was calculated from cubic equations that the concentrations of Na^+ and K^+ present

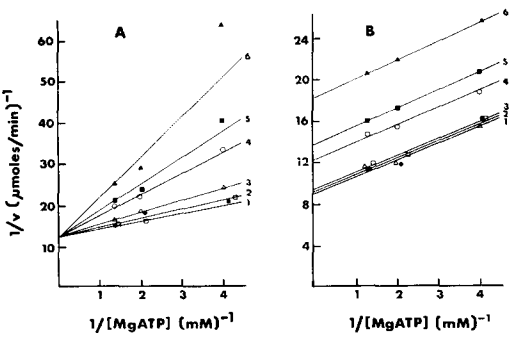


Fig. 2. The initial velocity of the reaction as a function of MgATP concentrations. The data presented were calculated from Fig. 1 as described in the text. A. MgATP varied at 6 concentrations of ATP_{free} (mM), 1: 0.2, 2: 0.5, 3: 1.0, 4: 5.0, 5: 7.0, 6: 10.0. B. MgATP varied at 6 concentrations of Mg_{free} (mM), 1: 0.2, 2: 0.5, 3: 1.0, 4: 5.0, 5: 7.0, 6: 10.0.

in the experiments did not alter the amount of MgATP present. Plots of $1/v$ *vs.* $1/[MgATP]$ calculated from the data in Fig. 1 are shown in Fig. 2. It can be seen that at concentrations above 1.0 mM both ATP_{free} and $Mg^{2+}{}_{free}$ act as inhibitors of MgATP. ATP_{free} is a competitive inhibitor and $Mg^{2+}{}_{free}$ an uncompetitive inhibitor. The K_i for free ATP calculated from a Dixon plot of the data[16] is $3.1 \cdot 10^{-3}$ M or about 10-fold higher than the K_m for MgATP. The K_m values calculated from the data are summarized in Table I. As can be seen, the apparent K_m for ATP_{52aa} at one concentration of

TABLE I

K_m VALUES FOR ATP_{free}, Mg_{free} AND MgATP AT DIFFERENT CONCENTRATIONS OF ATP_{free} AND Mg_{free}

ATP_{free} (mM)	K_m		Mg_{free} (mM)	K_m	
	Mg_{free} ($M \times 10^5$)	MgATP ($M \times 10^4$)		ATP_{free} ($M \times 10^5$)	MgATP ($M \times 10^4$)
0.2	3.00	1.54	0.2	3.57	1.69
0.5	1.48	1.54	0.5	1.37	1.65
1.0	0.77	1.88	1.0	0.63	1.49

$Mg^{2+}{}_{free}$ is equal to the apparent K_m for $Mg^{2+}{}_{free}$ at the same concentration of ATP_{free}. These data indicate that in the (Na^+-K^+-Mg^{2+})-ATPase reaction the free enzyme complexes with MgATP, the true nucleotide substrate, and ATP_{free} acts as a weak competitive inhibitor.

Effect of MgATP on the reaction velocity at several Na^+ and K^+ concentrations

FRIEDEN[17] has presented a generalized kinetic treatment of the effect of modifiers on enzymic reactions. By determining the effect of varying the modifier concentration on the $1/v$ *vs.* $1/[S]$ plots it is possible to gain information on the order of addition of modifier and substrate to the enzyme. The effect of Na^+ and K^+ concentrations on the reciprocal plots of $1/v$ *vs.* $1/[MgATP]$ are shown in Fig. 3. In both cases a series of parallel lines is obtained. Of the various possible mechanisms for cation activation presented by FRIEDEN[17], only a random addition of substrate and

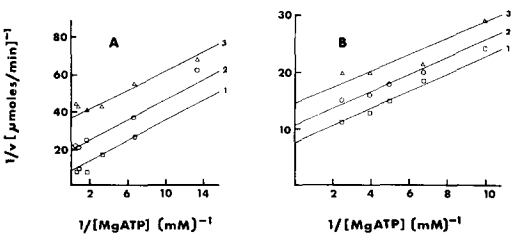


Fig. 3. The effect of MgATP concentration on the initial velocity of the reaction at several Na^+ and K^+ concentrations. The conditions and components are as described under METHODS except that MgATP, Na^+ and K^+ were varied as shown. The ratio of Mg^{2+} to ATP was 5. A. MgATP varied at 3 concentrations of Na^+ (mM), 1: 10, 2: 20, 3: 75. K^+ was constant at 15 mM. B. MgATP varied at 3 concentrations of K^+ (mM), 1: 0.3, 2: 0.6, 3: 1.0. Na^+ was constant at 75 mM.

