**Mg\(^{2+}\)-ATPase from Rabbit Skeletal Muscle Transverse Tubules Is 67-Kilodalton Glycoprotein**

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There exists a Mg\(^{2+}\)-ATPase in transverse tubules which has properties that are very different from other ATPases located in skeletal muscle cells. Several groups have suggested that a 100-kDa glycoprotein is the enzyme responsible for this Mg\(^{2+}\)-ATPase activity. In this study we have extended the methods utilized in the purification of this integral membrane glycoprotein. Although the apparent pI (isoelectric point) of this protein is pH 5, most of the net charge is due to the presence of sialic acid on the associated glycan chains. After these residues are removed, the behavior of this protein on an anion exchange column changes dramatically, allowing it to be further purified. Using a combination of the earlier procedures (Kirley, T. L. (1988) J. Biol. Chem. 263, 12682–12689 and Kirley, T. L. (1991) Biochem. J. 278, 375–400) and the one reported here, purification to specific activities of approximately 400,000 μmol of ATP hydrolyzed/mg of protein/h were obtained and all traces of the 100-kDa protein were removed. The digitonin-solubilized Mg\(^{2+}\)-ATPase appears to be a dimer of two identical 67-kDa subunits as assessed by high performance size exclusion chromatography. A single diffuse protein band is observed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis at approximately 67 kDa, which reduced to a single tight protein band at 52 kDa after deglycosylation with the following unique N-terminal amino acid sequence: Ala-Lys-Lys-Val-Leu-Pro-Leu-Leu-Leu-Pro-Leu-Val-X-Ala-Ala-Leu-Gly-Leu-Ala-X-Phe. Therefore, the Mg\(^{2+}\)-ATPase appears to be an enzyme of very high specific activity, present in small quantities in transverse tubule membranes and unrelated to the known classes of ATPases present in skeletal muscle. The data reported here on the orientation of the transverse tubule membrane preparations are consistent with the very recent report (Saborido, A., Moro, G., and Megias, A. (1991) J. Biol. Chem. 266, 23490–23498) that the Mg\(^{2+}\)-ATPase is an ecto-enzyme.

The Mg\(^{2+}\)-ATPase represents the major ATPase activity present in purified transverse tubule membranes from rabbit skeletal muscle. The physiological function of this enzyme is currently unknown, although the transverse tubule membrane system plays a crucial role in excitation-contraction coupling. It has been suggested that the Mg\(^{2+}\)-ATPase may be a proton pump, a calcium pump, or be involved in maintaining local concentrations of ATP. There has been no convincing evidence to favor any one of these postulated functions. Our laboratory has assayed the Mg\(^{2+}\)-ATPase for many types of biochemical functions including ion transport, phosphatase, and kinase activities, but no function other than the hydrolysis of nucleotide triphosphates has been found. One approach for determining function is to determine the primary structure of this enzyme and to compare it with other proteins whose sequence and function are known. The first step to this goal is to purify the protein to homogeneity and to obtain protein sequence. Several laboratories have been active in the purification of this enzyme including our own (2–7). This study reports such an effort and demonstrates that, unlike what was reported previously, the Mg\(^{2+}\)-ATPase activity does not reside in a protein of approximately 100 kilodalton (kDa), but that a single glycoprotein of 67 kDa represents the Mg\(^{2+}\)-ATPase. The solubilized functional enzyme appears to be composed of a dimer of two identical 67-kDa subunits. Furthermore, the purified Mg\(^{2+}\)-ATPase expresses an ATPase activity 200–400 times that of purified (Na,K)-ATPase, indicating a very fast hydrolysis rate. Enzyme assays on transverse tubule membranes indicate that the active site of the Mg\(^{2+}\)-ATPase is located on the exterior of the cell (in the lumen of the transverse tubule).

**EXPERIMENTAL PROCEDURES**

All buffer components were purchased from Fisher; Scientific or Sigma. Digitonin was obtained from Sigma and prepared as described previously (2). Neuraminidase Type X from Clostridium perfringens (240 units/mg of protein) was also obtained from Sigma. ATP and Peptide-N-glycosidase F (PNGase-F) were purchased from Boehringer Mannheim, and the colloidal gold solution used to measure protein concentrations was from Diversified Biotech (Newton Center, MA). Electrophoresis chemicals and SDS-PAGE standards were obtained from Bio-Rad. HPLC grade water was prepared inhouse using a Milli-Q (Millipore) system. Centricron 30 and centriprep 100 concentrators were purchased from Amicon (Beverly, MA). An LDC-Milton Roy HPLC system was used consisting of two constametric pumps, a Rheodyne injector, a spectrmonitor III variable wavelength UV monitor, a thermal printer-plotter and a dual disk drive, all controlled.

