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CHARACTERIZATION OF THE MEMBRANE BOUND Mg^{2+}-ATPase OF RAT SKELETAL MUSCLE

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A procedure was developed to isolate a membrane fraction of rat skeletal muscle which contains a highly active Mg^{2+}-ATPase (5–25 μmol P_i/mg min). The rate of ATP hydrolysis by the Mg^{2+}-ATPase was nonlinear but decayed exponentially (first-order rate constant $\geq 0.2\ s^{-1}$ at 37°C). The rapid decline in the ATPase activity depended on the presence of ATP or its nonhydrolyzable analog 5′-adenylyl imidodiphosphate (Ado*PP*[NH]*P*). Once inactivated, removal of ATP from the medium did not immediately restore the original activity. ATP- or Ado*PP*[NH]*P*-dependent inactivation could be blocked by concanavalin A, wheat germ agglutinin or rabbit antiserum against the membrane. Additions of these proteins after ATP addition prevented further inactivation but did not restore the original activity. Low concentrations of ionic and nonionic detergents increased the rate of ATP-dependent inactivation. Higher concentrations of detergents, which solubilize the membrane completely, inactivated the Mg^{2+}-ATPase. Cross-linking the membrane components with glutaraldehyde prevented ATP-dependent inactivation and decreased the sensitivity of the Mg^{2+}-ATPase to detergents. It is proposed that the regulation of the Mg^{2+}-ATPase by ATP requires the mobility of proteins within the membrane. Cross-linking the membrane proteins with lectins, antiserum or glutaraldehyde prevents inactivation; increasing the mobility with detergents accelerates ATP-dependent inactivation.

Introduction

The microsomal subcellular fraction of skeletal muscle contains both a Ca^{2+}-dependent ATPase and a Ca^{2+}-independent Mg^{2+}-ATPase. The Ca^{2+}-ATPase mediates the active transport of Ca^{2+} into the sarcoplasmic reticulum. The function of the Mg^{2+}-ATPase has not been identified. Fernandez et al. [1] demonstrated that vesicles containing the Mg^{2+}-ATPase from rabbit skeletal muscle can be separated from sarcoplasmic reticulum vesicles by first selectively loading the sarcoplasmic reticulum with calcium phosphate to increase their density and then fractionating the microsomes by sucrose density centrifugation. The Mg^{2+}-ATPase was associated with those vesicles which migrated to a density between 1.09 and 1.13 g/ml. There are various other reports in the literature on the preparation of a low density subfraction of skeletal muscle microsomes which have high Mg^{2+}-ATPase activity [2–6]. Antibodies specific to the low density vesicle fraction prepared from rabbit skeletal muscle were shown to bind in the region of the transverse tubule [4].

Abbreviations: ADA, *N*-(2-acetamido)-2-iminodiacetic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Ado*PP*[NH]*P*, 5′-adenylyl imidodiphosphate; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; Chapso, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate.

Malouf and Meissner [5] used cytochemical techniques to demonstrate a highly active Mg^{2+}-ATPase in the plasmalemma and transverse tubule of chicken pectoralis muscle.

The purpose of the studies reported here is to investigate the effect of perturbation of the membrane by detergents, lectins, antiserum and glutaraldehyde on the Mg^{2+}-ATPase. Investigations on the regulation of the Mg^{2+}-ATPase may lead to a better understanding of its function.

Materials

Adenosine 5′-triphosphate, 5′-adenylyl imidodiphosphate (Ado*PP*[NH]*P*), lactate dehydrogenase, pyruvate kinase, wheat germ agglutinin, concanavalin A, *N*-ethylmaleimide, 5,5′-dithiobis (2-nitrobenzoic acid), cytocholasin B, vinblastine, colchicine, phenylmethylsulfonylfluoride (PMSF), quercetin, sodium azide, sodium arsenate, *N*-acetylglucosamine, α-methyl mannoside, oligomycin, gramacidin, valinomycin, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 2,4-dinitrophenol, soybean lectin (*Glycine max*), lectin from *Bandeiraea simplicifolia*, Triton X-100, polyoxyethylene 20 oleoyl ether (Brij 9)), polyoxyethylene 10 stearyl ether (Brij 76), polyoxyethylene 23 lauryl ether (Brij 35), polyoxyethylene sorbitan monolaurate (Tween 20), polyoxyethylene sorbitan monopalmitate (Tween 40), polyoxyethylene sorbitan monostearate (Tween 60), polyoxyethylene sorbitan monooleate (Tween 80), sodium cholate, sodium deoxycholate, digitonin, phospholipase C, and cholesterol were obtainted from Sigma Chemical Company (St. Louis, MO). Zwittergent 3-8, Zwittergent 3-10, Zwittergent 3-12, Zwittergent 3-14, Zwittergent 3-16, monensin and A23187 were purchased from Calbiochem-Behring Corp. (La Jolla, CA). (γ-^{32}P)labeled adenosine 5′-triphosphate was provided by New England Nuclear (Boston, MA). Ruthenium Red was purchased from Aldrich Chemical Corp. (Milwaukee, WI). Lectins from *Limulus polythemus*, *Pisum sativum* and *Arachis hypogaea* were obtained from E-Y Laboratories (San Mateo, CA). Pierce Chemical Corp. (Rockford, IL) supplied the Chapso, Chaps, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD chloride), dansyl chloride, dimethyl adipimidate, dimethyl pimelimidate, dimethyl-3,3′-dithiobis(propionimidate), 2-iminothiolane, disuccinimidyl suberate, disuccinimidyl tartarate, ethylene glycol bis(succinimidyl succinate) and 4,4′-difluoro-3,3′dinitrophenylsulfone.

Methods

Preparation of muscle subcellular fractions

The back and hind leg muscles were removed from 300 g male Sprague-Dawley rats and trimmed of red muscle, connective and adipose tissue. After chopping the muscle into small pieces (about 0.25 cm^3), it was homogenized with a Polytron at low speed for 2 min in 0.15 M KCl, 10 mM *N*-(2-acetamido)-2-iminodiacetic acid (ADA), 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 6.8), and 5 mM $MgSO_4$ (KCl solution). Every 15 s the Polytron was stopped and the blade cleared of connective tissue. The homogenization and all subsequent steps were performed at 4°C. The muscle was further homogenized in a 50 ml Potter-Elvehjem homogenizer (five strokes) and then centrifuged at $1500 \times g$ for 10 min. The pellet was saved to prepare the crude myofibril fraction. The supernatant was further centrifuged at $10000 \times g$ for 15 min. The pellet was saved to prepare the crude mitochondrial fraction and the supernatant was centrifuged at $53000 \times g$ for 1 h to collect the microsomal fraction. The supernatant was saved to prepare the cytosol fraction. The microsomal pellet was resuspended in KCl solution and applied to a discontinuous sucrose gradient containing 10% (2 ml), 27% (9 ml), 30% (6 ml), 35% (6 ml), 40% (6 ml) and 45% (3 ml) sucrose made in KCl solution. The microsomes were fractionated by centrifugation at $130000 \times g$ for 3 h. The Mg^{2+}-ATPase containing vesicles were obtained at the 10–27% interphase (low density vesicles). The sarcoplasmic reticulum vesicles were removed from the lower part of the gradient (33–37% sucrose). The low density vesicles were diluted 10-fold in KCl solution and concentrated by centrifugation at $140000 \times g$ for 30 min. The vesicles were placed on another discontinuous sucrose gradient (6 ml 10% sucrose, 6 ml 27% sucrose made in KCl solution) and centrifuged 15 h at $130000 \times g$. The low density vesicles containing the Mg^{2+}-ATPase were removed from the 10–27% interphase.