cation leads to such plots. Two random mechanisms give rise to parallel plots of the type shown in Fig. 3: one in which *ES* does not go on to form product and one in which the ratio of the rate constants for breakdown of *ES* and *EMS* are equal. Since the enzyme prepared by the sodium iodide treatment does not catalyze the hydrolysis of ATP in the absence of Na^+ and K^+, it is assumed that the former type of random addition is operative.

Effect of Na^+ and K^+ concentrations on the reaction velocity

The effects of varying Na^+ concentration at several K^+ concentrations and varying K^+ concentration at several Na^+ concentrations, with the MgATP concentration held constant, are shown in Fig. 4. The nonlinear curves obtained when Na^+ was varied have been observed by others (see ref. 1, 18–23). The curves have been interpreted as either competition between the ion in excess and the ion in lesser

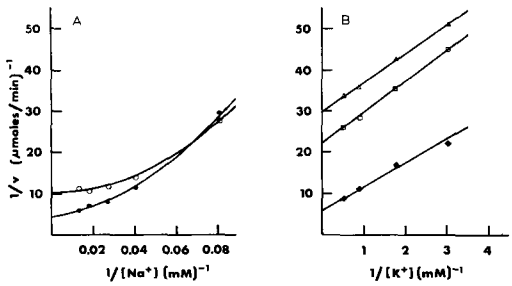


Fig. 4. The effect of Na^+ and K^+ on the reaction velocity. The conditions and components are as under METHODS except that Na^+ and K^+ concentrations were varied as shown. Mg^{2+} was 15 mM and ATP was 3 mM. A. Na^+ varied at 2 concentrations of K^+ (mM), 1: 0.6, 2: 5.0. B. K^+ varied at 3 concentrations of Na^+ (mM), 1: 10.0, 2: 20.0, 3: 75.0.

concentration[1,18,23] or the possibility that Na^+ exerts an allosteric effect on the enzyme[20,22]. The data of Fig. 4A was plotted according to the Hill equation[24] and gave values of n equal to 1.96 and 1.98 at K^+ concentrations of 0.6 and 5.0 mM, respectively. However, when K^+ was varied (Fig. 4B) at several Na^+ concentrations, no deviation from Michaelis–Menten kinetics was observed which is in contrast to the results reported by SQUIRES[20] and ROBINSON[22]. The K_m of K^+ under conditions where Na^+ and MgATP were present in saturating concentrations was $5 \cdot 10^{-4}$ M which is close to the value found by others[21,25].

Inhibition by ADP and P_i

Product inhibition studies can aid in determining the order of addition of substrates and release of products in multisubstrate or multiproduct reactions[26]. HSU *et al.*[27] have presented a discussion of the inhibition patterns expected for several mechanisms of a Uni-Bi-reaction[28]. For example, in a simple ordered reaction the first product to be released shows linear noncompetitive inhibition and the second product, linear competitive inhibition. If the release of products is random both products give the same type of inhibition, *i.e.* noncompetitive or competitive de-

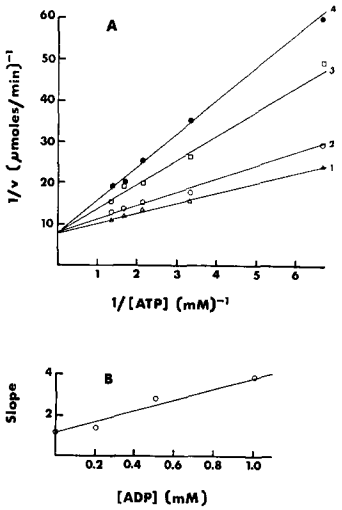


Fig. 5. Inhibition of the reaction velocity by ADP. The conditions and components are as described under METHODS except that the ATP concentrations were varied as shown. The ratio of Mg^{2+} to ATP was always 5. The NaCl and KCl concentrations were 75 mM and 15 mM, respectively. A. ATP varied at 3 concentrations of ADP (mM), 1: 0.0, 2: 0.2, 3: 0.5, 4: 1.0. B. Replot of the slopes from A as a function of ADP concentration.

pending on whether the conversion of the enzyme–substrate complex to the enzyme–products complex is rate limiting[27].

