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Kinetic properties of the purified Ca^{2+}-translocating ATPase from human erythrocyte plasma membrane

Antonio Villalobo, Laura Brown and Basil D. Roufogalis *

Laboratory of Molecular Pharmacology, Faculty of Pharmaceutical Sciences, University of British Columbia, 2146 East Mall, Vancouver, B.C., V6T 1W5 (Canada)

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The basic kinetic properties of the solubilized and purified Ca^{2+}-translocating ATPase from human erythrocyte membranes were studied. A complex interaction between the major ligands (i.e., Ca^{2+}, Mg^{2+}, H^+, calmodulin and ATP) and the enzyme was found. The apparent affinity of the enzyme for Ca^{2+} was inversely proportional to the concentration of free Mg^{2+} and H^+, both in the presence or absence of calmodulin. In addition, the apparent affinity of the enzyme for Ca^{2+} was significantly increased by the presence of calmodulin at high concentrations of $MgCl_2$ (5 mM), while it was hardly affected at low concentrations of $MgCl_2$ (2 mM or less). In addition, the ATPase activity was inhibited by free Mg^{2+} in the millimolar concentration range. Evidence for a high degree of positive cooperativity for Ca^{2+} activation of the enzyme (Hill coefficient near to 4) was found in the presence of calmodulin in the slightly alkaline pH range. The degree of cooperativity induced by Ca^{2+} in the presence of calmodulin was decreased strongly as the pH decreased to acid values (Hill coefficient below 2). In the absence of calmodulin, the Hill coefficient was 2 or slightly below over the whole pH range tested. Two binding affinities of the enzyme for ATP were found. The apparent affinity of the enzyme for calmodulin was around 6 nM and independent of the Mg^{2+} concentration. The degree of stimulation of the ATPase activity by calmodulin was dependent on the concentrations of both Ca^{2+} and Mg^{2+} in the assay system.

Introduction

The plasma membrane of animal cells contains a Mg^{2+}-stimulated, Ca^{2+}-dependent adenosine triphosphatase ((Mg^{2+} + Ca^{2+})-ATPase) responsible for the extrusion of calcium ion across the cell membrane against a steep electrochemical Ca^{2+} gradient [1]. The enzyme contributes to the maintenance of a local concentration of free Ca^{2+} in the cytoplasm in the order of $1 \cdot 10^{-7}$ M to $1 \cdot 10^{-8}$ M, against an extracellular concentration of about $1 \cdot 10^{-3}$ M. Although the enzyme is found in the cell membranes of a large number of different tissues [2], the enzyme from erythrocytes has been the most widely studied (for reviews, see Refs. 2–5). The erythrocyte Ca^{2+}-translocating ATPase is regulated by the Ca^{2+}-binding protein, calmodulin, which stimulates both ATP hydrolysis and Ca^{2+}-translocating activities [6,7]. As a first step towards a better understanding of the catalytic mechanism and the transport functions of the enzyme, as well as the regulatory properties induced by various ligands, we have purified the enzyme from human erythrocytes by a calmodu-

* To whom correspondence should be addressed.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; PMSF, phenylmethylsulphonyl fluoride; TLCK, N^2-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, L-tosylamido-2-phenylchloromethyl ketone.

lin-affinity chromatographic method [8] and studied its basic kinetic properties in the solubilized form. The effects of Ca^{2+} and Mg^{2+}, protons, ATP and calmodulin were considered in this study. The results show that even in a solubilized form, the catalytic cycle of the Ca^{2+}-translocating ATPase maintains multiple binding sites for Ca^{2+} and two affinities for ATP. In addition, the purified enzyme has the capacity to be regulated by calmodulin, and it shows competitive inhibitory behaviour between Ca^{2+} and H^+ or Mg^{2+}.

Materials and Methods

Chemicals. Rabbit muscle lactate dehydrogenase, EC 1.1.1.27 (type II), rabbit muscle pyruvate kinase, EC 2.7.1.40 (type II), bovine brain phosphodiesterase 3',5'-cyclic nucleotide activator-Agarose gel, cyanogen bromide activated Sepharose-4B gel, Triton X-100, dithiothreitol, Hepes, ATP (disodium salt and Tris salt), EGTA, β-NADH and phospho*enol*pyruvate were purchased from Sigma Chem. Co. (St. Louis, Missouri). Bovine brain calmodulin was obtained from Calbiochem (La Jolla, California), EDTA was purchased from BDH (Toronto) and asolectin from MCB Manufacturing Chemical Inc. (Cincinnati, Ohio). [γ-^{32}P]ATP (specific activity 20–40 Ci/mmol) was purchased from Amersham Canada (Montreal). All other chemicals used in this work were of the highest purity available.

Preparation of calmodulin-depleted erythrocyte membranes. Calmodulin-depleted erythrocyte membranes were prepared essentially as described by Carafoli et al. [9]. The whole preparation was carried out at 4°C. 1 U (approx. 250 g) of human packed red cells (4–5 days old), suspended in a citrate-phosphate-dextrose medium containing adenine was obtained from the Canadian Red Cross and washed three times in 1 l of isotonic solution containing 130 mM KCl, 20 mM Tris-HCl (pH 7.4) (buffer I). The cells were collected in 250 ml tubes by centrifugation at $3900 \times g_{max}$ for 5 min. The supernatant and a whitish-gray layer of white cells on top of the pellet were removed by aspiration. The washed red cells were then resuspended in 1 l of a hypotonic medium containing 1 mM EDTA and 10 mM Tris-HCl (pH 7.4) (buffer II) and centrifuged at $20000 \times g_{max}$ for 10 min to collect the membrane fraction. From this point on, the centrifugations were performed with the centrifuge brake off, to prevent excessive resuspension of the pellet. The membrane fraction was washed five more times in a total volume of 6 l of buffer II in order to remove haemoglobin, calmodulin and other soluble proteins. The calmodulin-depleted membranes were then transferred to 50 ml tubes and washed four times in 320 ml of 10 mM potassium-Hepes (pH 7.4) (buffer III) in order to remove the EDTA. The membranes were collected by centrifugation at $13000 \times g_{max}$ for 10 min and finally, they were resuspended in 320 ml of 130 mM KCl, 2 mM dithiothreitol, 0.5 mM $MgCl_2$, 50 μM $CaCl_2$ and 20 mM potassium-Hepes (pH 7.4) (buffer IV) and centrifuged at $13000 \times g_{max}$ for 15 min. The concentrated suspension of white membranes (approx. 50 ml containing a protein concentration of 1.5–2 mg/ml) was quick frozen in liquid nitrogen (−196°C) in aliquots of 5–10 ml and finally stored at −70°C until use.