The crude myofibril fraction was prepared from the $1500 \times g$ pellet by rehomogenizing the pellet in 10 volumes of KCl with the Polytron at medium speed. The suspension was then centrifuged $1500 \times g$ for 10 min. The pellet was kept as the crude myofibril fraction.

The crude mitochondrial fraction was prepared from the first $10000 \times g$ pellet by resuspending the pellet in 250 ml of KCl solution and centrifuging the suspension at $1500 \times g$ for 10 min. The supernatant was centrifuged again at $10000 \times g$ for 15 min. The pellet was resuspended in 250 ml of KCl and the two centrifugations were repeated. The final pellet was used as the crude mitochondrial fraction.

The cytosol fraction was obtained from the $53000 \times g$ supernatant. The supernatant was centrifuged $100000 \times g$ for 30 min and then dialyzed 24 h against KCl solution.

Assays

ATPase activity was measured using a coupled enzyme assay [7] or by monitoring the release of P_i from [γ-^{32}P]ATP [8]. Cholesterol was measured by the method of Courchaine et al. [9], following extraction of membrane lipids as described by Bligh and Dyer [10]. Total phospholipid was determined by measuring the amount of P_i released following the digestion of the lipids by the method of Bartlett [11]. Aldolase [12], pyruvate kinase [13], lactate dehydrogenase [14], and creatine phosphokinase [15] were assayed as previously described, except the assay medium was made in KCl solution. 5'-Nucleotidase was assayed by the method of Dixon and Purdam [16].

SDS-polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed following the method of Laemmli [17] using 6–12% acrylamide gradient gels. Gels were stained with 0.2% Coomassie brilliant blue R-125 in 40% methanol, 7% acetic acid and destained with 20% methanol, 7% acetic acid.

The binding of antibodies and lectins to the proteins separated by SDS-polyacrylamide electrophoresis was investigated by first transferring the proteins electrophoretically from the gel to a nitrocellulose sheet in 25 mM Tris-HCl (pH 8.3), 20% methanol at 60 V (about 200 mA) using a Bio-Rad Trans-Blot cell. The nitrocellulose sheet was incubated for 1 h with 1% bovine serum albumin in 10 mM phosphate buffered saline (pH 7.4) to block the remaining protein binding sites of the nitrocellulose sheet. To detect lectin binding sites, peroxidase conjugated lectins (100 ml, 1 μg/ml proteins) were incubated with the nitrocellulose for 2.5 h. The unbound lectin was removed by washing the nitrocellulose sheet five times with 30 ml of phosphate buffered saline containing 0.05% Tween 20. Bound lectin was localized by staining for peroxidase activity using 4-chloro-1-naphthol as described by Hawkes et al. [18]. After staining, the nitrocellulose sheets were washed with excess water.

Antibody binding was detected by first incubating the blocked nitrocellulose sheet with antiserum (or control serum) diluted 1 to 150 in phosphate buffered saline containing 0.01% bovine serum albumin for 3.5 h at room temperature. After the nitrocellulose was then washed three times with phosphate buffer saline, it was incubated with peroxidase conjugated goat antibody to rabbit IgG (0.3 μg/ml protein) in phosphate buffered saline containing 0.01% bovine serum albumin. After incubating 1 h at 25°C, the nitrocellulose sheet was washed and stained with 4-chloro-1-naphthol as described above.

Preparation of rabbit antiserum against the low-density vesicles

Before immunization, serum was collected from the rabbit to serve as control serum. Low density vesicles (1 mg protein emulsified with equal volume of Freund's complete adjuvant) were injected subcutaneously into the back of the rabbit. The injections were repeated after 2 and 4 weeks. Eleven days after the last injection, serum was collected from the rabbit and stored at −20°C.

Electron microscopy

Low density vesicles (0.25 mg of protein) were diluted into 0.1 M cacodylate buffer (pH 7.2) and centrifuged 30 min at $120000 \times g$. The pellet was fixed for 1 h at 4°C with 2.5% glutaraldehyde in 0.1 M cacodylate buffer containing 1% tannic acid. The fixed pellet was then rinsed with 0.1 M cacodylate and then treated with 2% OsO_4 in 0.1 M cacodylate buffer. After embedding in araldite, the pellet was sectioned and then stained with uranyl acetate and lead citrate.

Results

The highest specific activity of the detergent-sensitive, Ca^{2+}-independent Mg^{2+}-ATPase of rat skeletal muscle was found in the microsomal fraction (Table I). Low-density vesicles in the microsomal fraction which contained the Mg^{2+}-ATPase were separated from the sarcoplasmic reticulum vesicles by sucrose density centrifugation (Table I and Fig. 1). Following a second sucrose density centrifugation (Fig. 1), no detectable Ca^{2+}-dependent ATPase activity remained in the low-density vesicle fraction. The specific activity of the Mg^{2+}-ATPase of the low density vesicles was 26-times larger than that of the sarcoplasmic reticulum. The low density vesicles contained a much higher content of cholesterol (0.6 μmol/mg protein) and 5′-nucleotidase (16 nmol/mg per min) than the microsomal fraction (0.1 μmol/mg and 0.2 nmol/mg per min, respectively) indicating that the plasma membrane and/or the transverse tubule membrane is also enriched in the low-density vesicle fraction.

Analysis of the protein composition of the low-density vesicles by sodium dodecyl sulfate polyacrylamide electrophoresis showed a pattern quite distinct from that of the myofibril, mitochondrial, sarcoplasmic reticulum or cytosol fraction (Fig. 2A). The major unique protein band enriched in the low density vesicle fraction had an apparent molecular weight of about 56 000. When separated by isoelectric focusing, this band breaks up into a series of 5 spots with p*I* values ranging from 5.4 to 6 (Fig. 2B). Many of the proteins found in the low-density vesicles appear to be simply trapped inside the vesicles during their preparation. The protein with an apparent M_w of 69 000 and a p*I* of 5.8–6.0 had the same migration pattern as authentic rat serum albumin (Fig. 2B). Rosemblatt et al. [4] identified serum albumin as a major protein component in low-density vesicles isolated from rabbit skeletal muscle. The protein with an apparent M_w of 40 000 and a p*I* of 6.7–6.9 gave the same migration pattern as authentic creatine phosphokinase. The activity of several soluble cytoplasmic enzymes in the low-density fraction showed a 6–10-fold increase in activity upon addi-

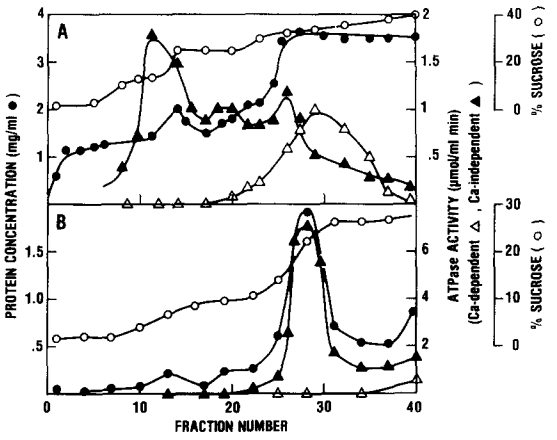

Fig. 1. Separation of microsomal vesicles from rat skeletal muscle. (A) First sucrose gradient. Microsomes (7 ml, 10 mg/ml protein) were placed on a discontinuous sucrose gradient (9 ml 27%, 6 ml 30%, 6 ml 40%, and 3 ml 45% sucrose in KCl medium). After centrifugation for 3 h at 130 000 × *g*, the gradient was fractionated into 0.9-ml aliquots and the protein, Ca^{2+}-dependent and Ca^{2+}-independent activity were measured as described in Methods. (B) Second sucrose gradient. Fraction 9–16 of the first sucrose gradient were pooled diluted with KCl medium and concentrated by centrifugation (140 000 × *g* for 0.5 h). The pellet was resuspended in KCl medium and applied to another discontinuous sucrose gradient (6 ml 10% sucrose, 6 ml 27% sucrose in KCl medium). After centrifugation at 130 000 × *g* for 15 h, the gradient was fractionated into 0.4-ml aliquots and the protein, Ca^{2+}-independent and Ca^{2+}-dependent ATPase activity of each fraction were measured.