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1 The abbreviations used are: (Na,K)-ATPase, sodium- and potassium-activated adenosine triphosphatase; TT, transverse tubule(s); HPLC, high performance liquid chromatography; MOPS, 3-(N-morpholino)propanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGTa, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HPSEC, high performance size exclusion chromatography; HPIEC, high performance ion exchange chromatography; WGA, wheat germ agglutinin; conA, concanavalin A; PNGase-F, peptide-N-glycosidase F; ACE, angiotension converting enzyme; CI2E9, polyoxyethylene 9-lauryl ether.
Characterization of HPIEC fractions analyzed by SDS-PAGE in Fig. 2

| Fraction number | Specific activity $\mu$mol P$_4$/mg/h | Total activity $\mu$mol P$_4$/h | Total protein in fraction $\mu$g | Protein loaded into each well $\mu$g |
|-----------------|---------------------------------------|-----------------------------|-------------------------------|-----------------------------------|
| 14a             | 1,410                                 | 6                           | 2.0                           | 6.6                               |
| 15a             | 42,300                                | 524                         | 12.4                          | 41.3                              |
| 16a             | 75,700                                | 1,298                       | 17.1                          | 57.1                              |
| 17a             | 38,900                                | 885                         | 22.8                          | 75.9                              |
| 18a             | 24,600                                | 374                         | 15.2                          | 50.7                              |
| 19a             | 12,600                                | 179                         | 14.2                          | 47.5                              |
| 20a             | 14,100                                | 176                         | 12.6                          | 41.8                              |
| 11b             | 330,000                               | 67                          | 0.2                           | 2.4                               |
| 12b             | 38,600                                | 578                         | 1.5                           | 17.5                              |
| 13b             | 193,000                               | 355                         | 1.8                           | 22.1                              |
| 14b             | 17,900                                | 101                         | 5.6                           | 67.6                              |
| 15b             | 8,670                                 | 53                          | 6.1                           | 73.6                              |
| 16b             | 5,570                                 | 46                          | 8.2                           | 98.2                              |

$^*$Fractions repurified by HPIEC (pH 9.1) after neuraminidase treatment.

$^*$Fractions repurified by native gel electrophoresis.

by a LDC chromatography control module. The N-terminal sequence of the Mg$^{2+}$-ATPase was determined on a Porton 2090E gas phase sequenator by the protein chemistry core facility of the department of Pharmacology and Cell Biophysics at the University of Cincinnati College of Medicine. Propac PA1 (4 × 250 mm and 9 × 250 mm) anion exchange (HPIEC) columns were purchased from Dionex Corporation (Sunnyvale, CA). Bio-Sil TSK gel 300 × 7.5 mm) HPLC (HPSEC) gel filtration columns were purchased from Bio-Rad.

The method of Fiske and SubbaRow (8) was used for the detection of inorganic phosphate (produced by Mg$^{2+}$-ATPase activity). Sample aliquots (typically 5 µl) were diluted into buffer containing 20 mM Tris malate, pH 7.0, with 100 mM KCl, 5 mM MgCl$_2$, 0.1% digitonin, and 1 mM EGTA. The reaction was started by the addition of a final concentration of 2.5 mM ATP and incubated at 37 °C for 3–10 min. The reaction was stopped, and the inorganic phosphate was determined colorimetrically.

