The Binding of Nucleotides and Bivalent Cations to the Calcium-and-Magnesium Ion-Dependent Adenosine Triphosphatase from Rabbit Muscle Sarcoplasmic Reticulum

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The binding of MgATP to purified Ca^{2+} - Mg^{2+} -dependent adenosine triphosphatase from rabbit muscle sarcoplasmic reticulum was studied by using a flow-dialysis method. Phosphoryl-enzyme formation and catalytic activity were also measured, and all three processes demonstrated negative co-operativity, with half-saturation of all three parameters at a MgATP concentration of 40–50 μM , and a Hill coefficient (h) of 0.8. The variation of binding constant with pH was measured and showed tighter binding of MgATP with increasing pH over the range 6.8–8.5. Binding parameters for ATP analogues were also measured. The binding of Ca^{2+} in the presence and absence of ATP analogues gave half saturation at a Ca^{2+} concentration of 1.2–1.3 μM . Hill plots of Ca^{2+} -binding data gave a slope of 0.8. These results show that the binding of MgATP and Ca^{2+} can occur in a random manner, with neither substrate influencing the affinity of the enzyme for the other.

The Ca^{2+} - Mg^{2+} -dependent ATPase† from rabbit skeletal muscle sarcoplasmic reticulum is the enzyme responsible for the active accumulation of Ca^{2+} by the sarcoplasmic reticulum, causing muscle relaxation. A crude enzyme preparation may be isolated from homogenized rabbit muscle by differential centrifugation; the resulting preparation contains about seven proteins and phospholipids. The ATPase enzyme responsible for Ca^{2+} transport may be further purified from the crude preparation by detergent treatment and ammonium acetate fractionation, the purified enzyme retaining about 100 molecules of phospholipid for each enzyme molecule (MacLennan *et al.*, 1972). The Ca^{2+} - Mg^{2+} -dependent ATPase will react with MgATP in the presence of Ca^{2+} to form a phosphoryl-enzyme complex (Yamamoto & Tonomura, 1968; Makinose, 1969; Martonosi, 1969), and this complex is stable when quenched in acid.

The present experiments have been performed to obtain data on the dissociation constants of the enzyme for its substrates and to see how these com-

pare with the K_m values of the enzyme for phosphorylation and for steady-state ATPase activity. This information is required to elucidate the mechanism of enzyme phosphorylation and dephosphorylation. Experiments are described in which the binding of Ca^{2+} , ATP and various ATP analogues are measured by a rapid-flow dialysis method (Colowick & Womack, 1969). Phosphoryl-enzyme formation is also studied together with rates of steady-state ATPase activity and the relevance of these results is discussed with respect to possible enzyme mechanisms.

Materials and Methods

Enzyme purification

The Ca^{2+} - Mg^{2+} -dependent ATPase was purified by the method of MacLennan (1970) from leg and back muscles of New Zealand White Rabbits obtained from Hyline Rabbits Ltd., Marston, Northwich, Cheshire, U.K. The procedure designated preparation A by MacLennan (1970) was followed. The soluble purified enzyme so obtained was converted into insoluble enzyme by removal of the deoxycholate by gel filtration. Samples (10 ml) of enzyme were passed through a column (2.5 cm $\times$ 30 cm) of Sephadex G-25 previously equilibrated with buffer containing 0.55 M-sucrose, 0.045 M-Tris/HCl, 0.001 M-histidine and 0.58 M-ammonium acetate, pH 8.0. The cloudy white vesicular enzyme eluted from the column was concentrated by centrifugation

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† Abbreviations: ATPase, adenosine triphosphatase; EGTA, ethanedioxybis(ethylamine)tetra-acetic acid; AMP-P(NH)P, adenylyl-5'-yl imidodiphosphate; AMP-PP(S), adenosine 5'-(3-thio)triphosphate; thio-ITP, 6-mercaptinosine 5'-triphosphate; FTP, formycin 5'-triphosphate; TNP-ATP, trinitrophenyladenosine 5'-triphosphate.

at 100000g for 30 min at 4°C in the 8×25 ml head of a MSE Superspeed 50 centrifuge. The pellet of enzyme was resuspended in the same buffer, by using a small glass homogenizer, to a concentration of 15mg/ml and stored at -20°C.

Enzyme purity was determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (MacLennan, 1970). One major band of protein was present, with an apparent mol.wt. of 95000-105000. Traces (<2%) of a minor component with a mol.wt. of 55000 were seen in some preparations, and this corresponds to the band identified by MacLennan (1970) as calsequestrin. Traces (<2%) of high-molecular-weight polymers (>200000 mol.wt.) were sometimes seen.

Gels run at pH2.4 after incubation with Mg[γ -³²P]-ATP by the methods of Jordon & Raymond (1969) and Avruch & Fairbanks (1972) were radioautographed on X-ray film (Kodirex KD 54T). The 100000-mol.wt. band was the only component to contain detectable ³²P radioactivity.

The enzyme preparation was taken to be over 95% pure by these criteria, and a suspension of 1mg of protein/ml as determined by the method of Lowry *et al.* (1951) was taken to be 10 μ M.