TABLE I

Mg^{2+}-ATPase ACTIVITY OF MUSCLE SUBCELLULAR FRACTIONS

Mg^{2+}-ATPase was assayed in medium containing 0.15 M KCl, 10 mM Mops, 5 mM $MgSO_4$, 16 U/ml pyruvate kinase, 5 U/ml lactate dehydrogenase, 0.5 mM phospho*enol*pyruvate, 0.15 mM NADH and 1 mM EGTA. The ATPase activity was determined from the initial rate NADH oxidation as monitored spectrophotometrically at 340 nm. Inhibition by Triton X-100 was measured by including 0.1% Triton X-100 in the assay medium.

Fraction	Initial Mg^{2+}-ATPase activity (μmol/mg min)	
	− Detergent	+0.1% Triton
1 Crude myofibril	0.19	0.19
2 Cytosol	0.03	0.006
3 Crude mitochondria	0.14	0.04
4 Microsome	0.25	0.007
5 Low-density vesicles	6.11	0.11

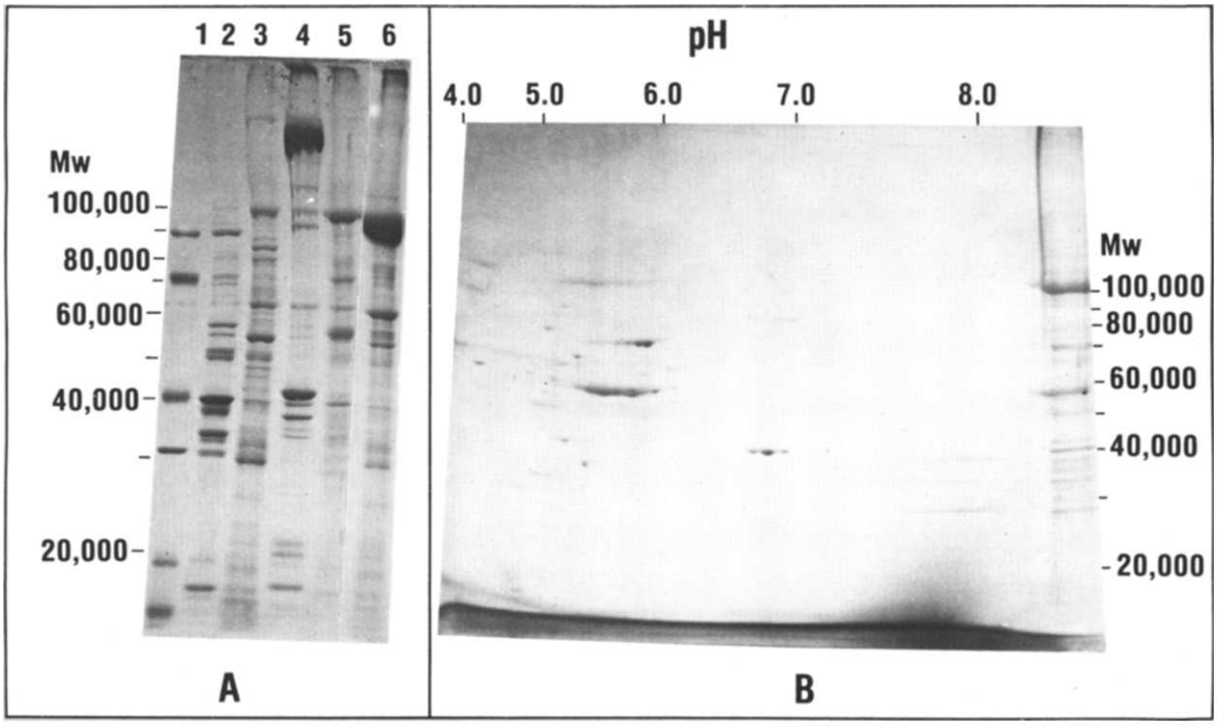

Fig. 2. Comparison of the protein composition of the low-density vesicle fraction with other subcellular fractions by SDS-polyacrylamide electrophoresis. (A) SDS-polyacrylamide gel electrophoresis was performed by the method described by Laemmli [17] using 6–12% acrylamide gradient gels. Each sample except the molecular weight standards (10 μg proteins) (phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and α-lactalbumin); lane 2, cytosol; lane 3, mitochondrial fraction; lane 4, myofibril fraction; lane 5, low density vesicles and lane 6, sarcoplasmic reticulum. (B) Two-dimensional electrophoresis of the low-density fraction. Procedure of O'Farrell [25] was followed. Low-density vesicles (200 μg protein were solubilized in 10% Triton X-100 for the first dimension, isoelectric focusing electrophoresis. A 6–12% acrylamide gradient gel was used for the second dimension, SDS polyacrylamide gel electrophoresis.

tion of 0.1% Triton X-100 (Table II). The latent, specific activity of these enzymes in the low-density vesicle fraction was 1/6 to 1/25 of the specific activity of these enzymes in the cytosol fraction indicating that up to 16% of the protein found in the low-density fraction may simply be trapped by the vesicles during their preparation.

The protein composition of the low-density vesicles was further characterized by identifying the wheat germ agglutinin binding proteins. Wheat germ agglutinin is a lectin which binds to glycoproteins containing *N*-acetylglucosamine. The proteins of each subcellular fraction were first separated by SDS-polyacrylamide electrophoresis and then transferred onto a nitrocellulose sheet. The binding of peroxidase conjugated wheat germ agglutinin to the protein bands was visualized using the peroxidase stain, 4-chloro-1-naphthol. This method was not sensitive enough to detect any wheat germ agglutinin binding proteins in mitochondrial, cytosol, myofibril, or sarcoplasmic reticulum fractions but at least 6 major bands and 15 minor bands appeared in the low-density vesicle fraction indicating an enrichment of glycoproteins in this fraction (data not shown). In the presence of 0.1 M *N*-acetylglucosamine, no wheat germ agglutinin binding to the low-density vesicle fraction was observed.

The location of the proteins in the low density vesicles was investigated using trypsin digestion and fluorescamine labeling of intact and solubilized membranes. Addition of trypsin to intact vesicles resulted in the rapid digestion of the 200000 and 100000 M_w proteins (Fig. 3). The

TABLE II

LATENT ACTIVITY OF CYTOSOL ENZYMES IN THE LOW-DENSITY VESICLES

Aldolase, lactate dehydrogenase, creatine phosphokinase and pyruvate kinase activities in the low density vesicles and cytosol fraction were assayed in KCl solution with and without 0.1% Triton X-100 as described in Methods.