Alternatively, the calmodulin-depleted membranes were prepared using a Millipore Pellicon Cassette System equipped with filters of 0.5 μm diameter pore size. The whole preparation was carried out between 4 and 10°C as described below. 1 U of packed red cells (4–5 days old) suspended in the same media described above were washed three times in 1 l of isotonic buffer I. The cells were collected by centrifugation at $3900 \times g_{max}$ for 5 min as described above. The washed red cells were suspended in 10 vols. of buffer II and stirred for 5–10 min to favour haemolysis. The haemolyzate was passed through the cassette system, operating in the concentration mode, until the volume was reduced to approx. 1 l. The concentrated membranes were washed with 15–20 l of buffer II, with the cassette system operating in the constant volume mode, until the membranes appeared white. The membranes were further washed with 10–15 l of buffer III in order to remove the EDTA and finally they were washed with 2 l of buffer IV. Some membrane preparations were made in the presence of 0.5 mM PMSF (see Figure legends). The membranes were finally concentrated by centrifugation at $20000 \times g_{max}$ for 20 min, with the centrifuge brake off. The concentrated membranes were stored as above.

Solubilization and purification of the Ca^{2+}-translocating ATPase. The solubilization and purification of the Ca^{2+}-translocating ATPase, performed at 4°C except where indicated, was carried out using the procedure of Graf et al. [8], with the following modifications: 100 ml of white calmodulin-depleted erythrocyte membrane suspension (150–200 mg protein) were concentrated by centrifugation at $125\,000 \times g_{max}$ for 30 min. The pellet was solubilized by resuspension in 50 ml of 0.5% (w/v) Triton X-100, 300 mM KCl, 10 mM potassium-Hepes, 1 mM $MgCl_2$, 100 μM $CaCl_2$ and 2 mM dithiothreitol (pH 7.4) and incubated for 10 min at 0°C. The solubilized membranes were centrifuged at $125\,000 \times g_{max}$ for 30 min, and the supernatant carefully removed by Pasteur pipette. To the supernatant (approx. 50 ml) containing the solubilized Ca^{2+}-translocating ATPase was added 0.1% (w/v) sonicated asolectin and an additional 0.05% (w/v) Triton X-100 and 2 mM dithiothreitol. The solution was passed at a low flow rate (0.5–1 ml/min) through a column of calmodulin-Sepharose-4B gel (6 cm high × 1.5 cm diameter, containing 7.5 mg covalently-bound calmodulin) or alternatively a calmodulin-Agarose gel (Sigma Chemical Co.) (11.3 cm high × 1.5 cm diameter, containing 18 mg covalently-bound calmodulin) equilibrated in 0.5% (w/v) Triton X-100, 200 mM KCl, 10 mM potassium-Hepes, 1 mM $MgCl_2$, 100 μM $CaCl_2$, 2 mM dithiothreitol and 0.1% (w/v) sonicated asolectin (pH 7.4). The calmodulin affinity column was washed overnight (16 h) with approx. 700 ml of 0.05% (w/v) Triton X-100, 300 mM KCl, 10 mM potassium-Hepes, 1 mM $MgCl_2$, 50 μM $CaCl_2$, 2 mM dithiothreitol and 0.1% (w/v) sonicated asolectin (pH 7.4). The Ca^{2+}-translocating ATPase was eluted from the column with 0.05% (w/v) Triton X-100, 300 mM KCl, 10 mM potassium-Hepes, 2 mM dithiothreitol, 0.1% (w/v) sonicated asolectin and 2 mM EDTA (pH 7.4). Fractions (2.5 ml) were collected with a fraction collector and the elution of the protein was recorded by a UV-light detector at 280 nm. Some preparations were performed in the presence of 0.5 mM PMSF in the solubilization medium only. The fractions with maximum activity were pooled, 2 mM $CaCl_2$ (final concentration) was added, and small aliquots were frozen in liquid nitrogen (−196°C) and finally stored at −70°C until use. The purified Ca^{2+}-translocating ATPase (300–800 μg protein) had a specific activity of 1–3.6 μmol P_i/min per mg protein.

The calmodulin-affinity column was treated after each use as follows: 100 ml of 20 mM potassium-Hepes/2 mM EDTA (pH 7.4) was passed through the column to remove phospholipids and Triton X-100. 100 ml of 2 M NaCl/3% (v/v) acetic acid followed by 100 ml of 6 M urea were added in order to remove nonspecifically bound proteins. Finally, the column was washed with 100 ml of 200 mM KCl, 50 mM potassium-Hepes, 50 μM $CaCl_2$, 0.02% (w/v) sodium azide (pH 7.4) and stored in this buffer at 4°C until used again.

Determination of the Ca^{2+}-translocating ATPase activity in the membranes. Calmodulin-depleted membranes (50–100 μg protein) were incubated at 37°C for 1 h in a total volume of 1 ml of 100 mM KCl, 50 mM potassium-Hepes, 10 mM $MgCl_2$, 20 μM $CaCl_2$, 0.1 mM dithiothreitol, 2 μg/ml calmodulin (when added) and 2 mM ATP (pH 7.4). The phosphate liberated to the medium was determined colorimetrically [10]. The activity of the Mg^{2+}-dependent ATPase (assayed in the absence of added $CaCl_2$ and in the presence of 2 mM EGTA) was subtracted from the total activity assayed in the presence of Ca^{2+}. The activity of the Ca^{2+}-translocating ATPase in the presence of calmodulin was in the range 20–100 nmol of P_i/min per mg protein and the ratio of the activities in the presence versus the absence of calmodulin was in the range 2.5–8.4, depending on the batch of membranes.