Adenylate kinase could possibly affect some of the results if present, and assays for this enzyme were performed as described below. No detectable activity could be found (<0.002 EC unit/mg).

The Ca²⁺+Mg²⁺-dependent ATPase preparation was tested for Ca²⁺ accumulation in the presence of oxalate, by using the conditions published by Weber *et al.* (1966). No Ca²⁺ accumulation could be detected, and the preparation was assumed to be freely permeable to Ca²⁺ when prepared in this manner.

Ca²⁺ depletion of reagents

Ca²⁺-depleted enzyme was prepared by adding buffered EGTA to a final concentration of 25 mM to the enzyme. Ca/EGTA and excess of EGTA were then separated from the enzyme by gel filtration of 1 ml samples on a column (30 cm×1 cm) of Sephadex G-25 pre-equilibrated with Ca²⁺-depleted buffer containing either 0.25 M-sucrose, 0.05 M-Tris/HCl and 0.001 M-histidine, pH8.0, or 0.1 M-Tris/HCl and 0.001 M-MgSO₄, pH8.0.

All reagents except for KCl and MgSO₄ were depleted of Ca²⁺ by passage through Chelex-100 resin columns (15 cm×1.5 cm) (from Bio-Rad Laboratories Ltd., Bromley, Kent, U.K.), in the K⁺ form. KCl and MgSO₄ were depleted of Ca²⁺ by recrystallization from solutions containing 10 mM-EGTA, followed by two recrystallizations from double-distilled water to lower EGTA contamination.

Ca/EGTA buffers were used to obtain solutions with a known and constant free Ca²⁺ ion concentration.

A 10 mM-Ca/EGTA buffer was prepared by mixing

20 mM solutions of EGTA and CaCl₂ (diluted from BDH standard 1 M-CaCl₂ solution) in the appropriate ratio to give a solution with the desired free Ca²⁺ concentration and the pH adjusted to 7.4 with NaOH before diluting to volume with water. The ratio of EGTA/CaCl₂ required was determined by using the dissociation constant for Ca/EGTA of 1.2×10^{-6} M at pH7 given by Weber *et al.* (1966). For a 26 μ M-free calcium solution the ratio of EGTA/CaCl₂ was 1:0.96. Unless otherwise defined this solution is referred to as Ca/EGTA buffer. The free Ca²⁺ concentration can be varied by altering the EGTA/CaCl₂ ratio.

Radiochemicals

Radioisotopes were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [γ -³²P]-ATP was prepared from [³²P]P_i by isotope exchange by the method of Glynn & Chappell (1964) and was further purified on a Whatman DE-52 DEAE-cellulose anion-exchange column (Cl⁻ form) pre-equilibrated with 0.05 M-Tris/HCl, pH8.0. The [γ -³²P]ATP was eluted with a linear KCl gradient (0.05-0.5 M). [γ -³²P]ATP was eluted at approx. 0.2 M-KCl and was stored frozen at -20°C.

Other reagents

ATP (disodium salt), ADP (free acid), adenylyl-5'-yl imidodiphosphate [AMP-P(NH)P] and adenosine 5'-(3-thio)triphosphate [AMP-PP(S)] (tetralithium salts), NADH, lactate dehydrogenase, pyruvate kinase and phosphoenolpyruvate were all obtained from The Boehringer (London) Corporation Ltd., Uxbridge Road, London, U.K. 6-Mercaptosine 5'-triphosphate (thio-ITP), trinitrophenyladenosine 5'-triphosphate (TNP-ATP) and formycin 5'-triphosphate (FTP) were kind gifts from Dr. J. F. Eccleston and Dr. D. R. Trentham, University of Bristol.

Sodium deoxycholate (BDH reagent grade) was recrystallized by the method of MacLennan (1970). The rare-earth elements gadolinium, europium and lanthanum were obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K., as their chloride salts. All other reagents were obtained from BDH Chemicals Ltd. (Poole, Dorset, U.K.) and were of AnalaR grade whenever available.

Protein determination

Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Enzyme activities

The Ca²⁺+Mg²⁺-dependent ATPase catalytic activity was measured either by pH-stat measurement or by linked-enzyme assay.

pH-stat assay. Protons released on ATP hydrolysis

were titrated with dilute NaOH by a Radiometer pH-stat assembly consisting of a type TTT1C titrator, type SBH 1a syringe burette and type SBR 2c pen recorder. Assays were performed at 20°C with an assay volume of 5 ml. A 0.5 ml burette containing 5 mM-NaOH allowed minimum rates of 0.02–0.03 μmol of ATP hydrolysed/min to be measured. The reaction vessel was flushed with N_2 to minimize interference from absorption of atmospheric CO_2 .

Linked enzyme assay. The ADP formed by the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase was rephosphorylated to ATP by using phosphoenolpyruvate with pyruvate kinase. Pyruvate was then converted into lactate with lactate dehydrogenase and NADH and the rate of NADH disappearance was measured in a spectrophotometer at 340 nm.

