Isolation and Partial Characterization of Magnesium Ion- and Calcium Ion-Dependent Adenosine Triphosphatase Activity from Bovine Brain Microsomal Fraction

By Torben SÆRMARK and Hans VILHARDT

Institute of Medical Physiology C, Panum Institute, University of Copenhagen, DK-2200 Copenhagen N, Denmark

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Microsomal fraction was prepared by ultracentrifugation of homogenates of cortical tissue from bovine brains. The preparation displayed ATPase (adenosine triphosphatase) activity in the presence of Mg^{2+} ($6.4 \mu\text{mol}$ of P_i/h per mg of protein) and Ca^{2+} ($3.4 \mu\text{mol}$ of P_i/h per mg of protein). Kinetic analysis of the activation of the enzyme preparation by Ca^{2+} resulted in the demonstration of two apparent K_m values for Ca^{2+} ($6.0 \times 10^{-8} \text{ M}$ and $1.2 \times 10^{-6} \text{ M}$). Treatment of the microsomal membranes with Triton X-100 resulted in solubilization of the ATPase, though with some loss of activity. The solubilized microsomal proteins were incorporated into liposomes. By incubation of the liposomes in media containing $^{45}\text{Ca}^{2+}$ an ATP-dependent uptake of Ca^{2+} was demonstrated. The solubilized preparation was subjected to preparative isoelectric focusing in granulated gel beds. Two distinct peaks of Mg^{2+} - and Ca^{2+} -dependent ATPase activity were observed at pH 4.8 (peak 4.8) and at pH 6.3 (peak 6.3). The material isolated in peaks 4.8 and 6.3 was focused in polyacrylamide gel with pH gradients. The material corresponding to peak 4.8 consisted of a single protein, whereas peak 6.3 contained one major and at least one minor protein. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis confirmed these results and indicated that the major component of peak 4.8 and the protein of peak 6.3 both had a molecular weight of 105 000. The material in peaks 4.8 and 6.3 was assayed for ATPase activity in the presence of various concentrations of Ca^{2+} . Kinetic analysis of the results for peak 4.8 demonstrated an apparent K_m value for Ca^{2+} of $4.1 \times 10^{-8} \text{ M}$. The enzyme isolated at pH 6.3 had an apparent K_m value of $3.8 \times 10^{-6} \text{ M}$. However, when the material from peak 4.8 was incubated in the presence of 1 mM-Mg^{2+} the ATPase could not be activated by Ca^{2+} .

The important role of calcium in cell function has become increasingly well documented over the last few years. Much attention has been paid to the involvement of Ca^{2+} in the secretory processes in a variety of tissues, including nervous tissue (Douglas & Poisner, 1964*a,b*; Katz & Miledi, 1965; Haycock & Meligeni, 1977). A feature common to the release process in these tissues is the uptake by the cells of Ca^{2+} from the surrounding medium (Douglas & Poisner, 1964*b*; Katz & Miledi, 1969; Blaustein, 1971). It has been suggested (Formby *et al.*, 1976; Duncan, 1976) that the entry of Ca^{2+} in the cell may result in activation of a Ca^{2+} -dependent ATPase. This hypothesis is particularly attractive, since it is known that the secretory processes are energy-dependent and can be abolished by inhibitors of ATP synthesis (Douglas *et al.*, 1965).

The presence of Ca^{2+} -dependent ATPase activity

Abbreviations used: ATPase, adenosine triphosphatase; Tes, 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulphonic acid

in subcellular fractions of homogenates of brain tissue has been demonstrated in a series of studies (Germain & Proulx, 1965; Hosie, 1965; Berl *et al.*, 1972; Duncan, 1976; Robinson, 1976). The results have shown not only that the preparations are able to hydrolyse ATP in the presence of Ca^{2+} but that activation of the ATPase also occurs in the presence of Mg^{2+} .

The object of the present work was to study the nature of ionic activation of the ATPase activity in a brain microsomal preparation. In particular we wished to investigate whether the effect of Ca^{2+} and Mg^{2+} on the microsomal ATPase activity could be ascribed to activation of a single enzyme with distinct affinities for the two ions or whether the preparation contained separate ATPases with specific ionic requirements for activation. To obtain such information it was decided to attempt to isolate the ATPase activity of the microsomal fraction. Preliminary studies had indicated that it was possible to solubilize the microsomal ATPase activity by incu-

bation of the membranes with Triton X-100. This particle-free preparation was used for further fractionation of the ATPase activity.

Materials and Methods

Biological material

Brains from adult cows were removed within 15 min of the killing of the animals by shooting and transported to the laboratory in an ice-bucket. The meninges were removed from the parietal lobes and 1–2-mm-thick slices were cut from the cortex. The slices were collected in ice-cold 0.3M-sucrose/1mM-EDTA. Approx. 25 g of tissue (wet wt.) was used for homogenization.

Homogenization and ultracentrifugation

Portions (3–4 g) of tissue were placed on a Perspex [poly(methyl methacrylate)] board and chopped to a fine mince with a stainless-steel knife. The mince was then homogenized in 12 ml of 0.3M-sucrose/1mM-EDTA in a Teflon/glass homogenizer with a radial clearance of 0.15 mm. Three strokes of the pestle slowly up and down at 1200 rev./min was the usual procedure.