Determination of the activity of the purified Ca^{2+}-translocating ATPase. Purified Ca^{2+}-translocating ATPase (2–9 μg protein) was incubated at 37°C for 1 h in a total volume of 1 ml of 124 mM KCl, 50 mM potassium-Hepes, 5 mM $MgCl_2$, 160 μM $CaCl_2$, 160 μM EDTA, 2 mM dithiothreitol, 0.05% (w/v) Triton X-100, 0.1% (w/v) sonicated asolectin, 2 μg/ml calmodulin (when added) enough EGTA to attain the desired free Ca^{2+} concentration and 2 mM ATP (pH 7.4). The liberated phosphate was assayed as above. Alternatively, the Ca^{2+}-ATPase activity was measured spectrophotometrically by following the initial rate of β-NADH oxidation at 340 nm or at 335–290 nm when measured with a dual-wavelength spec-

trophotometer (SLM-Aminco DW-2C). In a total volume of 1 or 2.5 ml, an ATP regenerating system consisting of 2 mM phospho*enol*pyruvate, 0.3 mM β-NADH, 20 U of lactate dehydrogenase (EC 1.1.1.27) and 16 U of pyruvate kinase (EC 2.7.1.40) was employed at 37°C in the following medium: 74 mM KCl, 50 mM potassium-Hepes, 5 mM $MgCl_2$, 160 μM $CaCl_2$, 160 μM EDTA, 2 mM dithiothreitol , 0.05% (w/v) Triton X-100, 0.1% (w/v) sonicated asolectin, 2 μg/ml calmodulin (when added), 10–18 mM $(NH_4)_2SO_4$, enough EGTA to attain the desired free Ca^{2+} concentration and 0.5–2 mM ATP (pH 7.4). Modifications of this basic protocol are indicated in the legends of the figures. 1 U of lactate dehydrogenase will reduce 1 μmol per min of L-pyruvate to L-lactate at pH 7.5 and 37°C. 1 U of pyruvate kinase will convert 1 μmol per min of phospho*enol*pyruvate to pyruvate at pH 7.6 and 37°C. The hydrolysis of ATP was found to be linear during the whole period of assay by either of the two methods used to monitor the ATPase activity. When the ATP regenerating system was used to monitor ATPase activity, preliminary tests determined that the activity of the lactate dehydrogenase and pyruvate kinase used in this assay were not limiting factors under any of the experimental conditions used. The activity of the Mg^{2+}-ATPase on all the purified preparations was virtually nonexistant; consequently, no corrections were necessary to calculate the activity of the Ca^{2+}-ATPase. The results shown are typical experiments repeated from two to five times.

Preparation of the calmodulin-Sepharose-4B gel. 3 g of cyanogen bromide activated Sepharose-4B was suspended in 80 ml of 1 mN HCl at room temperature for about 10 min, and filtered through a scintered funnel using Whatman filter paper No. 1 and washed three times with 100 ml of 400 mM NaCl, 50 μM $CaCl_2$, 100 mM H_3BO_3 (pH 8.2 at 4°C). The gel was transferred to 1 l of the same buffer containing 15 mg of bovine brain calmodulin and incubated at 4°C for 8 h with very gentle stirring. The gel was recovered in the scintered filter described above, and transferred to 100 ml of 0.5 M ethanolamine-HCl (pH 8.2) and incubated for 16 h at 4°C. The gel was packed in the column, washed, and stored at 4°C in 200 mM KCl, 50 mM potassium-Hepes, 50 μM $CaCl_2$, 0.02% (w/v) sodium azide (pH 7.4) until use.

It was determined that 50% of the calmodulin (7.5 mg) was covalently bound to the gel.

Protein determination. Protein concentration was determined according to a modification of the method of Lowry et al. [11]. Preparations were treated with 0.05% (w/v) deoxycholic acid and precipitated in a final concentration of 10% (w/v) trichloroacetic acid at room temperature [12]. Bovine serum albumin was used as a standard.

Preparation of dispersed phospholipids. A mixture of 4% (w/v) asolectin, 2% (w/v) Triton X-100 and 80 mM dithiothreitol was sonicated at room temperature for 5 min in 10 ml aliquots in a sonicator at 90–95 watts of power using the micro-probe, with alternate 30 s periods on and 30 s periods off, in order to obtain dispersion of the phospholipids. The sonicated mixture was divided in small aliquots and frozen at −20°C until use.

Polyacrylamide gel electrophoresis. Slab gel electrophoresis [13] was performed at 10 mA for 18 h in a linear gradient 5–20% (w/v) of polyacrylamide gel in the presence of 0.1% (w/v) sodium dodecyl sulfate. The purified enzyme was concentrated by precipitation at room temperature in 10% (w/v) trichloroacetic acid in the presence of 0.05% (w/v) deoxycholic acid. The precipitated protein was incubated in 62.5 mM Tris-HCl, 2% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) β-mercaptoethanol and 0.02% (w/v) Bromophenol blue for 3 min at 100°C (pH 6.8). As standards, the following proteins of known molecular weight were used: myosin (M_r 200 000), β-galactosidase (M_r 116 250), phosphorylase B (M_r 92 500), bovine serum albumin (M_r 66 200), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 31 000), soybean trypsin inhibitor (M_r 21 500) and lysozyme (M_r 14 400). The gels were stained with Coomassie blue R-250 or alternatively, by a silver staining method [14].

Calculation of the concentrations of free Ca^{2+} and Mg^{2+} and MgATP complex. Free Ca^{2+} and Mg^{2+} and MgATP complex concentrations were calculated by computer using a FORTRAN program (CATIONS.BC), described by Goldstein [15]. Association constants for Ca^{2+}, Mg^{2+} and H^+ to ATP, EGTA and EDTA were taken from Ref. 16, except in the case of monoprotonated ligands which were calculated as described by Blinks et al.

[17]. Prior to application of the program, constants were corrected for temperature using a BASIC program, LOGKTEMP, based on the formula given by Tinoco et al. [18] and using enthalpy values tabulated by Martell and Smith [16]. The constants were then adjusted for ionic strength [16,17].

Results

Purity of the purified Ca^{2+}-translocating ATPase

The Ca^{2+}-translocating ATPase preparations were analyzed by electrophoresis in a linear gradient (5–20%, w/v) of polyacrylamide gel in the presence of SDS. The gel profile was essentially similar to those published previously in other gel systems [8,19,20]. Fig. 1A shows a photodensitometric scan of the gel stained with Coomassie blue R-250. The prominent peak at approx. 132 kDa was identified as the Ca^{2+}-translocating ATPase by the formation of the phosphorylated catalytic intermediate when [γ-^{32}P]ATP was used in the assay medium (see autoradiogram, Fig. 1). A diffuse but fainter band of higher molecular mass, around 145 kDa, was also present (Fig. 1A) as reported by others [8,20]. This is less than the molecular mass of 200 kDa or more attributed to the dimer [2], consistent with the lack of phosphorylation we observed in this peak (Fig. 1). A few bands of lower molecular mass, accounting for only a few percent of the total protein, were detected, but the majority of these were also found in the electrophoresis of buffer containing only the commercial asolectin used in the purification procedure (Fig. 1B). A similar profile was observed in preparations solubilized in the presence of a cocktail of proteinase inhibitors of the following composition: 10 μM leupeptine, 100 μM TLCK, 100 μM TPCK, 500 μM PMSF and 0.25 mg/ml soybean Trypsin inhibitor. However, we also noted that the use fo the proteinase inhibitors prevented the appearance of a fainter band migrating just ahead of the Ca^{2+}-translocating ATPase, most probably a hydrolytic product of the enzyme.