Reaction mixtures in general contained 20 mM-Tris/HCl, 100 mM-KCl, 10 mM-Ca/EGTA giving a free Ca^{2+} concentration of 26 μM , 0.5 mM- MgCl_2 , 0.1 mM-phosphoenolpyruvate, 0.4 mM-NADH, 0.03 mg of pyruvate kinase/ml and 0.03 mg of lactate dehydrogenase/ml. A fivefold increase in pyruvate kinase concentration had no effect on the observed rate of NADH oxidation at all ATP concentrations used. Pyruvate kinase and lactate dehydrogenase were added as suspensions in $(\text{NH}_4)_2\text{SO}_4$.

Adenylate kinase assay

Adenylate kinase was assayed in the presence of ATPase by incubating enzyme with $[2\text{-}^3\text{H}]\text{ADP}$, acid quenching, chromatographing the supernatant and counting the radioactivity of the $[2\text{-}^3\text{H}]\text{AMP}$ formed. In a typical assay, 3.4 mg of enzyme was incubated in 1 ml of buffer containing 100 mM-triethanolamine/HCl, pH 7.5, 3 mM- $[2\text{-}^3\text{H}]\text{ADP}$ (specific radioactivity 3.33 $\mu\text{Ci}/\mu\text{mol}$), 3 mM- MgCl_2 and 30 μM - CaCl_2 . Samples (0.5 ml) were removed and quenched after 0 and 5 min, and the mono- and di-nucleotides separated by ion-exchange t.l.c. as described below. Radioactivity was measured by scintillation counting, after cutting out and eluting the spots.

Scintillation counting

Radioactivity was measured by liquid-scintillation counting in a Nuclear-Chicago Isocap 300 instrument. Samples were mixed with 15 ml of scintillation liquid, composed of 3 litres of toluene, 2 litres of 2-methoxyethanol, 400 g of naphthalene and 30 g of 2-(4-t-butylphenyl)-5-(4-biphenyl)-1-oxa-3,4-diazole (butyl-PBD). This liquid would accept up to 5% of aqueous sample without significant quenching of ^{32}P counts, but volumes of under 3% have been used in the experiments described here. Radioactivity was recorded in two channels, and these were programmed for each radioisotope used. Quenching was measured either from the ratio of counts in the two channels (sample-counts ratio) or from the distribution of counts from an external gamma source (9 μCi of

^{133}Ba), which produced a large number of counts compared with the sample (external-standard ratio). ^{32}P radioactivity was counted by using the ^{32}P programme with sample-counts-ratio quench correction, ^{45}Ca radioactivity was counted by using the ^{45}Ca programme with sample-counts-ratio quench correction, and ^3H radioactivity was counted by using the ^3H programme with external-sample-ratio quench correction. Quenching was normally insignificant for ^{32}P and ^{45}Ca , but counts for ^3H were calculated from the channels ratio with the external source with the aid of a quench curve prepared separately with a known amount of $^3\text{H}_2\text{O}$ and increasing proportions of water.

Phosphoryl-enzyme formation

Purified $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase was mixed rapidly with $\text{Mg}[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and Ca/EGTA buffer and the mixture quenched after 2 s by squirting an equal volume of trichloroacetic acid (0.612 M containing 2 mM-ATP and 5 mM-potassium phosphate) into the reaction mixture from a syringe. The precipitated protein was separated by filtration on 2.5 cm-diam. Whatman glass-fibre discs (GF/D) and washed with a further 30 ml of trichloroacetic acid (0.306 M) on a vacuum-filtration manifold. After being sucked dry, the filter discs were placed in scintillation vials containing 15 ml of scintillation fluid and counted for radioactivity. Controls were carried out with $\text{Mg}[\gamma\text{-}^{32}\text{P}]\text{ATP}$ being added after quenching to estimate ATP contamination of the washed pellet.

Ligand binding

The dissociation constants of MgATP , MgADP and Ca^{2+} from the enzyme were measured by the method described by Colowick & Womack (1969). A small flow-dialysis cell was used with a lower chamber volume of 2.8 ml and a membrane area of 3 cm^2 .

Enzyme (1.8 ml) and radioactive ligand in the appropriate buffer (see Figure legends) were added to the upper chamber and buffer alone was pumped through the lower chamber at a flow rate of 2.4 ml/min by using an LKB Vario-Perpex pump. Both chambers were stirred with magnetic stirring bars. A steady-state rate of dialysis was obtained 6 min after additions were made to the upper chamber.

Ion-exchange t.l.c.

Nucleoside mono-, di- and tri-phosphates were separated by ion-exchange t.l.c. on polyethyleneimine-cellulose thin-layer sheets (MN-Polygram Cel 300 PEI/UV, from Camlab Ltd., Cambridge, U.K.). Streaks (20 μl) of samples were applied to the sheets (10 cm $\times$ 5 cm) and the chromatograms were developed in 0.75 M- KH_2PO_4 , pH 3.4, until the solvent front had travelled 8 cm. This procedure is similar to that described by Goody & Eckstein (1971).

Nucleotide spots were detected as dark areas against a fluorescent background when irradiated with short-wave u.v. light.