The homogenate was centrifuged for 15 min at 800g_{av.} in an MSE Highspeed 18 refrigerated centrifuge. The supernatant was recovered and centrifuged at 10000g_{av.} for 20 min in a Beckman L5-65 ultracentrifuge with a 50.2Ti rotor. The resulting supernatant was further centrifuged at 100000g_{av.} for 60 min. The pellet thus obtained was resuspended in 5mM-Tris/HCl buffer, pH 7.5, and resedimented at 100000g_{av.} for 60 min. This procedure was repeated twice and the final pellet was taken up in 5 ml of 5mM-Tris/HCl buffer, pH 7.5 (washed microsomal fraction). All preparative procedures took place at 4°C. The washed microsomal fraction was either used directly or stored frozen at –20°C.

Solubilization with Triton X-100

Portions (usually approx. 2 mg of protein) of the microsomal preparation were made 1% (v/v) with respect to Triton X-100 and incubated with shaking for 20 min at 20°C. The mixture was then centrifuged at 100000g_{av.} for 60 min at 4°C and the supernatant was carefully removed. The extract displayed Mg²⁺- and Ca²⁺-dependent ATPase activity and could be stored frozen.

Assay of ATPase activity

The ATPase activity in particulate fractions was determined as described by Vilhardt & Hope (1974), with minor modifications. Samples (usually 50 µg of protein) were incubated in a final volume of 0.3 ml containing 20mM-Tes buffer, pH 7.5, 10mM-nitrilotriacetic acid, 0.6mM-ouabain, 0.5 µg of antimycin

A (Sigma Chemical Co., St. Louis, MO, U.S.A.) and Ca²⁺ at various concentrations calculated as described below. The tubes were placed in a water bath at 37°C for 10 min before addition of ATP to a final concentration of 1 mM. The incubation was carried out for 20 min with continuous shaking and the reaction was terminated by addition of 0.3 ml of 30% (v/v) HClO₄. If the reaction mixture contained Triton X-100 the stopping solution was made 1% (w/v) with respect to sodium dodecyl sulphate to prevent precipitation of detergent. Concentrations of HClO₄ below 15% caused precipitation if the sample contained Ampholine. The tubes were briefly placed in ice/water before centrifugation for 10 min at 1500g_{av.}. The supernatants were recovered for determination of P_i as described by Vilhardt & Hope (1974). P_i formation during the enzyme reaction was linear with time up to about 40 min. Suitable dilution of the samples ensured that ATP hydrolysis was kept below 10% of the total ATP concentration.

ATPase activity in solubilized preparations was assayed by a modification of the method described by Monk & Kellerman (1976). The assay medium consisted of 1mM-phosphoenolpyruvate, 0.2mM-NADH, 0.6mM-ouabain, 1mM-MgCl₂ and 8mM-KH₂PO₄ (adjusted to pH 7.5 with Tris) in a total volume of 3 ml containing 7 units of pyruvate kinase (EC 2.7.1.40) (from rabbit muscle; Sigma), 15 units of lactate dehydrogenase (EC 1.1.1.27) (from rabbit muscle; Boehringer G.m.b.H., Mannheim, W. Germany), 5 µg of antimycin A (Sigma) and 100 µl of solubilized microsomal material. The mixture was preincubated for 10 min at 30°C and the reaction was started by addition of ATP to a final concentration of 1 mM. The decrease in absorption at 340 nm was recorded continuously against a blank not containing microsomal ATPase. The presence of Triton X-100 in the medium did not interfere with the assay.

Determination of the activity of isolated ATPases at various concentrations of Ca²⁺ was done by incubating the samples with [γ -³²P]ATP (a modification of the method of Formby *et al.*, 1976). The assay medium contained 20mM-Tes buffer, pH 7.5, 10mM-nitrilotriacetic acid, 0.6mM-ouabain, 1mM-Na₂ATP and CaCl₂ concentrations calculated as described below. For the assay of Ca²⁺ activation in the presence of 1mM-MgCl₂ the medium contained 1mM-EGTA, 0.2mM-EDTA and CaCl₂ and MgCl₂ concentrations calculated as described below.

The assay was started by the addition of [γ -³²P]-ATP (100000 counts/min; The Radiochemical Centre, Amersham, Bucks., U.K.) to a final concentration of 1 mM. The total incubation volume was 100 µl. After 30–60 min of incubation at 30°C the reaction was stopped by adding 100 µl of 1% (w/v) sodium dodecyl sulphate in 30% HClO₄. A 50 µl sample was transferred to a glass test tube, followed

by addition of 150 μ l of 6.4 mM-ammonium heptamolybdate in 15% HClO_4 . Finally 200 μ l of isopropyl acetate was added and the mixture was stirred vigorously for 15 s. After centrifugation at 2500 g_{av} for 5 min, 100 μ l of the organic phase was pipetted off and its radioactivity counted in a Packard Tri-Carb model 3375 scintillation counter.