	Enzyme activity (μmol/mg min)			
	Aldolase	Lactate dehydrogenase	Creatine phosphokinase	Pyruvate kinase
Low-density vesicles				
−detergent	0.5	0.09	1.3	0.07
+0.1% Triton X-100	4.1	1.10	6.3	0.61
Cytosol				
−detergent	24.7	11.0	144	6.3
+0.1% Triton X-100	24.7	11.0	144	6.3

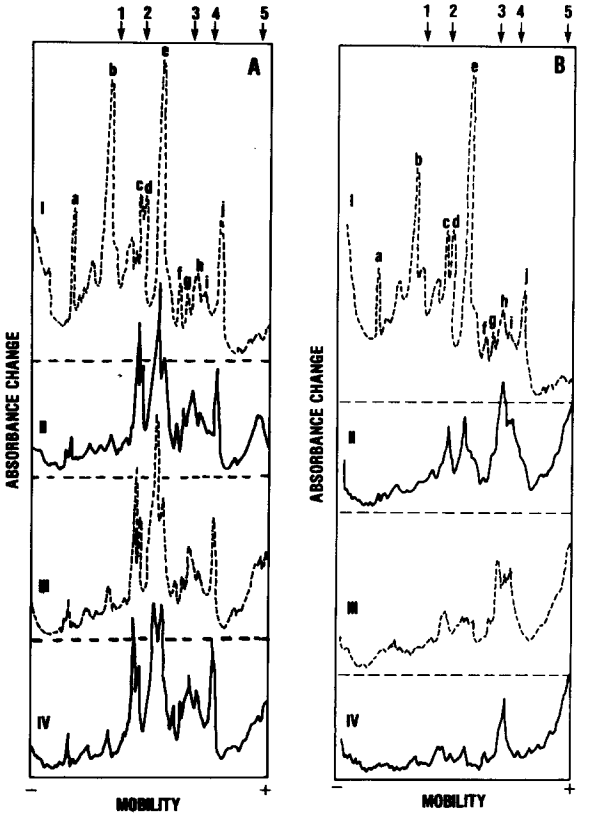

Fig. 3. Digestion of low-density vesicles with trypsin. (A) Low-density vesicles (4.5 mg/ml) were incubated in 0.15 M KCl, 10 mM Mops, 5 mM $MgSO_4$ at 25°C. Aliquots (50 μl) were removed before (I), or 1 min (II), 10 min (III), or 100 min (IV), after the addition of trypsin (2 μg/ml). PMSF (0.2 mM) was immediately added to the aliquots to inhibit the trypsin. After all the aliquots were collected, they were analyzed by SDS-polyacrylamide gel electrophoresis as described in Methods. The gels were stained with Coomassie blue, destained and scanned with a Gelford gel scanner at 550 nm. Mobilities of the molecular weight standards are indicated by the arrows: 1, phosphorylase *b*; 2, bovine serum albumin; 3, ovalbumin; 4, carbonic anhydrase; 5, soybean trypsin inhibitor. (B) Same as A, except low density vesicles were solubilized with 5 mg/ml deoxycholate before the trypsin addition.

56000 M_W protein appeared to be cleaved to form a protein of about 51000 M_W. Most of the other proteins seem to be unaffected by trypsin. The Mg^{2+}-ATPase activity was not influenced by trypsin even at a concentration of 1 mg/ml incubated for 30 min at 25°C. After solubilization the low-density vesicles with deoxycholate, all the proteins became susceptible to proteolytic digestion with the exception of a 35000 M_W protein. The addition of the nonpermeable cycloheptaamylose-fluorescamine complex to intact vesicles predominantly labeled the 100000 and 56000 M_W proteins (Fig. 4). Under the same conditions the Mg^{2+}-ATPase was inhibited 55%. All the proteins appeared to be labeled after the membranes were solubilized with sodium dodecyl sulfate (Fig. 4). These experiments indicate that the 100000 and 56000 M_W proteins are exposed to the external medium while most of the other proteins become exposed only after disruption of the vesicle membrane.

Since the specific activity of purified Mg^{2+}-ATPase is not known, the percentage of the vesicles in the low density vesicle fraction which actually contains the Mg^{2+}-ATPase cannot be estimated. The high content of cholesterol, 5′-nucleotidase and glycoproteins in the low-density vesicle fraction suggests that at least some of the vesicles are

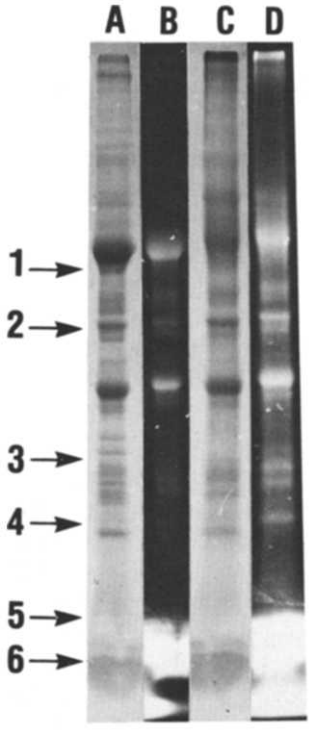

Fig. 4. Fluorescamine labeling of low-density vesicles. (Lanes A and B). Low-density vesicles (2 mg protein/ml) were incubated in 0.25 M sucrose, 10 mM imidazole (pH 7.5) and 2.5 mg/ml cycloheptaamylose-fluorescamine for 30 min at 37°C. After glycine (0.25 M) was added to react with the remaining fluorescamine, 5% SDS and 1% β-mercaptoethanol were added and the sample was heated at 100°C for 5 min. A 40-μl aliquot was then removed and analyzed by SDS-polyacrylamide electrophoresis. The gel was photographed under ultraviolet light with the shutter opened for 1 min to detect the proteins labeled with fluorescamine (lane B) and then the proteins stained with Coomassie brilliant blue R-115 (lane A). (Lanes C and D) Same as lanes A and B except 0.2% SDS was included in the fluorescamine incubation solution to solubilize the low-density vesicles. Mobilities of the molecular weight standards are indicated by the arrows: 1, phosphorylase *b* (94000); 2, bovine serum albumin (67000); 3, ovalbumin (43000); 4, carbonic anhydrase (30000); 5, soybean trypsin inhibitor (20000); and 6, α-lactalalbumin (14000).

derived from the plasma membrane and/or the transverse tubule. However, the low-density vesicle fraction may contain membranes from other sources, so the localization of the Mg^{2+}-ATPase is not possible at this time.

Electron micrographs of the low density fraction indicated a heterogeneous population of vesicles of various sizes present in the low-density fraction.

Rate of ATP hydrolysis by the Mg^{2+}-ATPase

The rate of ATP hydrolysis by the Mg^{2+}-ATPase as monitored by the enzyme coupled assay as nonlinear but declined exponentially (first-order rate constant $\cong 0.2\ min^{-1}$ at 37°C) (Fig. 5). Similar results were obtained when ATP hydrolysis was assayed by measuring the rate of P_i release from [γ-^{32}P]ATP. Addition of 2 mM K_2HPO_4 had no effect on the Mg^{2+}-ATPase activity, indicating that the decline in ATPase activity is not due to phosphate release from ATP. In the coupled enzyme assay, ATP is regenerated from ADP by pyruvate kinase and phospho*enol*pyruvate, so the decay in ATPase activity was not due to ADP accumulation. The initial rate of ATP hydrolysis was found to be directly proportional to the protein concentration of the low-density vesicles from 1 to 5 μg/ml using the coupled enzyme assay and from 10 to 100 μg/ml using the [γ-^{32}P]ATP assay. The rate of inactivation was independent of the protein concentration.

In the absence of ATP, the rate of inactivation was 600-times slower than that in the presence of ATP (Figs. 5 and 6). The rate of inactivation of the Mg^{2+}-ATPase was also accelerated by the addition of the non-hydrolyzable ATP analogue Ado*PP*[NH]*P* (Fig. 7). When added together with ATP, Ado*PP*[NH]*P* was a competitive inhibitor

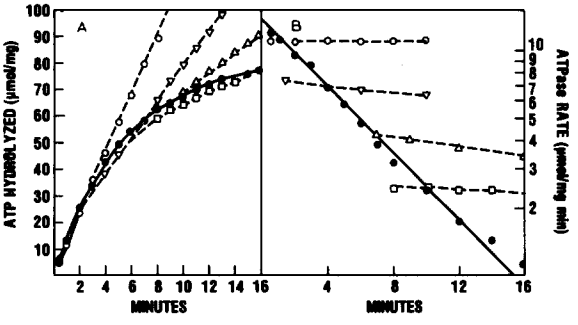

Fig. 5. Rate of ATP hydrolysis by the Mg^{2+}-ATPase. (A) Rate of ATP hydrolysis by Mg^{2+}-ATPase. ATP hydrolysis was measured by the enzyme coupled assay at 37°C in medium containing 0.15 M KCl, 10 mM ADA, 10 mM Mops (pH 6.8), 5 mM $MgSO_4$, 0.5 mM phospho*enol*pyruvate, 16 U/ml pyruvate kinase, 5 U/ml lactate dehydrogenase, 0.15 mM NADH, 2.0 mM ATP, 1.5 mM EGTA and 1 μg protein/ml low-density vesicles (●). Wheat germ agglutinin (20 μg/ml) was added before ATP (○) or 1.5 (▽), 6 (△), or 7 (□) min after ATP addition. (B) Data from panel A replotted on a logarithmic graph.