Results

Nucleotide binding

The dissociation constant of MgATP from the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase was determined by the flow-dialysis method of Colowick & Womack (1969). It was essential that MgATP hydrolysis did not occur during the time-course of the experiment; thus the binding was measured in the absence of Ca^{2+} , all the solutions used were depleted of Ca^{2+} before use and the experiments were carried out in the presence of 25 mM-EGTA. This concentration of EGTA complexed less than 1% of the added Mg^{2+} at pH 7.4. From the binding constants given by Schwarzenbach (1956), 98% of the ATP would be complexed by Mg^{2+} ions at 5 μM -ATP and more than 91% at 1 mM-ATP. K^{+} ions have negligible binding to EGTA or ATP under these conditions (KATP^{3-} is 30 μM at 1 mM-ATP). Typical results of such a determination are shown in Fig. 1(a), where the binding curve is shown with increasing MgATP concentration. These data cannot be analysed as a simple rectangular hyperbola, as shown by the Scatchard (1949) plot in Fig. 1(b), but replotting of the data as a Hill plot (Brown & Hill, 1922–23) gives the result shown in Fig. 1(c), where points from three different experiments are plotted. A straight line is obtained with a Hill coefficient (h) of 0.79 and a half-saturating

MgATP concentration of 51 μM . At the end of the binding experiments the enzyme was removed from the dialysis cell, the protein precipitated with trichloroacetic acid (0.306 M) and the denatured protein centrifuged off in a bench centrifuge. The clear supernatant was neutralized with 4 M-sodium acetate and then analysed by t.l.c. as described above. With the conditions stated above, less than 5% hydrolysis of MgATP occurred as judged by $[\gamma\text{-}^{32}\text{P}]$ phosphate concentrations in the enzyme supernatant.

The binding of MgADP was also measured by the rate-of-dialysis method, by using $\text{Mg}[2\text{-}^3\text{H}]\text{ADP}$ as the radioactive ligand. The addition of non-radioactive MgADP were made with a Hamilton syringe to the upper chamber of the dialysis cell. The binding curve and Hill plot of the results are shown in Figs. 2(a) and 2(b). The results were corrected for quenching by using a calibration curve obtained with $^3\text{H}_2\text{O}$ with the scintillation counter set for ^3H counting in the external-standard-ratio mode. The Hill plot was drawn by using a bound maximum of 35 μM obtained from extrapolation of a Scatchard plot (not shown) to the bound axis. The plot gives a Hill coefficient of 0.76, with half-maximum binding at a MgADP concentration of 120 μM .

Phosphoryl-enzyme formation

The amount of phosphoryl-enzyme formed at different MgATP concentrations was measured by the method given above, by quenching samples into trichloroacetic acid after 2 s. The proportion of total MgATP hydrolysed within this time period is negli-

Fig. 1. Binding of MgATP to the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase determined by the rate-of-dialysis method

(a) Binding curve for MgATP to the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase determined by the method of Colowick & Womack (1969). To a solution of 40 μM -ATPase, 100 mM-KCl, 25 mM-EGTA, 10 mM-Tris/HCl, 1 mM-MgSO₄ and 5 μM -Mg $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (specific radioactivity of 1.4 mCi/ μmol) at pH 7.4 and 20°C was added non-radioactive MgATP, increasing the concentration from 5 μM to 1 mM. The same mixture without enzyme and MgATP was pumped through the lower chamber. The radioactivity (c.p.m.) in each fraction was corrected for loss through dialysis. The radioactivity in the first fractions, before addition of non-radioactive MgATP, was subtracted from all subsequent fractions after correction for loss by dialysis, and results are plotted against the MgATP concentration. On average the increase in radioactivity over the baseline was approx. 30%. (b) The data in (a) were analysed as a Scatchard (1949) plot, [bound MgATP] versus [bound MgATP]/[free MgATP]. At a given MgATP concentration, the ratio of radioactivity/maximum radioactivity (determined at 1 mM-MgATP) represents the fraction of the MgATP unliganded. (c) A Hill plot of the data in (a). The maximum MgATP bound, [bound]_{max}, was determined by extrapolation of a double-reciprocal plot to an infinite MgATP concentration. The half-saturating concentration of MgATP, i.e. when $\log([bound]_{max} - [bound]) = 0.0$, was 51 μM , with a Hill coefficient (slope) of 0.79. Points from two additional experiments have been added to this Hill plot.

Fig. 2. Binding of MgADP to the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase determined by the rate-of-dialysis method

(a) A binding curve for MgADP to the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase determined by using the method of Colowick & Womack (1969) described in the Materials and Methods section. To a solution of $40 \mu\text{M}$ enzyme, 100 mM-KCl , 10 mM-Tris/HCl , 1 mM-MgCl_2 , 10 mM-Ca/EGTA giving a free $[\text{Ca}^{2+}]$ of $26 \mu\text{M}$, and $1 \mu\text{M-Mg}[2\text{-}^3\text{H}]\text{ADP}$ (specific radioactivity $4.5 \text{ mCi}/\mu\text{mol}$), at $\text{pH } 7.4$ and 20°C was added non-radioactive MgADP, varying the concentration from $1 \mu\text{M}$ to 1 mM . The same mixture without enzyme and MgADP was pumped through the lower chamber. The radioactivity in the fractions was corrected for quenching and loss through dialysis as described in the Materials and Methods section, and results are plotted against the MgADP concentration. The average maximum increase in radioactivity over the baseline was 20–25%. (b) A Hill plot of the data in (a) giving $K_D = 120 \mu\text{M}$ and Hill coefficient = 0.76.