Preparation of media with buffered concentrations of Ca^{2+}

Concentrations of free Ca^{2+} were obtained by computing concentrations of CaCl_2 in 10 mM-nitrilotriacetic acid/1 mM- Na_2ATP /20 mM-Tes buffer, pH 7.5 (Flodgaard & Fleron, 1974). Constants for complex-formation were taken from Sillén (1971). The protein sample was dialysed against three 5-litre portions of 1 mM-EGTA/0.2 mM-EDTA/20 mM-Tes buffer, pH 7.5, for 24 h before addition to the assay medium. The last volume of dialysis buffer did not contain EGTA and EDTA.

Determination of protein

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. The presence of Triton X-100 in the samples interfered with the assay. This difficulty was circumvented by addition of sodium dodecyl sulphate to a final concentration of 2% (w/v) before addition of the Folin-Ciocalteu reagent. This modification prevented precipitation in the assay medium (Wang & Smith, 1975). Samples containing Ampholines were first dialysed before the assay (5 litres of 5 mM-Tris/HCl buffer, pH 7.5, changed five times during 72 h).

Assay of pyrophosphatase activity

A modification of the method of Flodgaard & Torp-Petersen (1978) was used. Samples of the microsomal fraction were incubated in the presence of 1 mM-[8- ^3H]adenosine 5'-[β -imido]triphosphate (The Radiochemical Centre) diluted to a specific radioactivity of 2 mCi/mmol, 5 mM- MgCl_2 , 1 mM- CaCl_2 and 20 mM-Tes buffer (adjusted to pH 8.5 with HCl) in a total volume of 20 μ l. After various incubation periods up to 90 min, 50 nmol of AMP and 50 nmol of adenosine in a total volume of 10 μ l were added to the medium. Then 10 μ l of the mixed medium was immediately spotted on PEI- (polyethyleneimine-) cellulose thin-layer sheets (product no. 5579, from Merck A. G., Darmstadt, W. Germany) and the chromatogram was developed for 10 cm with 0.5 M- LiCl . The AMP+adenosine spot was cut out and extracted for 15 min in 1 ml of 4 M- NH_3 . The ^3H radioactivity was counted in 10 ml of Lumagel (Lumac Systems A.G., Basel, Switzerland) in a Packard Tri-Carb liquid-scintillation counter.

Preparation of liposomes

Liposomes were prepared basically by the method of Knowles & Racker (1974). A mixture of 35 μ mol of phosphatidylcholine [egg-yolk lecithin (BDH Chemicals, Poole, Dorset, U.K.) purified on 2 mm silica-gel plates developed with chloroform/methanol/aq. NH_3 (sp.gr. 0.880) (13:7:1, by vol.)], 1% Triton X-100 and 50 mM- or 0.4 M-sodium phosphate buffer (adjusted to pH 7.5 with Tris) was preincubated at 30°C for 30 min in a Micro test tube (polypropylene model 1.5 ml; Milian Instruments S.A., Geneva, Switzerland). The tube was placed in an ice/water bath and the contents were sonicated under N_2 to clarity from the outside. A Branson B12 sonifier was used at 50 W for periods of 5 s with 10 s intervals over a total period of 150 s. To 0.35 ml of this suspension was added 0.25 mg of protein solubilized with 0.75% Triton X-100 to a total volume of 500 μ l. The mixture was incubated for 20 min at 30°C. The suspension was then placed in a syringe containing 2 ml of dry Amberlite XAD-2 (BDH Chemicals) placed on a metal grid and left at 4°C. After 5 h the syringe was placed in a test tube and the liposomes were eluted by centrifugation at 60 g for 5 min in an MSE Coolspin refrigerated centrifuge with a swinging-bucket rotor. This procedure removed the Triton X-100 almost completely (values less than 10% of the critical micellar concentration of Triton X-100 were obtained in the eluate).

Incubation of liposomes with $^{45}\text{Ca}^{2+}$

Liposomes prepared as described above were incubated at 37°C in a medium containing 1 mM- MgCl_2 , 1 mM-ATP, 0.01 mM- $^{45}\text{CaCl}_2$ (10⁵ counts/min) (The Radiochemical Centre) and 50 mM-Tris/HCl buffer, pH 7.5, in a total volume of 250 μ l. The amount of liposomal ATPase activity added corresponded to the formation of 25 μ mol of P_i /h.

Immediately after the incubation 100 μ l of the incubation mixture was placed on a Millipore filter (GSWP 02500; pore size 0.22 μ m) clamped in a filter holder. The filter was washed with 10 ml of 100 mM- NaCl /50 mM- KCl . The washed filters were placed in 10 ml of Bray's solution (Bray, 1960) and their radioactivities counted in a Packard Tri-Carb scintillation counter. Blanks were obtained by incubating liposomes in the absence of ATP. Quenching by water and liposomes was assessed by the channels-ratio method.