Apparent affinity of the Ca^{2+}-translocating ATPase for Ca^{2+}

The activity of the purified Ca^{2+}-translocating ATPase was absolutely dependent on the presence of free Ca^{2+} in the assay medium. In Fig. 2, a semilogarithmic plot is presented of the activity of the Ca^{2+}-translocating ATPase as a function of the concentration of free Ca^{2+} in the assay media, in the presence and in the absence of the regulatory protein, calmodulin, at different concentrations of $MgCl_2$ (A to D). No ATP hydrolysis was detected in the absence of Ca^{2+} at any of the $MgCl_2$ concentrations examined. The stimulatory effect of calmodulin on the ATPase activity was most

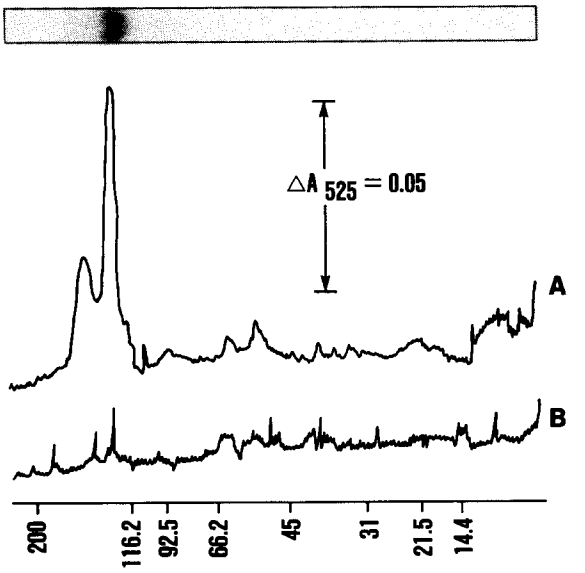

Fig. 1. Formation of the phosphorylated catalytic intermediate and purity of the Ca^{2+}-ATPase. (Top) The purified enzyme (2.6 μg protein) was incubated at 37°C in 0.2 ml of the following medium: 40 mM potassium-Hepes (pH 7.4), 0.1 mM $MgCl_2$, 0.92 mM $CaCl_2$, 0.8 mM EDTA, 2 mM dithiothreitol, 0.054% (w/v) Triton X-100, 0.1% (w/v) sonicated asolectin, and 0.6 μM calmodulin. The reaction was started by adding 10 μM Tris-ATP containing [γ-^{32}P]ATP (6.1 Ci/mmol). After 5 s of incubation, the reaction was terminated by adding ice-cold 10% (w/v) (final concentration) trichloroacetic acid. The precipitated protein was processed for electrophoresis at pH 7.0 and 3°C, and exposed to X-ray film for autoradiography. (Bottom) The purified enzyme (19 μg protein), suspended in 300 mM KCl, 10 mM K-Hepes (pH 7.4), 2 mM dithiothreitol, 0.05% (w/v) Triton X-100, 0.1% (w/v) sonicated asolectin, 2 mM $CaCl_2$ and 2 mM EDTA, was precipitated in ice-cold 10% trichloroacetic acid (w/v) (final concentration). The pellet was processed for electrophoresis at pH 8.3, and stained with Coomassie blue R-250. Densitometric traces shown are of track A, containing the purified enzyme and track B, without purified enzyme, in the presence of the same buffer and phospholipids.

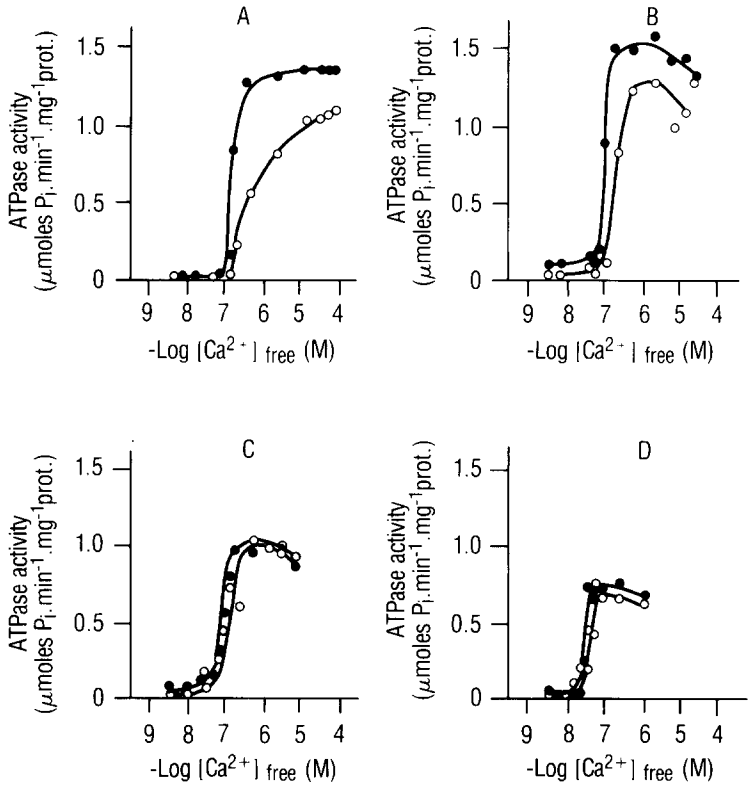

Fig. 2. Activation of the Ca^{2+}-translocating ATPase by Ca^{2+} at different concentrations of $MgCl_2$. The purified enzyme (5.6 μg protein) was assayed at 37°C for 1 h in a total volume of 1 ml of the following medium: 124 mM KCl, 50 mM K-Hepes (pH 7.4), 2 mM dithiothreitol, 0.054% (w/v) Triton X-100, 0.1% (w/v) sonicated asolectin, 2 mM ATP, 160 μM $CaCl_2$, 160 μM EDTA and 0–2 mM EGTA to yield the indicated concentrations of free Ca^{2+}. The experiment was performed in the absence (open circles) and in the presence (filled circles) of 120 nM calmodulin and at the following concentrations of $MgCl_2$: 5 mM (A), 2 mM (B), 1 mM (C) and 0.5 mM (D). The inorganic phosphate release was assayed as described in Materials and Methods.