TABLE III

EFFECT OF ATP PRETREATMENT ON THE Mg^{2+}-ATPase ACTIVITY

Low-density vesicles (20 ml, 13.5 μg protein/ml) were incubated 30 min at 25°C in 0.15 M KCl, 10 mM Mops (pH 6.8), 5 mM $MgSO_4$ 1 mM EGTA, 5 mM ATP. A control sample was incubated in medium lacking the ATP. The samples were then centrifuged 130000 × g for 30 min and the pellet was resuspended in KCl solution at a final protein concentration of 0.6 mg/ml. Aliquots were removed from the resuspended vesicles at various times to assay for ATPase activity as described in Fig. 5.

Preincubation medium	Time after preincubation (min)	Initial rate of ATP hydrolysis	Inactivation rate (min^{-1})
Control	5	21.9	0.21
	30	23.2	0.21
	150	19.2	0.19
+ATP	5	5.5	0.10
	30	5.0	0.10
	150	6.8	0.11

of ATP hydrolysis by the Mg^{2+}-ATPase (K_i = 0.26 mM, data not shown), but when added in the absence of ATP, Ado*PP*[NH]*P* caused inactivation of the Mg^{2+}-ATPase.

The K_m of the Mg^{2+}-ATPase for ATP was

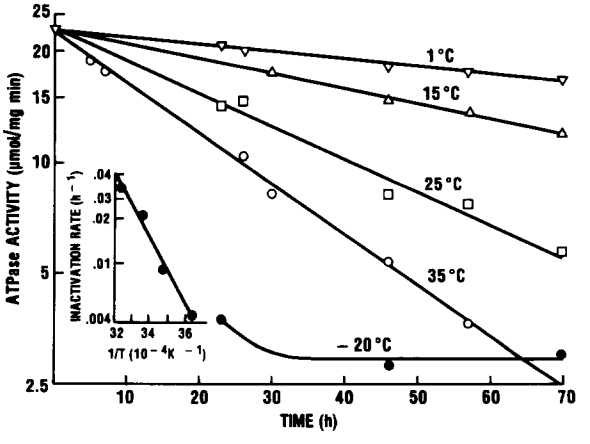

Fig. 6. Rate of inactivation of Mg^{2+}-ATPase in the absence of ATP. Low-density vesicles (26 μg protein/ml) were incubated at the indicated temperature in 0.15 M KCl, 10 mM ADA, 10 mM Mops (pH 6.8), 5 mM $MgSO_4$, 5 mM sodium azide. Aliquots (50 μl) were removed at various times and the initial ATPase activity was measured by the coupled enzyme assay at 25°C.

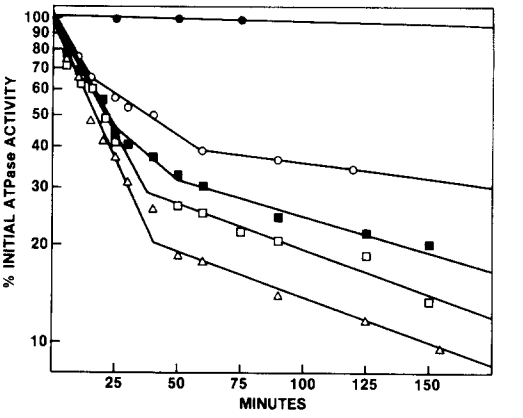

Fig. 7. Rate of inactivation of the Mg^{2+}-ATPase in the presence of Ado*PP*[NH]*P*. Low-density vesicles (0.6 mg/ml) were incubated at 25°C in 0.15 M KCl, 10 mM Mops, 10 mM ADA (pH 6.8). 5 mM $MgSO_4$ and either 0.1 mM (○), 0.2 mM (■), 0.4 mM (□) or 2.0 mM (△) Ado*PP*[NH]*P*. The control lacked Ado*PP*[NH]*P* (●). At various times aliquots (10 μl) were removed and assayed for ATPase activity using the coupled enzyme assay at 37°C. The data is reported as the ratio of the initial rate of ATP hydrolysis at the various times to the initial rate of ATP hydrolysis at $t = 0$ of the sample lacking Ado*PP*[NH]*P*. The vesicle suspension was diluted 200-fold into the assay medium so that the highest concentration of Ado*PP*[NH]*P* in the assay medium was 10 μM which did not significantly effect the ATPase activity (Ado*PP*[NH]*P* is a competitive inhibitor of the Mg^{2+}-ATPase with a K_i = 0.26 mM).

determined to be 0.2 mM. The rate of ATP hydrolysis and the rate of ATP-dependent inactivation did not vary between pH 5.5 and 8.5. The rate of ATP hydrolysis and the rate of ATP-dependent inactivation assayed with [γ-^{32}P]ATP was not significantly altered when the 0.15 M KCl in the assay medium was replaced with any of the following: 0.15 M NaCl, 0.15 M LiCl, 0.15 M RbCl, 0.15 M CsCl, 0.15 M sodium acetate, 0.15 M sodium bicarbonate, 0.15 M sodium aspartate, 0.15 M disodium maleate, 0.15 M sodium isothiocyanate, 0.15 M lysine chloride or 0.3 M sucrose. Various ionophores (gramicidin, valinomycin, monensin, A23187, CCCP, FCCP, dinitrophenol) and inhibitors of other ATPase enzymes (oligomycin, ouabain, vanadate, quercetin, sodium azide, sodium arsenate) had no significant effect on the effect on the activity of the Mg^{2+}-ATPase. Neither the rate of ATP hydrolysis nor the rate of ATP-dependent inactivation was significantly influenced

by 0.1 mM cytocholasin B, 0.1 mM vinblastin or 10 μM colchicine. No apparent alteration of the protein composition of the low-density vesicles as analysed by SDS-polyacrylamide electrophoresis was observed after treatment with ATP (data not shown). Neither the serine protease inhibitor phenylmethylsulfonyl fluoride nor the sulfhydryl reagents 5,5′-dithiobis(2-nitrobenzoic acid) acid and *N*-ethylmaleimide had any effect on the ATP-dependent inactivation rate of the Mg^{2+}-ATPase.

The effect of temperature on ATP hydrolysis by the Mg^{2+}-ATPase is shown in Fig. 8. The energy of activation for ATP hydrolysis and ATP-dependent inactivation determined from an Arrhenius plot of the data in Fig. 8 was 4.0 kcal/mol and 14.4 kcal/mol, respectively.