Isoelectric focusing in polyacrylamide gels

Analytical isoelectric focusing was performed by using a modification of the methods described by Vesterberg (1975) and Davies (1975). Slabs of gel (2 mm $\times$ 105 mm $\times$ 230 mm between two glass plates) were made up of 5% (w/v) acrylamide monomer, 0.15% (w/v) NN' -methylenebisacrylamide, 27 μ M-riboflavin, 12.5% (w/v) sucrose, 2% (w/v) Ampholine (pH 4-7 or 3-10) and 0.1% (v/v) Triton X-100. The gel was polymerized by u.v. radiation for 16 h. Samples

were applied to pieces of Whatman 3MM filter paper (10mm × 10mm) placed on the surface at the cathodal end of the gel. Focusing was performed at 1400 V at 30 W constant power in an LKB water-cooled Multiphor apparatus (LKB Produkter, Bromma, Sweden) with wicks of filter paper (obtained through LKB) soaked in 1 M-NaOH (cathode) or 1 M-H₃PO₄ (anode) for 2.5 h. The gels were stained with 0.2% (w/v) Coomassie Blue in methanol/water/acetic acid (5:5:1, by vol.) for 15 min at 60°C (Vesterberg, 1975), followed by destaining in ethanol/water/acetic acid (5:5:1, by vol.). In certain experiments the gels were cut into 0.5 cm × 10 cm strips for densitometry. Scanning was performed at 600 nm in a Pye-Unicam SP.1700 spectrophotometer equipped with an SP.1809 scanning unit.

Isoelectric focusing in granulated gels

Preparative isoelectric focusing was performed in granulated gels by a modification of the method described by Radola (1973) with the LKB Multiphor apparatus. The final gel composition was 5% Sephadex G-75 (superfine grade), 1% (w/v) Ampholine (pH 4–6) plus 1% (w/v) Ampholine (pH 5–7), 1% (v/v) Triton X-100 and 10 mM-β-mercaptoethanol. Membrane proteins solubilized in 1% (v/v) Triton X-100 were mixed with the gel slurry to a volume of 33 ml, which was poured into the gel bed and dried under a fan to a final volume of 25 ml. This procedure yielded a gel of 1 mm thickness and with a focusing distance of 23 cm. The load of protein was usually 0.5 mg/ml of gel slurry. Electrodes were paper strips obtained through LKB and electrode buffers were 1 M-H₃PO₄ (anode) and 1 M-NaOH (cathode). Focusing was carried out with water cooling (4°C) for 16 h at 1500 V at 8 W constant power.

After focusing the gel bed was fractionated into 30 parallel strips by using a metal grid. Then 10% of each fraction was removed with a spatula and mixed with 1 ml of CO₂-free 10 mM-NaCl for pH determination. The remainder of each fraction was placed in a 2 ml disposable plastic syringe mounted with a 0.45 μm Millipore filter. Centrifugation of the tubes at 60 g for 5 min (4°C) caused the gel to pack on top of the filter. Elution from the gel was performed by addition to each tube of 1 ml of 50 mM-Tris/HCl buffer, pH 7.5, 1% (v/v) Triton X-100 and 10 mM-β-mercaptoethanol followed by centrifugation of the tubes at 60 g for 5 min.

Location of proteins in granulated gels

The Ampholines interfere with the colorimetric assay for proteins, and the method of Lowry *et al.* (1951) can thus be applied only after extensive dialysis of each fraction. Furthermore both Triton X-100 and Ampholines absorb u.v. light at 280 nm. An estimation of the location of proteins in the gel

bed could, however, be obtained before fractionation of the gel by placing a strip of Whatman 3MM filter paper on top of the gel for 2 min. The paper was then dried at 100°C for 5 min and washed three times for 15 min in 15% (w/v) trichloroacetic acid, followed by staining for 30 min in 0.2% (w/v) Coomassie Blue in methanol/water/acetic acid (5:5:1, by vol.). Destaining was performed in ethanol/water/acetic acid (5:5:1, by vol.).

Determination of molecular weights of ATPases

The molecular weights of the ATPases isolated by isoelectric focusing were determined by comparing the electrophoretic mobilities the enzymes with those of proteins of known molecular weight.

Protein samples were taken up in a medium containing 10 mM-sodium phosphate buffer, pH 6.5, 1% (w/v) sodium dodecyl sulphate and 6% (w/v) sucrose. The marker proteins were β-galactosidase (EC 3.2.1.23), phosphorylase *a* (EC 2.4.1.1), lactate dehydrogenase (EC 1.1.1.27), pyruvate kinase (EC 2.7.1.40), cytochrome *c* and bovine serum albumin (all from Sigma, except for lactate dehydrogenase, which was obtained from Boehringer).

The gels were made up by a modification of the method of Weber & Osborn (1969) as described by Vilhardt *et al.* (1975) with 6% (w/v) Cyanogum 41 (BDH Chemicals) and 1% (w/v) sodium dodecyl sulphate in 0.1 M-sodium phosphate buffer, pH 6.5. Polymerization was catalysed by 0.07% (w/v) ammonium persulphate in the presence of 0.035% (v/v) *NNN'*-tetramethylethylenediamine. The gels were pre-run at 5 mA/tube for 2 h in 0.1% sodium dodecyl sulphate in 0.1 M-sodium phosphate buffer, pH 6.5. Then 50 μl of sample was placed on top of each gel with 10 μl of 0.05% Bromophenol Blue and electrophoresis was carried out at 8 mA/tube until the dye had reached the bottom of the gel. After staining with Coomassie Blue the migration distances of the proteins were measured and compared with the migration distance of Bromophenol Blue. After destaining the gels were scanned at 600 nm.