prominent, and occurred over a wider concentration range of free Ca^{2+}, when $MgCl_2$ was in excess of the concentration of ATP (2 mM) present in the assay system (Fig. 2A). The estimated maximum rate of ATP hydrolysis (V_{max}) progressively decreased as the concentration of $MgCl_2$ in the assay system was lowered. When the concentration of $MgCl_2$ was equal to or lower than the concentration of ATP (2 mM), inhibition of the rate of ATP hydrolysis was observed at concentrations of free Ca^{2+} higher than the optimum concentrations (Fig. 2 B–D).

The ATP hydrolytic activity of the enzyme does not follow a typical Michaelis-Menten model with respect to the activation induced by Ca^{2+}. A double-reciprocal plot of the rate of ATP hydrolysis versus the concentration of free Ca^{2+} was markedly concave up, both in the presence and in the absence of calmodulin, at all the concentrations of $MgCl_2$ tested (results not shown). Consequently, the Ca^{2+} concentration for half-maximal activation of the enzyme, $K_{0.5[Ca^{2+}]}$, was calculated from the plots in Fig. 2. In Fig. 3, the $-\log K_{0.5[Ca^{2+}]}$ is plotted as a function of the $-\log [Mg^{2+}]_{free}$ in the assay system. The apparent affinity of the enzyme for Ca^{2+} became progressively higher when the concentration of free Mg^{2+} was decreased, both in the presence and in the absence of calmodulin. The $K_{0.5[Ca^{2+}]}$ changed from $3 \cdot 10^{-8}$ M to $8 \cdot 10^{-7}$ M in the absence of calmodulin, and from $2 \cdot 10^{-8}$ M to $2 \cdot 10^{-7}$ M in the presence of calmodulin, in the range of free Mg^{2+} concentration studied (see Fig. 3). Similar $K_{0.5[Ca^{2+}]}$ values were obtained when the experiment of Fig. 2A was repeated maintaining a constant concentration of EGTA (1 mM) and varying the total amount of calcium added to the system. The $K_{0.5[Ca^{2+}]}$ found under these conditions was $5 \cdot 10^{-7}$ M in the presence of calmodulin and $1.1 \cdot 10^{-6}$ M in its absence (results not shown), i.e., similar to those found when EGTA was varied at constant total $CaCl_2$ in the assay system (see Fig. 2).

Ca^{2+}-induced positive cooperativity that is modified by calmodulin

The complex behaviour of the ATPase activity

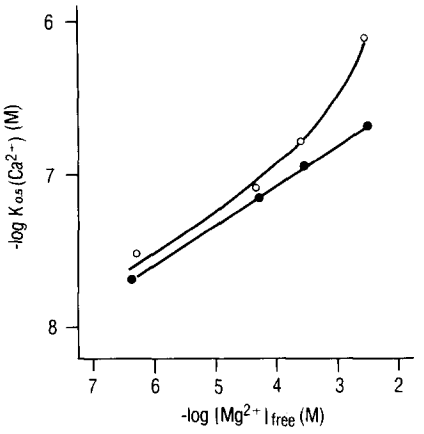

Fig. 3. Effect of free Mg^{2+} concentration on the apparent affinity of the enzyme for Ca^{2+}. The concentration of Ca^{2+} for half-maximal activition was calculated from the plots in Fig. 2, measuring the concentration of free Ca^{2+} needed to attain half of the estimated maximum rate of ATP hydrolysis in the absence (open circles) and in the presence (filled circles) of calmodulin. The concentration of free Mg^{2+} in the system was calculated by the computer program.

as a function of the concentration of Ca^{2+} prompted an investigation of the cooperativity for Ca^{2+} activation of the enzyme, both in the presence and in the absence of calmodulin. Fig. 4

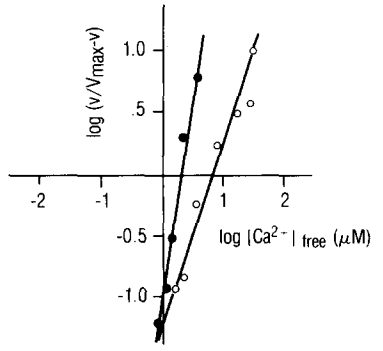

Fig. 4. Positive cooperativity of Ca^{2+} activation of the Ca^{2+}-translocating ATPase. Hill plots of the activity of the enzyme at different concentrations of free Ca^{2+} were performed. The purified enzyme (2.24 μg protein) was assayed at 37°C for 1 h in a total volume of 1 ml of the following medium: 124 mM KCl, 50 mM potassium-Hepes (pH 7.4), 2 mM dithiothreitol, 0.054% (w/v) Triton X-100, 0.1% (w/v) sonicated asolectin, 2 mM ATP, 5 mM $MgCl_2$, 160 μM $CaCl_2$, 160 μM EDTA and 0–2 mM EGTA to yield the indicated concentrations of free Ca^{2+}. The assay was performed in the absence (open circles) or in the presence (filled circles) of 120 nM calmodulin. The inorganic phosphate released was assayed by the colorimetric method.

shows a Hill plot of the rate of ATP hydrolysis as a function of the concentration of free Ca^{2+} present in the assay system at pH 7.4 and 5 mM $MgCl_2$. The calculated Hill coefficient was 3.9 in the presence of calmodulin and 1.5 in its absence.