Effect of lectins on the Mg^{2+}-ATPase activity

The ability of lectins to influence the activity of the Mg^{2+}-ATPase was investigated. Of the lectins tested, only wheat germ agglutinin and concanavalin A altered the Mg^{2+}-ATPase activity. Wheat germ agglutinin and concanavalin A bind *N*-acetylglucosamine and D-mannose residues, respectively. Lectins from *Limulus polyhemus, Pisum sativum, Arachis hypogaea* and *Glycine max* had no effect on the Mg^{2+}-ATPase concentrations up to 50 μg/ml protein. Wheat germ agglutinin and concanavalin A were able to completely block ATP-dependent inactivation (Table IV). They were also able to block the inactivation of the Mg^{2+}-ATPase induced by Ado*PP*[NH]*P* (Table V). If wheat germ agglutinin was added to the assay medium following ATP addition, further inactivation was prevented, but the initial rate of ATP hydrolysis was not restored (Fig. 5). Similar results were obtained with concanavalin A. Inhibition of ATP-dependent inactivation of the Mg^{2+}-ATPase by lectins was completely reversible. Addition of 0.1 M *N*-acetylglucosamine to the assay medium eliminated the effect of wheat germ agglutinin on the Mg^{2+}-ATPase activity even when added after ATP hydrolysis is initiated. Similar results were obtained when α-methyl mannoside was added to the assay medium containing concanavalin A. These results indicate that wheat germ agglutinin and concanavalin A must bind to the membrane in order to prevent the inactivation of the Mg^{2+}-ATPase and that the effect of these lectins on the Mg^{2+}-ATPase is completely reversible.

Concanavalin A and wheat germ agglutinin shifted the K_m of the Mg^{2+}-ATPase for ATP from 0.2 mM to 0.34 mM.

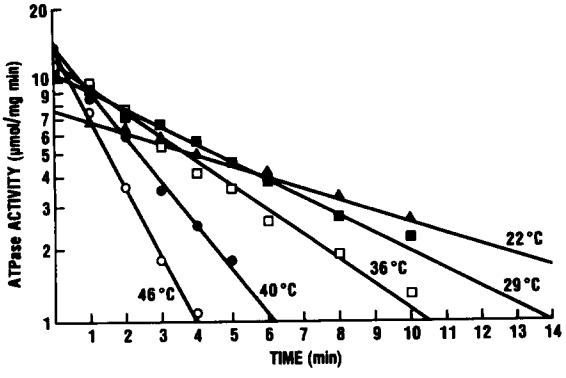

Fig. 8. Rate of inactivation of the Mg^{2+}-ATPase in the presence of ATP. ATPase activity was measured by the coupled enzyme assay at the indicated temperature in medium containing 0.15 M KCl, 10 mM ADA, 10 mM Mops (pH 6.8), 5 mM $MgSO_4$, 0.5 mM phospho*enol*pyruvate, 16.8 U/ml pyruvate kinase, 4.8 U/ml lactate dehydrogenase, 0.15 mM NADH, 2.0 mM ATP, 1.5 mM EGTA and 1.7 μg protein/ml low-density vesicles. The rate of ATP hydrolysis was determined from the change in the NADH absorbance at 340 nm.

TABLE IV

EFFECT OF WHEAT GERM AGGLUTINIIN, CONCANAVALIN A AND RABBIT ANTISERUM ON THE Mg^{2+}-ATPase ACTIVITY

Mg^{2+}-ATPase was assayed as described in Fig. 5. Low-density vesicles (1.8 μg/ml) were added to the assay medium 1 min before the addition of 2 mM ATP. When tested, the lectins, sugars, antiserum or control serum were added to the assay medium before the addition of the low-density vesicles.

Addition	Initial rate ATP hydrolysis (μmol/mg per min)	Inactivation rate (min^{-1})
1 Control	8.9	0.26
2 + wheat germ agglutinin (70 μg/ml)	5.9	0
3 + wheat germ agglutinin (70 μg/ml) and *N*-acetylglucosamine (0.1 M)	7.3	0.29
4 + concanavalin A (40 μg/ml)	6.9	0
5 + concanavalin A (40 μg/ml) and α-methylmannoside (0.1 M)	7.6	0.35
6 + rabbit antiserum (1 : 700)	5.8	0
7 + control serum (1 : 700)	10.1	0.23

TABLE V

EFFECT OF WHEAT GERM AGGLUTININ, CONCANAVALIN A AND RABBIT ANTISERUM ON Ado*PP*[NH]*P*-INDUCED INACTIVATION OF THE Mg^{2+}-ATPase

Low-density vesicles (0.38 mg protein/ml) were incubated at 25°C in KCl solution with the indicated additions for 60 min. The vesicles were then diluted 400-fold into Mg^{2+}-ATPase assay medium and the initial rate of ATP hydrolysis was determined as described in Fig. 5.

Incubation medium	ATPase activity (% of control)
Control (no Ado*PP*[NH]*P*)	100
+2 mM Ado*PP*[NH]*P*	26
+2 mM Ado*PP*[NH]*P* and wheat germ agglutinin (1 mg/ml)	91
+2 mM Ado*PP*[NH]*P* and wheat germ agglutinin (1 mg/ml) and *N*-acetylglucosamine (33 mM)	49
+2 mM Ado*PP*[NH]*P* and concanavalin A (2.5 mg/ml)	84
+2 mM Ado*PP*[NH]*P* and concanavalin A (2.5 mg/ml) and α-methyl mannoside (33 mM)	26
+2 mM Ado*PP*[NH]*P* and rabbit antiserum (1 : 100)	85
+2 mM Ado*PP*[NH]*P* and control serum (1 : 100)	30

Effect of rabbit antiserum to the low-density vesicles on the Mg^{2+}-ATPase activity

The effect of rabbit antiserum produced against the low density vesicles on the Mg^{2+}-ATPase activity was similar to that of wheat germ agglutinin and concanavalin A. The initial rate of ATP hydrolysis was slightly decreased by the antiserum, but the ATP-dependent inactivation was completely blocked (Table VI). Control serum had little effect on the Mg^{2+}-ATPase activity. Rabbit antiserum also prevented inactivation of the Mg^{2+}-ATPase by Ado*PP*[NH]*P* (Table VI). Like the wheat germ agglutinin, rabbit antiserum was found to bind to a variety of proteins in the low density fraction (data not shown).

Effect of glutaraldehyde on the Mg^{2+}-ATPase activity

Glutaraldehyde is a cross-linking reagent which is able to react with amine groups of protein and lipids. Immediately after adding 2.5% glutaraldehyde to a suspension of low-density vesicles at 1°C, the initial rate of ATP hydrolysis and the rate of ATP-dependent inactivation decreased 36% and 54%, respectively (Fig. 9). After a 30 min incuba-

TABLE VI

EFFECT OF WHEAT GERM AGGLUTININ, CONCANAVALIN A, AND RABBIT ANTISERUM ON THE INCUBATION OF THE Mg^{2+}-ATPase IN THE PRESENCE OF DETERGENTS

Mg^{2+}-ATPase activity was measured as described in Fig. 10 except wheat germ agglutinin (20 μg/ml), concanavalin A (50 μg/ml) and rabbit antiserum (1 : 700) were included in the assay medium where indicated. Not determined, N.D.