Results

ATPase activity in the microsomal preparation

The specific ATPase activity in the presence of various cations is shown in Table 1. Activation occurred in the presence of Ca²⁺ or Mg²⁺ or Mg²⁺ + Na⁺ + K⁺, whereas Ba²⁺, Sr²⁺ and Be²⁺ were inactive. The Na⁺ + K⁺-dependent activity could be abolished completely by 0.5 mM-ouabain. Addition to the incubation medium of equimolar amounts of Ca²⁺ and Mg²⁺ gave values intermediate between those found with either Ca²⁺ or Mg²⁺ alone. The ATPase activity was stable when the microsomal fraction was kept frozen.

Table 1. *Specific ATPase activity of bovine brain microsomal fraction incubated in the presence of various ions*
 Samples of the microsomal fraction were incubated with 1 mM-ATP after addition of various salts. The results are means $\pm$ s.d. for the numbers of experiments given in parentheses. n.d., Not detected.

Addition	Specific activity ($\mu\text{mol of P}_i/\text{h per mg of protein}$)
CaCl ₂ (1 mM)	3.4 $\pm$ 1.0 (6)
MgCl ₂ (1 mM)	6.4 $\pm$ 1.1 (6)
CaCl ₂ (1 mM) + MgCl ₂ (1 mM)	5.3 $\pm$ 0.8 (6)
MgCl ₂ (1 mM) + NaCl (100 mM) + KCl (10 mM)	9.2 $\pm$ 1.6 (6)
MgCl ₂ (1 mM) + NaCl (100 mM) + KCl (10 mM) + ouabain (1 mM)	6.5 $\pm$ 0.9 (4)
BaCl ₂ (1 mM)	n.d.
SrCl ₂ (1 mM)	n.d.
BeCl ₂ (1 mM)	n.d.
EDTA (2 mM)	n.d.

Fig. 1. *ATPase activity of bovine brain microsomal fraction incubated in the presence of various concentrations of Ca²⁺*
 Portions of the microsomal fraction were assayed for ATPase activity after incubation with ATP and various concentrations of Ca²⁺ in media buffered by nitrilotriacetate at pH 7.5 as described in the Methods section. Results are given as means $\pm$ s.d. for three experiments. The inset shows a double-reciprocal plot of the results. The apparent K_m values found were 6.0×10^{-8} M and 1.2×10^{-6} M.

Other phosphatases in the microsomal preparation

The microsomal preparation contained adenylate kinase activity. It was therefore necessary to measure the content of pyrophosphatase in the fraction, since this enzyme can generate AMP from ATP, which would interfere with the ATPase assay method used in the present study (the coupled enzyme assay method described by Monk & Kellerman, 1976).

Incubation of the microsomal fraction with 1 mM-Mg²⁺ and 1 mM-[8-³H]adenosine 5'-[β -imido]triphosphate demonstrated the presence of only minute amounts of pyrophosphatase in the preparation. The specific activity found with this substrate was 17 nmol of substrate hydrolysed/h per mg of protein and the error introduced in the ATPase assay by this enzyme is thus well below 1%.

Dependence of ATPase activity on various concentrations of Ca²⁺

The dialysed microsomal preparation was incubated with ATP and Ca²⁺ in nitrilotriacetate-stabilized media as described in the Methods section. Measurable activation of ATPase occurred at a concentration of 0.01 μM -Ca²⁺ (the lowest concentration tested).

When the results are displayed in a double-reciprocal plot it is revealed that the activation of the enzyme preparation by Ca²⁺ does not follow simple first-order kinetics (inset in Fig. 1). By using this plot two apparent K_m values for Ca²⁺ of 1.2×10^{-6} M and 6.0×10^{-8} M are found. Incubation of the microsomal fraction with increasing concentrations of Ca²⁺ in the presence of 1 mM-Mg²⁺ resulted in increasing total

ATPase activity at Ca^{2+} concentrations up to $5\text{ }\mu\text{M}$. Further increase in free Ca^{2+} concentration resulted in a decrease in total ATPase activity (results not shown).

Effect of Triton X-100 on ATPase activity

Even after repeated washings of the microsomal fraction with either dilute buffer (5 mM-Tris/HCl buffer, $\text{pH} 7.5$) or 0.6 M-KCl the ATPase activity remained firmly membrane-bound. In an attempt to solubilize the ATPase activity the preparation was incubated with a variety of detergents (sodium dodecyl sulphate, deoxycholate, Empigen BB, Triton X-100, Lubrol PX, Brij-23). The detergents all inhibited the enzyme activity, but least so, however, with Triton X-100. Even at a Triton X-100 concentration of 1% (v/v) the microsomal fraction retained approx. 60% of its ATPase activity.