Modifications of the apparent affinity of the enzyme for Ca^{2+} and the degree of cooperativity induced by Ca^{2+} at different pH values

The optimum pH for the ATP hydrolytic activity of the purified Ca^{2+}-translocating ATPase both in the presence and in the absence of calmodulin was between 7.0 and 7.25 (results not shown). We also have studied the effects of the medium pH both on the apparent affinity of the enzyme for Ca^{2+} and on the degree of cooperativity induced by Ca^{2+}. Fig. 5A shows a plot of the Ca^{2+} concentration for half maximal activation ($K_{0.5[Ca^{2+}]}$) of the enzyme versus pH in the range 6.0–8.25. It was observed that the apparent affinity of the enzyme for Ca^{2+} is highest at a pH around 8, both in the presence and in the absence of calmodulin, and it decreases progressively as the pH approaches 6. Furthermore, Fig. 5B shows that the degree of cooperativity induced by Ca^{2+} is strongly modified by pH in the presence of calmodulin (Hill coefficient of 4, at alkaline pH and below 2 at acid pH). However, little modification of the Hill coefficient was found in the absence of calmodulin, remaining around 2 or below, in the range of pH studied.

Effect of free Mg^{2+} on the Ca^{2+}-translocating ATPase

In Fig. 2, it was observed that the rate of ATP hydrolysis at saturating concentrations of Ca^{2+} progressively increased when the concentration of $MgCl_2$ was also increased. However, to test the effect of $MgCl_2$ in excess of the concentration of ATP in the system, a series of experiments was performed in which the free Ca^{2+} concentration was maintained constant at 2 μM, and the concentration of $MgCl_2$ was varied systematically below and above the total concentration of ATP in the system (2 mM). Fig. 6 presents the results of an experiment performed both in the absence and in the presence of calmodulin. It was observed that the rate of ATP hydrolysis increased with increasing concentrations of added $MgCl_2$, until the

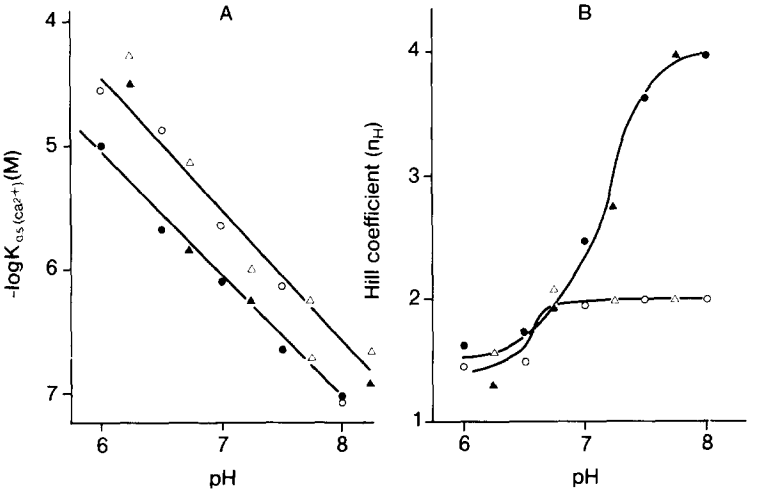

Fig. 5. Effects of pH on the apparent affinity of the enzyme for Ca^{2+} and the degree of cooperativity induced by Ca^{2+}. A. The purified enzyme (1.2 or 1.4 μg protein) (prepared in the presence of PMSF) was assayed for 1 h at 37°C in a total volume of 0.5 ml of the following medium: 124 mM KCl, 100 mM Tris-maleate buffer (triangles) or 100 mM Tris-Hepes buffer (circles) at the indicated pH values, 2 mM dithiothreitol, 0.054% (w/v) Triton X-100, 0.1% (w/v) sonicated asolectin, 2 mM ATP, 5 mM $MgCl_2$, 160 μM $CaCl_2$, 160 μM EDTA and 0–2 mM EGTA to yield variable concentrations of free Ca^{2+}, in the absence (open symbols) or the presence (filled symbols) of 120 nM calmodulin. The inorganic phosphate released was assayed colorimetrically. The values for $K_{0.5[Ca^{2+}]}$ were calculated from a plot as in Fig. 2. B. The Hill coefficient was calculated from the same set of experiments from a plot as described in Fig. 4.

$MgCl_2$ concentration exceeded the concentration of ATP in the system (2 mM), whereupon a strong inhibition of the rate of ATP hydrolysis was ob-

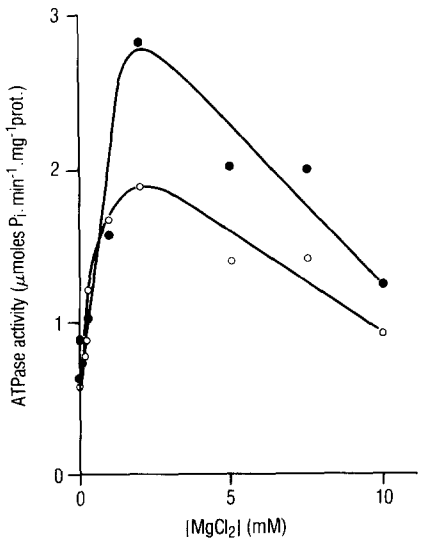

Fig. 6. Effect of $MgCl_2$ on the activity of the Ca^{2+}-translocating ATPase. The purified enzyme (3.4 μg protein) was assayed at 37°C for 1 h in a total volume of 1 ml of the following medium: 124 mM KCl, 50 mM K-Hepes (pH 7.4), 2 mM dithiothreitol, 0.054% (w/v) Triton X-100, 0.1% (w/v) sonicated asolectin, 2 mM ATP, 200 μM EDTA, 200 μM EGTA, and sufficient $CaCl_2$ to attain a concentration of 2 μM free Ca^{2+}. The experiment was performed in the absence (open circles) and in the presence (filled circles) of 120 nM calmodulin at the indicated concentrations of $MgCl_2$. The inorganic phosphate released was assayed colorimetrically.