Fig. 3. Formation of phosphoryl-enzyme by $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase as a function of MgATP concentration

(a) The saturation curve was obtained by mixing $5 \mu\text{M}$ enzyme with $0.5\text{--}48 \mu\text{M-Mg}[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (specific radioactivity $6.4 \mu\text{Ci}/\mu\text{mol}$) in a solution of 100 mM-KCl , 10 mM-Tris/HCl and 10 mM-Ca/EGTA giving a free Ca^{2+} concentration of $33 \mu\text{M}$ at $\text{pH } 7.4$ and 20°C . The reactions were terminated with trichloroacetic acid (0.306 M)/ $2 \text{ mM-ATP}/5 \text{ mM-P}_i$ after zero and 2 s, and the concentration of phosphoryl-enzyme, $[\text{E-P}]$ was measured as described in the Materials and Methods section. (b) A Hill plot of the data in (a) giving $K_D = 37 \mu\text{M}$ and a Hill coefficient of 0.76.

Fig. 4. Rate of ATP hydrolysis by $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase as a function of ATP concentration monitored as NADH oxidation by the linked assay system

(a) A double-reciprocal plot of activity against MgATP concentration obtained by using reaction mixtures containing 100 mM-KCl , 10 mM-Ca/EGTA giving a free Ca^{2+} concentration of $26 \mu\text{M}$, 20 mM-Tris/HCl , 0.5 mM-MgCl_2 , $0.1 \text{ mM-phosphoenolpyruvate}$, 0.4 mM-NADH , 0.1 mg each of pyruvate kinase and lactate dehydrogenase, $0.3 \mu\text{M-Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase and MgATP concentrations ranging from $1.25 \mu\text{M}$ to 1.22 mM . Reactions were followed by measuring the disappearance of NADH extinction at 340 nm at $\text{pH } 7.4$ and 20°C . Two possible K_m values were calculated, 10 and $44 \mu\text{M}$, the latter from an expanded plot at the higher MgATP concentrations. (b) A Hill plot of the data in (a), where v is the rate measured at the various MgATP concentrations and V_{max} is the rate determined from plot (a) at an infinite MgATP concentration. An apparent K_m of $47.8 \mu\text{M}$ and a Hill coefficient of 0.77 were calculated from this plot.

gible. Fig. 3(a) shows a typical phosphorylation curve. Analysis of these data by a double-reciprocal plot yields a non-linear graph but a Hill plot gives a straight line (Fig. 3b), with a Hill coefficient of 0.76 and half-maximum phosphorylation at $37\text{ }\mu\text{M}$ -MgATP. These values are close to those obtained for MgATP binding.

Ca²⁺ + Mg²⁺-dependent ATPase activity

Enzyme catalytic activity was measured both by a pH-stat assay and by using a MgATP-regenerating system as described in the Materials and Methods section. Although data from both methods gave similar results, those presented in Fig. 4(a) were obtained by the linked-assay system, as this gave greater accuracy with low MgATP concentrations. They are presented as a double-reciprocal plot and it is again evident that the data cannot be analysed as a simple rectangular hyperbola. Reprocessing these results as a Hill plot gives the result shown in Fig. 4(b), where a straight line is obtained with a Hill coefficient of 0.77 and a half-maximal rate at a MgATP concentration of $48\text{ }\mu\text{M}$.

The MgATP concentration giving half-maximal activity ($K_{0.5}$) at different pH values was determined by the pH-stat assay. The results are shown in Table 1. Hill coefficients for assays at pH 7.8 and 8.2 are of limited accuracy, as all concentrations used were above the $K_{0.5}$ value.

Ca²⁺ + Mg²⁺-dependent ATPase activity on ATP analogues

Ca²⁺ + Mg²⁺-dependent ATPase activity was assayed in the presence of various chromophoric ATP analogues by the pH-stat assay procedure to measure nucleotide hydrolysis. Those analogues that were not substrates were tested for inhibitory activity against the hydrolysis of MgATP. The results of these assays are summarized in Table 2, which gives the values of

k_{cat} , and K_i or $K_{0.5}$ for the analogues used. The data were analysed as Lineweaver-Burk plots, because the limited availability of these analogues allowed the concentration to be varied over only a limited range.