Incubation with shaking of the microsomal fraction in the presence of 1% Triton X-100 for 20 min at 20°C and centrifugation for 1 h at $100\,000g$ at 4°C was used as a routine for solubilization of ATPase activity. About 50% of the ATPase activity of the original membranes was recovered in the supernatant and 5% in the pellet by this procedure. The specific

activity of the solubilized ATPase was $3.1\text{ }\mu\text{mol}$ of P_i/h per mg of protein in the presence of 1 mM-Mg^{2+} .

The inactivation of the ATPase activity by Triton X-100 was partially reversible. Incubation of the Triton X-100 extract with Amberlite XAD-2 resin as described in the Methods section (under 'Preparation of liposomes') increased the ATPase activity in the presence of 1 mM-Mg^{2+} by $55 \pm 3\%$ (four experiments). Addition to the solubilized preparation of a lipid extract of bovine brain microsomal fraction [with chloroform/methanol ($2:1$, v/v) (Vilhardt & Højlmer, 1972); protein/lipid ratio $1:4.8$] followed by sonication (three 10 s periods; 50 W) caused a 67% increase of Mg^{2+} -dependent ATPase activity as compared with controls (sonication without addition of lipid).

ATPase stability with time

The ATPase activity of the microsomal fraction remained unchanged for months if the preparation was stored frozen. The activity was found to be stable for at least 24 h when the fraction was kept at 4°C .

ATPase solubilized by Triton X-100 could also be stored frozen for weeks without loss of activity. However, the stability of the solubilized enzyme was found to be remarkably influenced by Ca^{2+} . During 100 h of storage at -20°C the solubilized fraction lost more than 60% of its activity when 1 mM-Ca^{2+} was present.

Fig. 2. Uptake of $^{45}\text{Ca}^{2+}$ by liposomes

Liposomes prepared in the presence of microsomal proteins solubilized by Triton X-100 were incubated for various periods in the presence of $^{45}\text{Ca}^{2+}$ as described in the Methods section. The liposomes were then isolated and washed, and the uptake of $^{45}\text{Ca}^{2+}$ was measured. The curves show the uptake of $^{45}\text{Ca}^{2+}$ in the presence of 1 mM-ATP by liposomes prepared in a solution of 50 mM-phosphate (□) or 0.4 M-phosphate (○). Also shown is the uptake by the two preparations of liposomes in the absence of ATP (■, ●).

Fig. 3. Distribution of ATPase activity after isoelectric focusing of solubilized bovine brain microsomal proteins in a granulated gel bed

Bovine brain microsomal fraction solubilized by 1% Triton X-100 was mixed in a Sephadex G-75 (super-fine grade) gel bed and electrofocused as described in the Methods section. The lower part of the Figure shows a photograph of stained paper print of the gel surface. The top of the Figure shows the distribution of ATPase activity (Δ) measured after fractionation of gel. The pH of the fractions is also indicated (●).

⁴⁵Ca²⁺ uptake by liposomes

Liposomes enriched in ATPase were incubated with ⁴⁵CaCl₂ in the absence or presence of ATP as described in the Methods section. Fig. 2 demonstrates that the uptake of ⁴⁵Ca²⁺ by the liposomes is dependent on the presence of ATP in the medium and on the amount of phosphate trapped in the liposomes. The total amount of ⁴⁵Ca²⁺ taken up by the liposomes increases with time. After 60min the liposomes prepared in the presence of 0.4M-phosphate had accumulated 55% of the Ca²⁺ of the incubation medium. Omission of Na⁺ from the incubation medium or addition of ouabain (1mM) did not influence the Ca²⁺ uptake. No accumulation of Ca²⁺ was found when the liposomes were incubated with adenosine 5'-[βγ-imido]triphosphate.

Preparative fractionation of solubilized proteins by iso-electric focusing

Initial efforts to purify the ATPase by gel filtration proved unsuccessful, partly owing to the multitude of proteins present in the Triton X-100-solubilized preparation (see densitometric scan of isoelectric focused preparation, Fig. 4a). Instead it was decided to apply preparative isoelectric focusing in an attempt to fractionate the solubilized membrane proteins. Triton X-100-solubilized preparations of the microsomal fraction were added to a gel bed consisting of Sephadex G-75 (superfine grade) containing Ampholines in the range pH4-7 (chosen after pilot runs in

Fig. 4. Isoelectric focusing in polyacrylamide gels of solubilized bovine brain microsomal proteins. Samples of microsomal fraction (a) and material isolated by preparative isoelectric focusing at pH4.8 (b) and at pH6.3 (c) were solubilized by Triton X-100 (1%) and subjected to isoelectric focusing in polyacrylamide gels (pH3-10). The gels were stained and scanned at 600nm. The arrows indicate where the samples were applied to the gel surface.