Detergent	Control		Wheat germ agglutinin		+Concanavalin A		+Rabbit antiserum	
	Initial ATPase rate(min^{-1}) (%)	Inactivation rate	Initial ATPase rate (%)	Inactivation rate (min^{-1})	Initial ATPase rate (%)	Inactivation rate (min^{-1})	Initial ATPase rate (%)	Inactivation rate (min^{-1})
–	100	0.28	75	0	95	0	102	0
25 μg/ml Brij 35	96	0.75	83	0	95	0	87	0
100 μg/ml Brij 35	85	1.76	83	0	94	0	75	0
2000 μg/ml Brij 35	120	1.54	83	0.08	94	0.07	N.D.	N.D.
100 μg/ml Chapso	93	0.41	65	0	88	0	91	0
250 μg/ml Chapso	82	1.62	65	0.05	79	0	70	0.05
1000 μg/ml Chapso	–	< 3	56	0.14	41	0.1	58	0.12
1 μg/ml Triton X-100	94	0.29	75	0	90	0	88	0
10 μg/ml Trison X-100	85	0.42	63	0	92	0	96	0
50 μg/ml Triton X-100	60	0.76	47	0.06	77	0.09	94	0.10

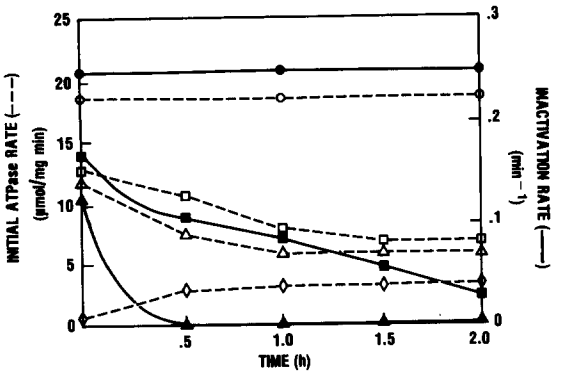

Fig. 9. Effect of glutaraldehyde on Mg^{2+}-ATPase activity. Low-density vesicles (0.2 mg protein/ml) were incubated at 1°C in 0.15 M KCl, 10 mM Mops (pH 6.8), 5 mM $MgSO_4$ and 2.5% (▲, △, ◇) or 0.25% (■, □) glutaraldehyde. Controls lacked glutaraldehyde (●, ○). Aliquots (5 μl) were removed at various times and diluted into 2 ml of ATPase assay medium to determine the initial rate of ATP hydrolysis (○, △, □, ◇) and the rate of inactivation (●, ▲, ■) as described in Fig. 5. In one experiment (◇), the vesicles incubated in 2.5% glutaraldehyde were assayed in medium containing 0.1% Triton X-100 in order to measure detergent-resistant ATPase.

tion in 2.5% glutaraldehyde, the Mg^{2+}-ATP showed no ATP-dependent inactivation but the initial rate of ATP-hydrolysis was reduced by 58%. Similar results were obtained in 0.25% glutaraldehyde, except the rate at which ATP-dependent inactivation was inhibited was greatly reduced (Fig. 9).

The Mg^{2+}-ATPase of low-density vesicles not treated with glutaraldehyde was completely inhibited by 0.1% Triton X-100. But after an hour incubation with 2.5% glutaraldehyde at 1°C, 1% Triton X-100 inhibited the Mg^{2+}-ATPase by only 42% (Fig. 9). In conclusion, cross-linking the membrane components with glutaraldehyde of the low-density vesicles (1) completely blocked ATP-dependent inactivation while inhibiting the initial rate of ATP hydrolysis up to 68%, and (2) increased the resistance of the Mg^{2+}-ATPase to detergents.

Several other cross-linking reagents which were tested (dimethyl pimelimidate, dimethyl 3,3′-dithiobis(propionimidate), 2-iminothiolane, disuccinimidyl suberate, and ethylene glycol bissuccinimicyl succinate) also prevented inactivation. When incubated with the low density vesicles at a concentration of 50–5 mM in 0.1 M triethylamine (pH 8.0) for 30 min at 25°C, they reduced the initial rate of ATP hydrolysis of the Mg^{2+}-ATPase 5–90% and completely eliminated ATP-dependent inactivation. Glutaraldehyde (50 mM) under the same conditions reduced the initial rate by 44%.

Effect of detergents on the Mg^{2+}-ATPase activity

At low concentrations, detergents maybe incorporated into biological membranes affecting membrane fluidity and protein-lipid interaction. At higher concentrations, some detergents can totally disrupt the membrane leading to the formation of micelles containing varying amounts of lipid, protein and detergent. At low concentrations of detergents and short incubation times, the initial rate of Mg^{2+}-ATPase was only slightly altered, while the rate of ATP-dependent inactivation was greatly accelerated. Fig. 10 shows the effect of Chapso, Brij 35 and Triton X-100 on the Mg^{2+}-ATPase activity. Similar results were obtained with Tween 20, Tween 40, Tween 60, Tween 80, Nonidet P-40, cholate, Chaps, Digitonin, Brij 76, Brij 99, Zwittergent 3-08, Zwittergent 3-10, Zwittergent 3-12, Zwittergent 3-14, Zwittergent 3-16, octylglucoside

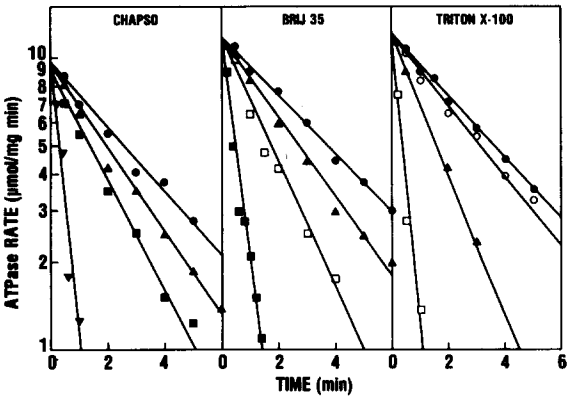

Fig. 10. Effect of Chapso, Brij 35 and Triton X-100 on Mg^{2+}-ATPase activity was measured by the coupled enzyme assay at 37°C in medium containing 0.15 M KCl, 10 mM ADA, 10 mM Mops (pH 6.8), 5 mM $MgSO_4$, 16 U/ml pyruvate kinase, 5 U/ml lactate dehydrogenase, 0.5 mM phospho*enol*pyruvate and 0.15 M NADH. Chapso, Brij 35 or Triton X-100 was added to the assay medium at a final concentration of 0.00025% (○), 0.001% (△), 0.0025% (□), 0.01% (■) or 0.025% (▼). The control (●) lacked detergent. Low-density vesicles (3.4 μg protein/ml) were added 1 min before the reaction was initiated with 2 mM ATP. The rate of ATP hydrolysis was determined from the rate of NADH oxidation as monitored spectrophotometrically at 340 nm.

and lysophosphatidylcholine. Some detergents such as Brij 35 and Tween 80 were unable to solubilize the Mg^{2+}-ATPase even at high concentrations (the Mg^{2+}-ATPase was still precipitable by centrifugation at $100000 \times g$ for 10 min). These detergents increased the inactivation rate 10-fold at a concentration of 0.05% (3.4 μg protein/ml low-density vesicles) but additional increases in the detergent concentration had no effect. Wheat germ agglutinin, concanavalin A or antiserum prevented the ATP-dependent inactivation of the Mg^{2+}-ATPase even in the presence of 2% Brij 35 (Table VI). Other detergents such as Chapso, Triton X-100, Nonidet P-40, cholate, octylglucoside and Zwittergent 3-16 completely solubilized the membrane at high detergent to protein ratios, at which no Mg^{2+}-ATPase activity was observed. At low detergent concentrations, the inactivation rate was increased with little change in the initial rate of ATP hydrolysis. Wheat germ agglutinin, concanavalin A or antiserum could still block the ATP-dependent inactivation of the Mg^{2+}-ATPase at low concentrations of Chapso and Triton X-100, but inactivation still occurred at higher concentrations (Table VI).