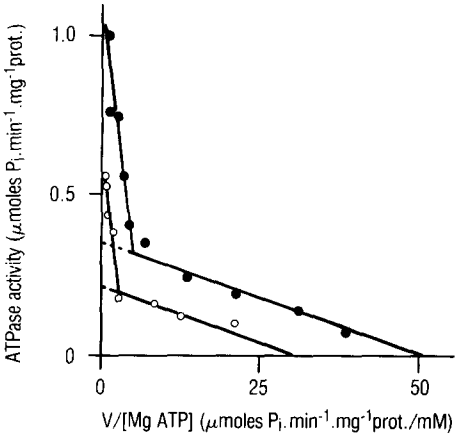

Fig. 7. Apparent affinity of the Ca^{2+}-translocating ATPase for the MgATP complex. Eadie-Hofstee plots were made of the activity of the enzyme at different concentrations of MgATP complex. The purified enzyme (9.5 μg protein) was assayed at 37°C in a total volume of 2.5 ml of the following medium: 124 mM KCl, 25 mM potassium-Hepes (pH 7.4), 0.8 mM dithiothreitol, 0.024% (w/v) Triton X-100, 0.08% (w/v) sonicated asolectin, 5 mM $MgCl_2$, 200 μM EDTA, 200 μM EGTA, sufficient $CaCl_2$ to attain a concentration of 2 μM free Ca^{2+}, 1 mM phospho*enol*pyruvate, 0.3 mM β-NADH, 19.6 U/ml lactate dehydrogenase, 6.4 U·ml^{-1} pyruvate kinase, and 17.2 mM $(NH_4)_2SO_4$. The experiment was performed in the absence (open circles) and in the presence (filled circles) of 120 nM calmodulin at the indicated concentrations of MgATP complex. The initial rate of β-NADH oxidation was followed spectrophotometrically at 335–290 nm wavelength pairs.

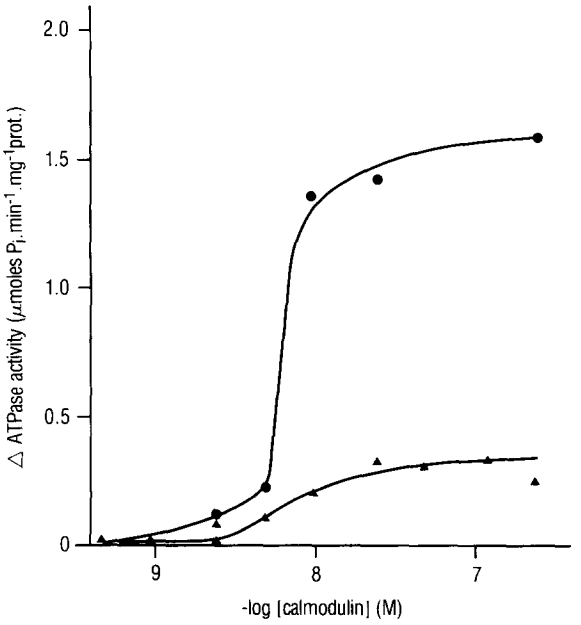

Fig. 8. Apparent affinity of the Ca^{2+}-translocating ATPase for calmodulin at different concentrations of $MgCl_2$. The purified enzyme (9.5 μg protein) was assayed at 37°C in a total volume of 2.5 ml of the following medium: 124 mM KCl, 25 mM potassium-Hepes (pH 7.4), 0.8 mM dithiothreitol, 0.024% (w/v) Triton X-100, 0.08% (w/v) sonicated asolectin, 5 mM (circles) or 0.5 mM (triangles) $MgCl_2$, 200 μM EDTA, 200 μM EGTA, sufficient $CaCl_2$ to attain a concentration of 2 μM free Ca^{2+} ion, 1 mM phospho*enol*pyruvate, 0.25 mM β-NADH, 24 U/ml lactate dehydrogenase, 23.5 U/ml pyruvate kinase, 10.75 mM $(NH_4)_2SO_4$, at the indicated concentrations of calmodulin. The initial rate of β-NADH oxidation was followed spectrophotometrically at 335–290 nm wavelength pairs.

served. It should be noted that there was a basal ATP hydrolysis in the absence of any $MgCl_2$ added to the medium, which contains EDTA. When the concentration of ATP was increased to 5 mM in another series of experiments (not shown), inhibition of the rate of ATP hydrolysis took place only at concentrations of $MgCl_2$ above 5 mM, as expected, indicating again that the inhibitory effect resulted from an increase in free Mg^{2+}.

Apparent affinity of the Ca^{2+}-translocating ATPase for the ATP complex

Fig. 7 presents an Eadie-Hofstee plot of the rate of ATP hydrolysis at different concentrations of the MgATP complex in the presence and in the absence of calmodulin and at a fixed concentration of free Ca^{2+} (2 μM) in the assay system. The plot is biphasic in both cases. Two apparent Michaelis constants for the MgATP complex ($K'_{m[MgATP]}$) were calculated. The high-affinity site has a $K'_{m[MgATP]}$ value of $7 \cdot 10^{-6}$ M, and the low-affinity site has a $K'_{m[MgATP]}$ value of $1.4 \cdot 10^{-4}$ M. The biphasic trend was also observed (results not shown) when the experiment was performed at lower concentrations (0.5 mM) of $MgCl_2$ in the system, with $K'_{m[MgATP]}$ values of the same order of magnitude as those at the higher concentrations (5 mM) of $MgCl_2$ (see Fig. 7).

Apparent affinity of the Ca^{2+}-translocating ATPase for calmodulin

The purified Ca^{2+}-translocating ATPase activity was stimulated by calmodulin at high concentrations of free Mg^{2+}, as shown in Fig. 2A. To test the influence of Mg^{2+} on the apparent affinity for calmodulin, in the experiment represented in Fig. 8, the activity of the calmodulin stimulated ATPase (i.e., ATPase activity in the presence minus the absence of calmodulin) was plotted against the −log[calmodulin]. The experiment was performed at constant concentrations of total ATP (2 mM) and free Ca^{2+} (2 μM), and at the indicated concentrations of $MgCl_2$. The concentration of calmodulin for half-maximal activation ($K_{0.5[calmodulin]}$) was $6.3 \cdot 10^{-9}$ M at both 0.5 mM and 5 mM $MgCl_2$.

Discussion

The membrane bound Ca^{2+}-translocating ATPase from erythrocytes has been reported to have high and low-affinity sites for Ca^{2+} [21–23], their distribution depending on the method of membrane preparation [23], the conditions of assay [24] and the concentration of calmodulin in the assay [23,25–31]. The present results with the purified enzyme confirm that the Ca^{2+}-translocating ATPase exhibits high-affinity site(s) for Ca^{2+} in the presence of calmodulin, and their apparent affinity is decreased in the absence of calmodulin (Fig. 2). The generally lower stimulatory effect of calmodulin found in this study, compared to that reported by others [29] in enzyme reconstituted in phosphatidylcholine, is most likely due to the presence of acidic phospholipids in the asolectin preparation used during the purification and assay of

the enzyme in this work. Nevertheless, we found that increasing free Mg^{2+} concentrations in the range of $4 \cdot 10^{-7}$–$3 \cdot 10^{-3}$ M decreased the apparent affinity of the purified enzyme for Ca^{2+}, both in the presence and absence of added calmodulin (see Fig. 3). This effect could be interpreted as a competition between Mg^{2+} and Ca^{2+} at the high Ca^{2+} affinity sites on the enzyme, as proposed by Penniston [32]. The decrease in apparent affinity for Ca^{2+} in the absence of calmodulin is more marked at higher levels of free Mg^{2+}, so that the increase in apparent Ca^{2+} affinity induced by calmodulin is most evident at these high Mg^{2+} concentrations. The calmodulin concentration used in these experiments was saturating at all $MgCl_2$ concentrations tested (see Fig. 8).