Binding of cations to the Ca²⁺ + Mg²⁺-dependent ATPase

The binding of Ca²⁺ to the Ca²⁺ + Mg²⁺-dependent ATPase was studied by the rate-of-dialysis method of Colowick & Womack (1969) with ⁴⁵Ca. Binding was measured both in the absence of nucleotide and in the presence of the MgATP analogues MgAMP-PP(S) and MgAMP-P(NH)P. MgATP could not be used because the catalytic rate of the enzyme was too great. MgAMP-P(NH)P was not hydrolysed by the enzyme, but MgAMP-PP(S) was hydrolysed slowly. To ensure that the MgAMP-PP(S) was not totally hydrolysed during the course of the experiment, the enzyme was removed from the dialysis cell at the end of the experiment, precipitated with trichloroacetic acid and the solution clarified by centrifugation.

Table 1. *Effect of pH on the $K_{0.5}$ of the Ca²⁺ + Mg²⁺-dependent ATPase for MgATP*

Assays were performed by the pH-stat procedure with assay mixtures containing 100mM-KCl, 10mM-Ca/EGTA giving a free Ca²⁺ concentration of $33\text{ }\mu\text{M}$, and MgATP concentrations varying from 0.075 to 0.575 mM. Reactions were started by the addition of $0.4\text{ }\mu\text{M}$ enzyme. $K_{0.5}$ values were obtained from Hill plots of the data at each pH value.

pH	$K_{0.5}$ (mM)	Hill coefficient (<i>h</i>)
6.8	0.131	0.60
7.1	0.102	0.79
7.4	0.047	0.88
7.8	0.031	0.83
8.2	0.014	1.02

Table 2. *Catalytic parameters of ATP analogues with the Ca²⁺ + Mg²⁺-dependent ATPase*

Assays were performed by the pH-stat procedure with reaction mixtures containing 100mM-KCl, 10mM-Ca/EGTA giving a free Ca²⁺ concentration of $26\text{ }\mu\text{M}$ and various concentrations of substrates and inhibitors. Inhibition constants were determined by using MgATP as the substrate. Reactions were initiated by the addition of $0.5\text{ }\mu\text{M}$ enzyme.

ATP analogue	$K_{0.5}$ (mM)	K_i (mM)	k_{cat} (s ⁻¹)	Concentration range used (mM)
MgATP, pH 7.4	0.047		4.0	0.075–0.575
MgATP, pH 8.0	0.011		2.0	0.050–0.575
ATP, pH 7.4		8.5		0.340–8.340
MgAMP-PP(S), pH 7.4	0.065		0.43	0.156–0.935
MgAMP-PP(S), pH 8.0	0.016		0.42	0.050–0.190
Mg thio-ITP, pH 8.0	0.166		0.69	0.050–0.190
MgFTP-P, pH 8.0	0.154		2.0	0.033–0.108
MgAMP-P(NH)P, pH 7.4		0.280		0.270
MgTNP-ATP, pH 7.4		0.039		0.100

Fig. 5. Ca^{2+} binding to the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase measured by the rate-of-dialysis method

Ca^{2+} -binding curves were obtained by the rate-of-dialysis method of Colowick & Womack (1969) as described in the Materials and Methods section, in solutions containing 12 μM enzyme, 100 mM-KCl, 10 mM-Tris/HCl, 5 mM- MgSO_4 , 2 μM - $^{45}\text{CaCl}_2$ (specific radioactivity 1 mCi/ μmol), (a) without MgAMP-PP(S) and (b) with 5 mM-MgAMP-PP(S) at pH 7.4 and 20°C. The Ca^{2+} concentration was increased to 1.5 mM by the addition of portions of non-radioactive CaCl_2 . A similar solution without enzyme and CaCl_2 was pumped through the lower chamber. Scatchard plots of these data without MgAMP-PP(S) and with MgAMP-PP(S) are shown in (c) and (d) respectively. The maximum value for bound calcium, $[\text{bound}]_{\text{max}}$, was taken from the intercept of the extrapolated line of the points for tight binding with the bound Ca^{2+} -concentration axis of the Scatchard plots, at 24 μM . Dissociation constants of 1.29 and 1.23 μM and Hill coefficients of 0.82 and 0.83 were determined from Hill plots (not shown).

Fig. 6. Effect of Ca^{2+} on the steady-state phosphoryl-enzyme concentration during ATP hydrolysis by $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase

A double-reciprocal plot of the results obtained when 5 μM enzyme was mixed with 50 μM - $\text{Mg}[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in 100 mM-KCl/10 mM-Tris/HCl and either 10 mM- Ca/EGTA giving free Ca^{2+} concentration of 1.8–26 μM , or for higher Ca^{2+} concentrations CaCl_2 , at pH 7.4 and 20°C. The reactions were quenched after 0 or 2 s with trichloroacetic acid and the phosphoryl-enzyme concentrations measured as described in the Materials and Methods section. A dissociation constant of 8.9 μM was calculated from the intercept on the abscissa.

Samples of the supernatant were subjected to t.l.c. on polyethyleneimine-cellulose plates and inspected at 254 nm. The results showed less than 50% hydrolysis of the MgAMP-PP(S) during the time-course of the experiment. MgADP and thiophosphate pro-

duced by hydrolysis would not bind to a significant degree at these concentrations, and this is demonstrated by the good agreement between results in the presence of MgAMP-PP(S) (slowly hydrolysed) and those in the presence of MgAMP-P(NH)P (not hydrolysed). At the Mg^{2+} concentration used, nucleotides would be fully liganded with Mg^{2+} and less than 1% of Ca^{2+} added would be bound by nucleotide.