Reciprocal plots of the velocity *vs.* MgATP concentration at several concentrations of ADP are shown in Fig. 5. This figure shows that ADP is a competitive inhibitor of the enzymic reaction. A replot of the slopes versus the concentration of ADP is linear as shown in Fig. 5B. The K_i for ADP is $4.5 \cdot 10^{-4}$ M and can be compared to the K_m for MgATP of $1.0–2.0 \cdot 10^{-4}$ M. Inhibition by P_i is shown in Fig. 6. It can be seen from this figure that P_i is a noncompetitive inhibitor of the reaction. Replots of both slopes and intercepts against P_i concentration are linear as shown in Figs. 6B

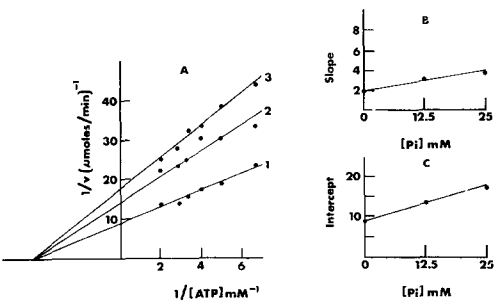


Fig. 6. Inhibition of the reaction velocity by P_i. The conditions and components are as described under METHODS except that the ATP concentrations were varied as shown. The ratio of Mg^{2+} to ATP was always 5. The NaCl and KCl concentrations were 75 mM and 15 mM, respectively. A. ATP varied at two concentrations of P_i (mM), 1: 0.0, 2: 12.5, 3: 25.0. B. Replot of the slopes from A as a function of P_i concentration. C. Replot of the intercepts from A as a function of P_i concentration.

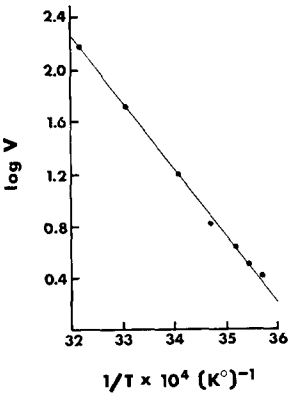


Fig. 7. An Arrhenius Plot of the effect of temperature on the maximum initial velocity. The conditions and components are as described under METHODS except that the reactions were conducted at the temperatures shown. At each temperature the ATP concentration was varied keeping a Mg^{2+}/ATP ratio of 5. The Na^+ and K^+ concentrations were 75 mM and 15 mM, respectively. The V values were obtained from Lineweaver–Burk plots of the data.

and 6C. The K_i for P_i is $2.3 \cdot 10^{-2}$ M. The inhibition data rule out a random release of products and are consistent with a mechanism in which P_i is released before ADP. However, certain mechanisms which involve the ordered release of products with dead end complex formation or a mechanism which involves an isomerization of free enzyme and no central complex are not ruled out[27].

The effect of temperature and pH on K_m and V

The effect of varying concentrations of MgATP at constant Na^+ and K^+ concentrations on the maximal initial velocity at several temperatures was determined and plotted in the form of an Arrhenius plot (Fig. 7). The maximal initial velocities shown are those obtained from extrapolated $1/v$ *vs.* $1/[MgATP]$ plots. The energy of activation calculated from the slope of the line is 23 150 cal/mole. As can be seen, there is no indication of deviation from linearity over this temperature range. Nonlinear Arrhenius plots for rat brain preparation have been reported by other workers[29]. Over the temperature range studied the K_m of MgATP was found to be constant.