Increasing $MgCl_2$ also inhibited ATP hydrolytic activity; this affect appeared to be due to free Mg^{2+}, as it was observed only when total $MgCl_2$ exceeded the total ATP concentration in the medium (Fig. 6). However, we cannot exclude the possibility that excess $MgCl_2$ inhibited hydrolysis by lowering the CaATP or free ATP concentration (see Ref. 33 for further discussion). It is not clear whether the effect of Mg^{2+} on both Ca^{2+} affinity and ATP hydrolytic activity occur at the same site and by induction of one single low activity state, or whether these are separate effects of Mg^{2+}. The latter is more likely, since the inhibition of the ATP hydrolysis occurs at millimolar concentrations, while the effects of Mg^{2+} on Ca^{2+} affinity also became apparent at micromolar concentrations. In addition, an increase of the H^+ concentration in the assay system also caused a strong decrease of the apparent affinity of the enzyme for Ca^{2+}, both in the absence and in the presence of calmodulin (see Fig. 5). These results suggest that the binding of Ca^{2+} to the enzyme occurs at Ca^{2+}-binding site(s) containing groups which dissociate over the pH range studied, and which preferentially bind Ca^{2+} and produce maximum activation of the enzyme in the dissociated state. The affinity of the purified enzyme for calmodulin, at a constant concentration of $Ca^{2+}_{(free)}$, was approximately the same ($6.3 \cdot 10^{-9}$ M) at 0.5 and 5.0 mM $MgCl_2$ (Fig. 8), in contrast to an earlier report from a preliminary study [34], in which some differences were found. This has been confirmed in four separate preparations using both the inorganic phosphate assay and the spectrophotometric assay with the ATP regenerating system.

The activation of the purified enzyme by Ca^{2+} occurred exponentially over a narrow range of Ca^{2+} concentration, indicating a cooperative mechanism of Ca^{2+} activation. The positive cooperative effect has also been observed previously in the membrane-bound form of the enzyme (Hill coefficient ranging from 1.5 to 3) [23,26]. It was further reported that solubilization of the enzyme increased its cooperativity induced by Ca^{2+} [35]. We show in the present study that calmodulin modulates the degree of cooperativity expressed, from around 2 in its absence to near 4 in its presence, at high pH values. However, no significant differences in the presence or absence of calmodulin was found at low pH values (Hill coefficient below 2). The value of the Hill coefficient close to 4 might indicate the participation of at least four Ca^{2+} ions in the enzyme activation. While the stoichiometry of Ca^{2+}-translocation to ATP hydrolysis is uncertain, varying from 2 to 1 in various studies [3], there is general agreement that two Ca^{2+} ions bind to the Ca^{2+} pump during transport [5]. Binding of two Ca^{2+} ions per ATPase cycle [36] and a cooperative Ca^{2+} activation (Hill coefficient = 2) [37] have also been demonstrated for the sarcoplasmic reticulum Ca^{2+}-translocating ATPase. In this latter enzyme, two Ca^{2+} ions have been demonstrated to bind cooperatively to each monomer, the binding of Ca^{2+} to the first site being followed by higher affinity binding to a second site [37]. A similar analysis is not available for the erythrocyte Ca^{2+}-translocating ATPase. Since the erythrocyte Ca^{2+}-translocating ATPase apparently functions as a dimer in the erythrocyte membrane [38], it may be that the cooperative activation in the presence of calmodulin involves interactions between two ATPase monomers, each associated with the binding of two Ca^{2+} ions. Another possibility is that the Hill slope reflects the well-known binding of four Ca^{2+} ions to calmodulin [39], although it has been argued that the Ca^{2+} transported is unlikely to be that associated with calmodulin [40]. Modulation of the Ca^{2+} sensitivity of the Ca^{2+}-translocating ATPase by calmodulin could have a physiological role in enhancing the efficiency of removal of cellular Ca^{2+} following its transient elevation in cells [26].

A number of reports have demonstrated that activation of the Ca^{2+}-translocating ATPase by ATP shows two apparent Michaelis constants for the MgATP complex ($K'_{m(MgATP)}$), one in the order of $1-4 \cdot 10^{-6}$ M, and another of lower apparent affinity, in the order of $1.2-3.3 \cdot 10^{-4}$ M [25,41–43]. In our experiments, the purified Ca^{2+}-translocating ATPase demonstrated similar biphasic behaviour with respect to ATP activation both in the presence and in the absence of calmodulin (Fig. 7). The two values of $K'_{m(MgATP)}$ differed by nearly two orders of magnitude under both conditions. This result therefore, differs from the membrane-bound enzyme, where the low-affinity ATP activation occurs only [25] or more markedly [44] in the presence of calmodulin. The reason for this difference is unclear, but may reflect either a difference in conformation of the enzyme in the membrane-bound and solubilized forms or masking of the calmodulin effect by acidic phospholipids or other components in the purified enzyme preparation. For instance, only a single high-affinity ATP site is observed when the purified Ca^{2+}-ATPase is assayed at a high Ca^{2+} concentration (1 mM) [45]. It is considered that the activation by ATP at the low affinity site plays a regulatory function [42] and that this site is probably the site of hydrolysis of *p*-nitrophenyl phosphate [45]. Recent experiments, however, suggest that the low- and high-affinity sites for ATP may be located on alternating conformational states (E_1 and E_2) of the enzyme [46], which may account for the dependence of the biphasic ATP kinetics on the enzyme effectors present.

In conclusion, we have shown that the purified ($Ca^{2+} + Mg^{2+}$)-ATPase from human erythrocytes has regulatory properties similar to those of the native enzyme in the membrane. These observations in turn suggest that this purified enzyme should be an appropriate system to study in reconstituted artificial phospholipid vesicles in order to elucidate the mechanisms of Ca^{2+} translocation.

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