The results of Ca^{2+} -binding experiments in the presence and absence of MgAMP-PP(S) are shown in Figs. 5(a) and 5(b). From Scatchard plots (Figs. 5c and 5d), two binding components are recognized, a tight-binding component with K_D about 1 μM and a weaker component with K_D about 200 μM . From the intercepts on the 'bound' axis, the tight-binding component represents 2 mol of Ca^{2+} /mol of enzyme, and the weaker component represents about 25 mol of Ca^{2+} /mol of enzyme. The points for the tight-binding component can be replotted as a Hill plot, giving half-maximal Ca^{2+} binding at concentrations of 1.29 and 1.23 μM without and with MgAMP-PP(S) respectively. The slopes of the Hill plots gave Hill coefficients of 0.82 and 0.83. Similar results were obtained by using MgAMP-P(NH)P as the nucleotide with half-maximal binding occurring at 3.3 μM - Ca^{2+} .

Phosphoryl-enzyme formation at different Ca^{2+} concentrations

The response of the steady-state phosphoryl-enzyme concentration to different Ca^{2+} concentrations is shown in Fig. 6. To obtain these results the enzyme was incubated with 50 μM - $\text{Mg}[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and various concentrations of Ca^{2+} adjusted with $\text{Ca}/$

Fig. 7. Effect of Ca^{2+} on the steady-state catalytic activity of the Ca^{2+} -depleted ATPase

The enzyme activity was measured by the pH-stat method by using Ca^{2+} -depleted enzyme and reagents. Reaction mixtures contained 100mM-KCl, 5mM-MgATP, 10mM-EGTA and various amounts of CaCl_2 to give final free Ca^{2+} concentrations ranging from 0.1 to 100 μM , and 0.5 μM enzyme at pH7.4 and 20°C. From the double-reciprocal plot, half-maximal activity was obtained at a Ca^{2+} concentration of 4.4 μM .

EGTA buffers. The reaction mixtures were quenched after 2s with trichloroacetic acid containing ATP and P_i as described in the Materials and Methods section. The results are expressed as a double-reciprocal plot, giving half-maximal phosphorylation with a Ca^{2+} concentration of 8.9 μM .

A Hill plot (results not shown) gave a similar $K_{0.5}$ for phosphorylation, with a Hill coefficient of 0.9. If phosphoryl-enzyme concentrations were measured with respect to time, they decreased as ATP was hydrolysed.

Effect of cation concentration on ATPase activity

The effect of Ca^{2+} on ATPase activity was measured by monitoring the enzyme activity in the pH-stat and by using Ca^{2+} -depleted enzyme and reagents. The results of these assays are shown in Fig. 7, where they are expressed as a double-reciprocal plot. Half-maximal MgATP hydrolysis occurs with a Ca^{2+} concentration of 4.4 μM . At Ca^{2+} concentrations greater than 35 μM the ATPase is inhibited, as shown by the upward curve in the double-reciprocal plot.

The chemical similarity between Ca^{2+} and the rare-earth elements has led to their use as calcium analogues in several enzymes, including α -amylase (Smolka *et al.*, 1971), thermolysin (Colman *et al.*, 1972) and trypsinogen (Darnall & Birnbaum, 1970). Some experiments were performed to investigate the effect of the rare-earth elements lanthanum, gado-

linium and europium on the ATPase activity of the Ca^{2+} -depleted Ca^{2+} -Mg $^{2+}$ -dependent ATPase. A pH-stat assay was used with reaction mixtures containing 100mM-KCl, 5mM-MgATP and 3.33–33.3 μM - Ca^{2+} or rare earth at pH7.4 and 20°C. Reactions were started with 0.5 μM (final concentration) Ca^{2+} -Mg $^{2+}$ -dependent ATPase. The concentrations of metals giving half-maximal rates were 4.4 μM for Ca^{2+} , 13 μM for gadolinium, 31 μM for europium and 15 μM for lanthanum. It is possible that a significant proportion of these cations are bound by ATP.

Discussion

Nucleotide binding

The binding of MgATP to the sarcoplasmic-reticulum ATPase has been studied by flow dialysis and the results described in this paper show that MgATP binds to the enzyme in the absence of Ca^{2+} . The shape of the binding curve is not a simple rectangular hyperbola, but gives a curved Scatchard plot, as shown in Fig. 1. These data could be interpreted as representing two binding constants for MgATP (Yamamoto & Tonomura, 1968), but an alternative explanation would be weak negative co-operative interaction between the protein molecules, and, if this is assumed, the data can be expressed as a Hill plot (Fig. 2), giving half-maximal binding with 44 μM -MgATP and a Hill coefficient of 0.82. The enzyme has been shown to exist as a single polypeptide chain with mol.wt. 100000 (Martonosi & Halpin, 1970) and the MgATP-binding data in Fig. 1 indicate that one molecule of MgATP is bound per polypeptide chain. It is therefore difficult to envisage two different binding sites for MgATP on two identical polypeptide chains, unless there is interaction between adjacent molecules in the membrane. The MgADP-binding data show a similar non-linear Scatchard plot (Fig. 2), with an affinity for MgADP which is about one-third of that for MgATP.