The effects of varying concentrations of MgATP on the initial velocity at constant cation concentrations were examined over a pH range of 6.0 to 8.8. The buffers used for the pH ranges of 7.1 to 8.8 and 6.0 to 6.7 were 50 mM Tris–HCl and 50 mM imidazole–HCl, respectively. Preliminary experiments showed that the reaction velocity is constant at the pH extremes and that the enzyme does not undergo inactivation at any of the pH values used. Plotting the data as pK_m versus pH and log V *vs.* pH produces the curves shown in Fig. 8. The plot of pK_m against pH shows one upward bend of the curve which indicates the presence of an ionizing group with a pK of 7.5 in the ES complex[30]. The plot of log V against pH shows two bends in the graph, indicating the presence of two ionizing groups in the ES complex with pK values of about 7.1 and 7.6. These groups control catalysis and thus account for the rather narrow pH optimum of the reaction shown in Fig. 8C. A pK for the ES complex near 7.1 is not seen in Fig. 8A. However, in this pH region the situation becomes very

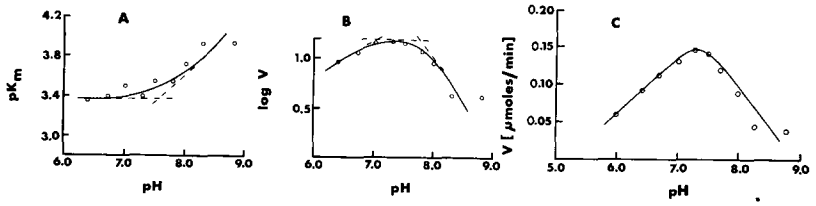
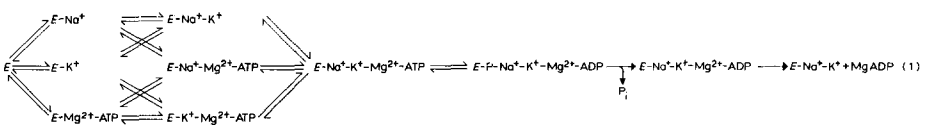


Fig. 8. pK_m, log V and V as functions of pH. The conditions and components are as described under METHODS except that the pH was varied as shown. The buffers used for the pH ranges of 7.1–8.8 and 6.0–6.7 were 50 mM Tris–HCl and 50 mM imidazole–HCl, respectively. V and K_m were determined varying the concentration of Mg^{2+} and ATP in a 5/1 ratio. A. pK_m as a function of pH. B. Log V as a function of pH. C. V as a function of pH.

complex due to the various ionized forms of ATP and MgATP. Since maximal initial velocities are obtained at an infinite substrate concentration, the data in Fig. 8B are not affected by the ionization of the substrate. It would appear that two ionizing groups are controlling the catalysis of (Na^+-K^+-Mg^{2+})-ATPase. The pK values of these groups are close to those for several amino acid residues, including histidine, cysteine and α-amino groups. It has been shown that the enzyme is inhibited by sulfhydryl reagents[31].

DISCUSSION

The results of initial velocity studies presented here show that in the reaction catalyzed by (Na^+-K^+-Mg^{2+})-ATPase the divalent cation complexes with ATP before the nucleotide binds to the enzyme. If there is a large excess of Mg^{2+}_{free} or ATP_{free}, inhibition occurs. A ratio of Mg^{2+}_{total} to ATP_{total} of 2 to 5 should be maintained to insure that all of the ATP is in the form of the real substrate MgATP. The data from the experiments in which MgATP was varied at several concentrations of K^+ and Na^+ (Fig. 3) indicate that the addition of the monovalent cations and MgATP to the enzyme occurs in a random fashion. The results of the product inhibition studies (Figs. 5 and 6) are consistent with an ordered mechanism in which P_i is released before ADP. The mechanism shown in Eqn. 1 represents a summary of these findings. This mechanism is different from that which is usually written[32] and which shows an ordered reaction where ATP binds before K^+. The latter mechanism is based on the finding that the enzyme preparation is phosphorylated by ATP in the presence of Na^+ and dephosphorylated upon the addition of K^+. These two mechanisms are not mutually exclusive if one assumes that the E–Na^+–Mg^{2+}–ATP complex can go on to E–P–Na^+–Mg^{2+}–ADP at a rate which is slow compared to the rate in the presence of K^+.



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