Discussion

The Mg^{2+}-ATPase of rat skeletal muscle had the unusual property of ATP-dependent inactivation. Inactivation could also be induced by the nonhydrolyzable ATP analogue Ado*PP*[NH]*P*. The rate of ATP-dependent inactivation was increased by detergents (Fig. 10) and temperature (Fig. 8). Inactivation was inhibited by wheat germ agglutinin (Fig. 5), concanavalin A (Table IV), glutaraldehyde (Fig. 9) and rabbit antiserum produced against the low-density vesicles (Table IV). Since ATP-dependent inactivation is blocked by cross-linking components of the low-density vesicle membrane with lectins, antibodies or glutaraldehyde, mobility of the membrane proteins may be required for inactivation (Fig. 11). The active Mg^{2+}-ATPase may be a protein complex which dissociates to give inactive monomers. Dissociation would be prevented by cross-linking the complex with wheat germ agglutinin, concanavalin A, antibodies or glutaraldehyde. ATP or Ado*PP*[NH]*P* would promote dissociation. Detergents would increase the rate of dissociation. Alternatively, the Mg^{2+}-ATPase may be regulated by a separate protein which must interact with the Mgl^{2+}-ATPase to inactivate it. Preventing protein mobility with cross-linking reagents, lectins or antiserum would block the interaction of the regulatory protein with the Mg^{2+}-ATPase. Inactivation of the Mg^{2+}-ATPase by the regulatory protein would require ATP or Ado*PP*[NH]*P*. A third possibility is that the Mg^{2+}-ATPase exists in two conformational states, active and inactive. The inactive state would be stabilized by ATP or Ado*PP*[NH]*P*, while the active state would be stabilied by the binding of lectins or antibodies to the Mg^{2+}-ATPase. At the present time, it is not possible to eliminate any of these proposed mechanisms for the regulation of the Mg^{2+}-ATPase without further experimental data.

Since there is an abundance of ATP in the muscle cell, something must prevent the inactivation of the Mg^{2+}-ATPase in vivo. One possibility is that the Mg^{2+}-ATPase is not normally exposed to ATP. There are numerous reports in the literature on the existence of ecto-ATPase enzymes which act on extracellular substrates [19–24]. If the vesicles which contain the Mg^{2+}-ATPase are nonpermeable to ATP and lectins, then the ATP and lectin binding sites must be on the same side of the membrane. Since the extracellular surface of muscle cells is heavily glycosylated, this would be the most likely site of lectin binding. Therefore it is possible that the active site of the Mg^{2+}-ATPase faces the extracellular medium rather than hydrolyzing intracellular ATP. A large fraction of the low density vesicles appear to be nonpermeable since (1) most of the proteins associated with the intact vesicles were protected from trypsin digestion (Fig. 3) and from labeling with cyclohepatamylose-fluorescamine (Fig. 4), (2) a 6–10-fold increase in the activity of cytoplasmic enzymes trapped inside the low-density vesicles was observed after disrupting the membrane with detergent (Table II), and (3) the efflux rate of ^{86}Rb (0.5 min^{-1}) from the vesicles was relatively slow (data not shown). It is not known, however, if the Mg^{2+}-ATPase is associated with these tight vesicles.

Another possible way in which the Mg^{2+}-ATPase retains its activity in vivo is that inactiva-

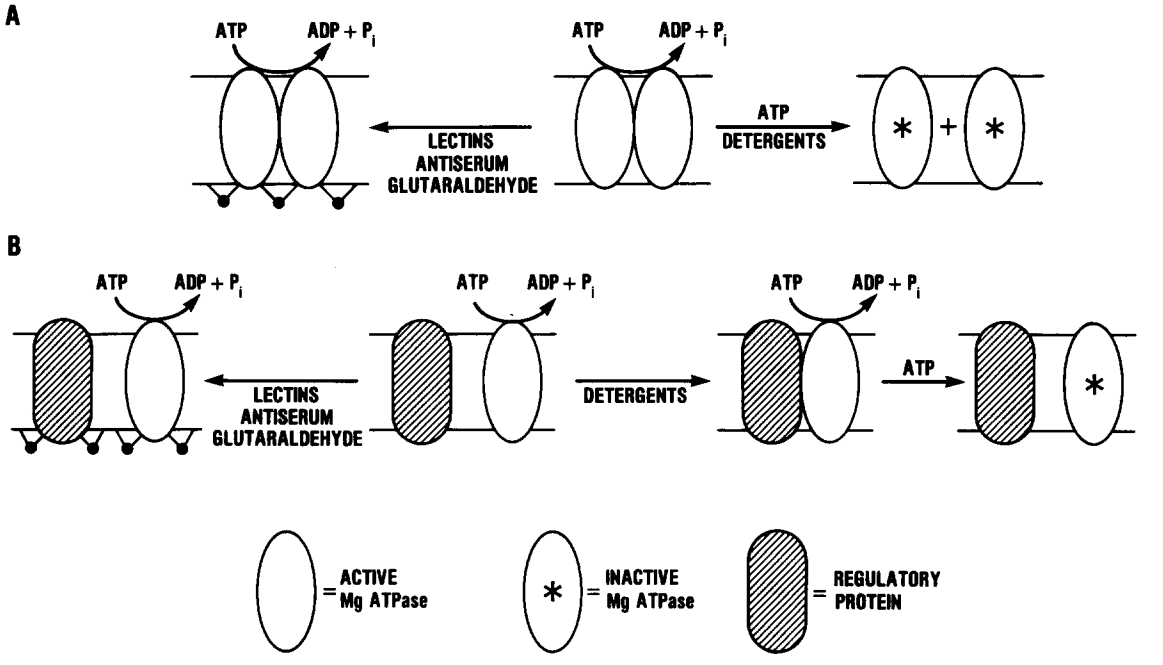

Fig. 11. Regulation of Mg^{2+}-ATPase. Mechanism A. Active Mg^{2+}-ATPase is a protein complex. Dissociation of the protein complex is promoted by ATP and detergents and leads to inactivation of the ATPase. Lectins, antiserum and glutaraldehyde prevent dissociation of the protein complex. Mechanism B. Mg^{2+}-ATPase is inactivated by a regulatory protein in the presence of ATP. Lectins, antiserum and glutaraldehyde immobilize membrane proteins preventing the interaction between the Mg^{2+}-ATPase and the regulatory protein. Low concentrations of detergents increase the mobility of proteins in the membrane and therefore increase the rate of inactivation.

tion may be blocked by a naturally occurring regulatory protein in a similar manner in which lectins, antiserum or glutaraldehyde prevent inactivation. No such regulatory protein, however, was detected in the muscle cytosol fraction or rat serum.

During the preparation of the low density vesicles, inactivation is partially prevented by working at 4°C, but it is likely that some inactivation does occur. When the Mg^{2+} concentration of the isolation medium was maintained below 1 μM with EDTA to inhibit the Mg^{2+}-ATPase, the specific activity of the Mg^{2+}-ATPase in the low density vesicles was increased 3-fold. EDTA was not routinely added to the isolation medium since this also caused an increase in the contamination of the low-density vesicle fraction with sarcoplasmic reticulum.

The Mg^{2+}-ATPase found in the low-density vesicles was not specific for skeletal muscle (unpublished data). A Mg^{2+}-ATPase with very similar properties as the skeletal muscle enzyme was also found in low density vesicles isolated from heart, spleen, lung, kidney, liver, brain and adipose tissue. The Mg^{2+}-ATPase activity of low density vesicles from heart, spleen and adipose tissue had a specific activity equal to or greater than that from skeletal muscle.

The function of the Mg^{2+}-ATPase is not known. We have found no evidence that the Mg^{2+}-ATPase is involved in ion transport. The enzyme requires millimolar amounts of Mg^{2+} or Ca^{2+} for activity but no other ionic requirements were observed. The activity of the ATPase was not influenced by a variety of anions, monovalent cations or ionophores. The efflux rate of ^{86}Rb from the low-density vesicles was not influenced by ATP nor was ATP-dependent Ca^{2+} uptake into the vesicles observed. The rate of ATP hydrolysis by the low-density vesicles was not influenced by preimposed negative or positive membrane potentials generated with Cl^- or K^+ gradients.

We are presently involved in developing techniques to solubilize and purify active Mg^{2+}-ATPase from skeletal muscle in order to better characterize this enzyme.

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