Phosphorylation of the enzyme could be measured by quenching the enzyme 2s after mixing with Mg- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of Ca^{2+} . It has been established by independent experiments (V. C. Duance & D. W. Yates, unpublished work) that phosphoryl-enzyme concentrations remain constant for at least 2s after the initial rapid phosphorylation. The results in Figs. 3(a) and 3(b) show that the saturation curve for enzyme phosphorylation has a similar shape and half-saturation point to the binding curve for MgATP, with half-maximal phosphorylation at 37 μM -MgATP. This suggests that the equilibrium for the phosphoryl-transfer reaction:

must be near to or less than unity, with the rate-limiting step being subsequent to phosphorylation.

As a consequence of the equilibrium of the phosphoryl-transfer reaction being less than unity the

ternary complex $\text{E}-\text{Ca}_2\text{MgATP}$ is a major constituent of the steady-state complex. It then follows that the response of the steady-state catalytic activity to the MgATP concentration should be similar to that found in the binding and phosphorylation experiments (Figs. 2 and 3). This was confirmed as shown in Figs. 4(a) and 4(b), half-maximal activity being attained at $48 \mu\text{M}$ MgATP . Besides the similarity in the MgATP concentration required for half-maximal saturation, phosphorylation and catalytic activity, all three parameters showed similar negative co-operative effects with respect to MgATP , as shown by the slopes of the Hill plots, 0.82, 0.76 and 0.77 for MgATP binding, phosphorylation and catalytic activity respectively.

The rate of phosphoryl-enzyme formation is fast compared with the hydrolysis of MgATP by the enzyme (300 s^{-1} for the observed rate of phosphorylation at high ATP compared with 4 s^{-1} for enzyme catalytic activity; V. C. Duanee & D. W. Yates, unpublished work). As the equilibrium of the phosphoryl-transfer reaction is near to unity, the reverse reaction, phosphorylation of MgADP by phosphoryl-enzyme, must also be fast, as suggested by results of Froehlich & Taylor (1975) and V. C. Duanee & D. W. Yates (unpublished work).

As the half-maximal phosphorylation and ATPase catalytic activity occur at the same MgATP concentration required for half saturation, with the former two being determined in the presence of Ca^{2+} and the latter in its absence, it can be concluded that Ca^{2+} does not influence the binding of MgATP .

Cation binding to the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase

Fig. 5 shows the binding of Ca^{2+} to the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase in the presence and absence of a bound nucleotide. In both cases the stoichiometry of Ca^{2+} binding indicates that 2Ca^{2+} ions are tightly bound per enzyme molecule, in agreement with the published data of Hasselbach (1964) and Yamada *et al.* (1970). MgAMP-PP(S) was chosen as the analogue, as its hydrolysis rate is much slower than that of MgATP ; thus an appreciable amount remained at the end of the experiment. The results show that the presence of a nucleotide [AMP-PP(S) or AMP-P(NH)P] does not affect the affinity of the enzyme for Ca^{2+} and that the enzyme shows negative co-operativity for Ca^{2+} binding in both cases. The degree of co-operativity is similar to that found by Meissner (1973) for Ca^{2+} binding.

Although the presence of a bound nucleotide does not affect the binding of Ca^{2+} to the enzyme, the binding of Ca^{2+} to the phosphoryl-enzyme may be different. The catalytic rate of hydrolysis of MgAMP-PP(S) has been shown to be similar at pH 7.4 and 8.0 (Table 2), differing from that of MgATP in that the rate at pH 7.4 is double that measured at pH 8.0. A possible explanation for this could be a change in the rate-limiting step, as was found with the myosin ATPase (Bagshaw *et al.*, 1972), where the hydrolysis rate of the MgAMP-PP(S) is rate-limiting. If this is the case with the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase, the steady-state concentration of phosphoryl-enzyme would be very low when hydrolysing MgAMP-PP(S) ; thus any change in affinity for Ca^{2+} between the enzyme and phosphoryl-enzyme would not be observed. The rather higher concentrations of Ca^{2+} required for half-maximal phosphorylation of the enzyme may suggest a lower affinity of the phosphoryl-enzyme for Ca^{2+} than the free enzyme, but more data are required to test this suggestion.

The data presented in this paper suggest that bind-

Scheme 1. Reversible formation of phosphoryl-enzyme from enzyme, MgATP and Ca^{2+}

ing of Ca^{2+} and MgATP to the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase is a random process, as indicated by Scheme 1, with affinities for Ca^{2+} and MgATP being unaffected by the presence of liganded MgATP and Ca^{2+} respectively. The data are consistent with the resulting ternary complex being able to react to give the phosphoryl-enzyme in a reversible manner, with the equilibrium being in favour of the ternary complex with MgATP and calcium.

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