Table 2. ATPase activity of material isolated by isoelectric focusing of bovine brain microsomal fraction solubilized by Triton X-100

Triton X-100 (1%) extracts of bovine brain microsomal fraction were subjected to isoelectric focusing in granulated gel beds. The load was 0.5mg/ml of gel bed in a total volume of 25 ml. Recovery was 65 ± 15% (mean ± s.d. for three experiments). The material focusing at pH4.8 (peak 4.8) and pH6.3 (peak 6.3) displayed ATPase activity. The Table shows the specific activity (μmol of P_i/h per mg of protein) in the presence of 1mM-MgCl₂ (means ± s.d. for the numbers of experiments given in parentheses) or 1mM-CaCl₂. Also shown is the specific activity of the microsomal preparation solubilized by 1% Triton X-100. The recovery of ATPase activity was 60-70% and that of protein 60-100%.

Material	ATPase activity (μmol of P _i /h per mg of protein)	
	MgCl ₂	CaCl ₂
Solubilized microsomal preparation	3.1 ± 10. (3)	1.9 ± 0.8 (4)
Peak 4.8	17.3 ± 4.5 (3)	8.1 ± 2.0 (3)
Peak 6.3	21.0 ± 2.0 (3)	13.1 ± 3.0 (3)

the range pH3-10). After the focusing the gel was fractionated, and the fractions were eluted and assayed for ATPase activity (Fig. 3). Two distinct peaks of activity were discovered at pH4.8 ± 0.2 (peak 4.8) and at pH6.3 ± 0.2 (peak 6.3) (means ± s.d. for five experiments).

Determination of protein in all the fractions was not attempted, since Triton X-100 absorbs heavily at 280nm and since the Ampholines interfered with the standard assay method for protein (Lowry *et al.*, 1951). The fractions containing ATPase activity were, however, dialysed (5 litres of 5mM-Tris/HCl buffer, pH7.5, changed five times during 72h) and assayed for protein. Samples (100μl) from eluates of all the other fractions were pooled. The pooled sample was likewise

dialysed and assayed for protein and ATPase activity. The recovery for ATPase activity was 60–70% and for protein 60–100% of the material placed on the gel.

The specific ATPase activities in the presence of Mg^{2+} and Ca^{2+} in the two peaks are given in Table 2. The material corresponding to peaks 4.8 and 6.3 were freeze-dried and taken up in 150 μ l of 50 mM-Tris/HCl buffer, pH 7.5, containing 10 mM- β -mercaptoethanol and used for polyacrylamide-gel electrofocusing.

Isoelectric focusing in polyacrylamide gels of proteins from peaks 4.8 and 6.3

Samples of the material corresponding to peaks 4.8 and 6.3 were subjected to isoelectric focusing in polyacrylamide gels. The stained gels were cut into strips and scanned as described in the Methods section. As shown in Fig. 4, peak 4.8 contained a single protein whereas peak 6.3 contained one major and at least one minor component. These results were confirmed when the two fractions were electrophoresed in sodium dodecyl sulphate/polyacrylamide gels (see below).

Determination of molecular weights of the ATPases from peaks 4.8 and 6.3

Samples of material from peaks 4.8 and 6.3 were subjected to polyacrylamide-gel electrophoresis in

Fig. 5. Dependence of ATPase activity of material isolated at pH 4.8 and at pH 6.3 on various concentrations of Ca^{2+} . Triton X-100 (1%) extracts of bovine brain microsomal fraction were subjected to isoelectric focusing in granulated gel beds. The material focusing at pH 4.8 and at pH 6.3 was assayed for ATPase activity in the presence of various concentrations of Ca^{2+} buffered by nitrilotriacetate as described in the Methods section. The results are given as a double-reciprocal plot. The apparent K_m value found was $4.1 \times 10^{-8} M$ for peak 4.8 (●) and $3.8 \times 10^{-6} M$ for peak 6.3 (▲).

Fig. 6. Dependence of ATPase activity of material isolated at pH 4.8 and at pH 6.3 on various concentrations of Ca^{2+} in the presence of 1 mM Mg^{2+} .

Triton X-100 (1%) extracts of bovine brain microsomal fraction were subjected to isoelectric focusing in granulated gel beds. The material focusing at pH 4.8 (▲) and at pH 6.3 (●) was assayed for ATPase activity in the presence of 1 mM- Mg^{2+} and various concentrations of Ca^{2+} . The results are expressed as percentages of the activity found in the presence of 1 mM- Mg^{2+} without addition of Ca^{2+} .

the presence of sodium dodecyl sulphate as described in the Methods section. For comparison different proteins of known molecular weight were run simultaneously. The stained gels were scanned and the distance of migration was measured for each protein. Molecular weights were then calculated as described by Weber & Osborn (1969). The ATPase from peak 4.8 migrated as one band and had a molecular weight of 105 000. The material from peak 6.3 showed the presence of one major protein with a molecular weight of 105 000 and one minor component with a molecular weight of 118 000.

Dependence of ATPase activity of peaks 4.8 and 6.3 on various concentrations of Ca^{2+}

Samples of the material corresponding to peaks 4.8 and 6.3 were incubated in stabilized media containing labelled ATP and various concentrations of Ca^{2+} . It was found that even the lowest concentration of Ca^{2+} tested (0.01 μM) was able to activate the enzyme isolated at pH 4.8. The enzyme isolated at pH 6.3 was activated at 0.8 μM free Ca^{2+} . Increasing concentrations of Ca^{2+} caused an increase in the rate of hydrolysis of ATP that apparently followed first-order kinetics. Double-reciprocal plots of the ATP hydrolysis versus the concentration of Ca^{2+} showed that the ATPase activity in peaks 4.8 had an apparent K_m value for Ca^{2+} of $4.1 \times 10^{-8} M$ (Fig. 5). The enzyme isolated at pH 6.3 had a somewhat higher apparent K_m value of $3.8 \times 10^{-6} M$.

The material from peaks 4.8 and 6.3 was incubated

with various concentrations of Ca^{2+} in the presence of 1 mM-Mg^{2+} . Under these conditions the ATPase of peak 4.8 was not activated by Ca^{2+} (Fig. 6). The ATPase of peak 6.3 was activated by small amounts of Ca^{2+} . However, increasing the concentration of Ca^{2+} caused a pronounced inhibition of the Mg^{2+} -dependent activity.

Discussion

The microsomal preparation used in the present study was able to hydrolyse ATP in the presence of Mg^{2+} or Ca^{2+} . Brain microsomal fraction has been shown to accumulate Ca^{2+} in the presence of Mg^{2+} and ATP (de Meis *et al.*, 1970; Trotta & de Meis, 1975; Robinson, 1976), and we therefore intended to examine the possible role of the microsomal Mg^{2+} - and Ca^{2+} -dependent ATPase in the transport of Ca^{2+} .

The solubilized enzymic activity could be reconstituted into liposomes prepared from phosphatidylcholine. This preparation was able to accumulate Ca^{2+} in the presence of ATP. Since it has been shown (Flodgaard & Torp-Petersen, 1978) that the enzyme pyrophosphatase may be involved in cellular translocation of Ca^{2+} , we incubated the liposomes in the presence of adenosine 5'-[β -imido]triphosphate instead of ATP. Under these conditions no Ca^{2+} uptake was demonstrated. Blaustein & Ector (1976) have suggested that Ca^{2+} uptake may be part of an exchange mechanism between Ca^{2+} and Na^+ . In the present study omission of Na^+ from the incubation medium or addition of ouabain did not influence the ATP-dependent uptake of Ca^{2+} from the liposomes. We therefore conclude that Ca^{2+} uptake into liposomes reconstituted with Mg^{2+} - and Ca^{2+} -dependent ATPase is coupled to ATP hydrolysis. The molar ratio between Ca^{2+} transported and ATP hydrolysed was calculated to be close to 1 : 3.

To investigate the kinetics of the activation of the ATPase by Ca^{2+} the microsomal fraction was incubated at various concentrations of free Ca^{2+} obtained by buffering the media with nitrilotriacetic acid (Sillén, 1971). Under these conditions we found two apparent K_m values for Ca^{2+} activation of the preparation ($6.0 \times 10^{-8}\text{ M}$ and $1.2 \times 10^{-6}\text{ M}$).

The K_m values for Ca^{2+} activation found for the isolated enzymes differ slightly from the values for the membrane preparation. There may be several explanations for this discrepancy. First, the kinetics of the membrane-bound enzyme (as compared with those of the soluble enzyme) may be influenced by limitations in the free access of substrate and activators to the enzyme. Secondly, neural tissue has been shown to contain membrane-bound calcium-binding proteins (Watterson *et al.*, 1976; Russell & Thorn, 1977). The presence in the assay system of such proteins could change the concentration of free Ca^{2+} in the incubation medium. Finally, solubiliza-

tion of the ATPases by Triton X-100 leads to a partial inactivation of the enzyme, and this process could well include a change in the affinity of the enzymes for Ca^{2+} .

Solubilization of the ATPase activity by Triton X-100 made it possible to fractionate the proteins by preparative isoelectric focusing. By this technique the presence of two Ca^{2+} -activated ATPases in the solubilized material could be demonstrated. The enzyme isolated at pH 4.8 displayed the highest affinity for Ca^{2+} ($4.1 \times 10^{-8}\text{ M}$). This enzyme could not be activated by Ca^{2+} in the presence of excess concentrations of Mg^{2+} . The enzyme isolated at pH 6.3 could be activated by low concentrations of Ca^{2+} even in the presence of 1 mM-Mg^{2+} . When Mg^{2+} was present in the assay medium it was found that higher concentrations of Ca^{2+} had an inhibitory effect on the ATPase activity. Such an inhibition was not seen when Mg^{2+} was omitted from the medium. One hypothetical model of the enzyme that might explain these findings could be that the ATPase contains two sites for ionic activation. One site may be activated by Ca^{2+} , the other by Mg^{2+} . Ca^{2+} may also have affinity for the latter site, however, with little or no activating ability (intrinsic activity). Increasing concentrations of Ca^{2+} would thus displace Mg^{2+} from this site and thereby diminish the activation of the ATPase. Other reports on the ionic dependence of neural-tissue ATPases have shown that Ca^{2+} may depress the Mg^{2+} -dependent activity (Nakamaru *et al.*, 1967; Poisner & Douglas, 1968; Vilhardt & Hope, 1974; Duncan, 1976). These observations fit the model described above.

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