The Mg$^{2+}$-ATPase was purified according to the flow diagram in Fig. 1. Briefly, transverse-tubule (TT) membranes were either solubilized and purified using lyssolecithin and ConA-Sepharose as described previously by Kirley (3) or using digitonin and WGA-Sepharose 6 M8 as described by Nakayama et al. (9). The most active Mg$^{2+}$-ATPase fractions from either the ConA or WGA columns were then purified on the HPLC anion exchange column in 20 mM MOPS, pH 7.1, containing 5 mM MgCl$_2$, and 0.1% digitonin as described previously (3). The three fractions with the highest ATPase activity from the Propac PA1 anion exchange column were buffer exchanged into 20 mM Tris Cl, pH 9.1, containing 5 mM MgCl$_2$ and 0.1% digitonin (buffer A) and concentrated to less than 1 ml. The concentrated sample was injected onto the 4 × 250-mm Propac PA1 anion exchange column equilibrated with buffer A at 1.0 ml/min and eluted with a NaCl gradient in buffer A. One-m1 fractions were collected and assayed for ATPase activity. The two most active fractions, according to the amount of inorganic phosphate released, from the first high pH anion exchange column (fractions 16a and 17a, see Fig. 2 and Table I) were buffer exchanged into 50 mM sodium acetate, pH 5.5, containing 2 mM EDTA and 0.1% digitonin (desialylation buffer) using centrifcon 30 microconcentrators. 100 µl of neuraminidase Type X (0.4 units) was added, and the total volume was adjusted to 700 µl with 0.1 M sodium acetate, pH 5.0. The reaction mixture was incubated at 37 °C for 2 h. After desialylation, the sample was exchanged into buffer A and reinserted onto the same anion exchange column, equilibrated, and eluted under the same conditions as before. The two most active fractions (fractions 12b and 13b, see Fig. 2 and Table I) were concentrated to ~100 µl using a centrifcon 30 microconcentrator. The concentrated protein was collected by back flushing with 100 µl of centricon 30 microconcentrator.

The concentrated protein was collected by back flushing with 1.5 mM thick modified native Laemmli (10) gel (Bio-Rad Mini Protein II), and the gel was run for 15 min at 80 V and for 180 min at 180 V at room temperature. The running buffer was replaced with cold running buffer after 105 min into the run, all remaining steps were as described previously (2). The protein composition of each fraction from the two anion exchange columns and the electroeluted native gel were assessed using gradient minigels (see Fig. 2). Table I characterizes selected fractions from the first and second pH 9.1 HPIEC columns in terms of specific activity, total activity present in each fraction, total protein in each fraction, and the total protein loaded into each well of the SDS-PAGE gel in Fig. 2. Protein yields and total recovery of ATPase activity from the solubilization, lectin affinity chromatography, and pH 7.1 HPIEC column steps have been published previously (2).
The electroeluted Mg\(^{2+}\)-ATPase from the native gel was acetone-precipitated (11) and dissolved in 25 \(\mu\)l of 0.2% SDS, sonicated for 5 min, and heated for 10 min at 60 °C. The SDS concentration was adjusted to 0.1% with 20 \(\mu\)l of deglycosylation buffer yielding a final concentration of 0.06% octyl glucoside, 10 mM EDTA, 0.5% 2-mercaptoethanol, and 20 mM sodium phosphate at pH 7.3. 2 ml of water and 3 \(\mu\)l (0.6 units) of PNGase-F were added to start the deglycosylation reaction. The sample was incubated at room temperature for 24 h, diluted with 50 \(\mu\)l of SDS-PAGE sample buffer, and analyzed by SDS-PAGE and stained by Coomassie Blue (see Fig. 3).

SDS-PAGE was performed according to Laemmli (10). The native gels were 1.5 mm thick, and the analytical gels were either 1.5 or 0.75 mm thick. The analytical gels consisted of a 4% stacking gel and a 5–15% gradient separating gel which were silver stained as described by Ansorge (12). Native gel electrophoresis was carried out using a gel thickness of 0.2 M NaCl, and eluted at 0.5 ml/min with the same buffers. Molecular weight standards were run on the same two TSK 400 HPLC columns, and the retention times were used for generating standard curves (Fig. 4). The crude solubilized or desialylated Mg\(^{2+}\)-ATPase were injected onto the column, and 1-ml fractions were collected. The retention time for the Mg\(^{2+}\)-ATPase was determined by assay ing each fraction.

The sidedness and sealedness of the TT vesicles (4) were evaluated essentially as described by Saborido et al. (13). A final concentration of 0.02% saponin was used to permeabilize membranes to evaluate the degree of membrane integrity. (Na,K)-ATPase activity was determined by the linked-enzyme assay of Schwartz et al. (14) with/without the inhibitor ouabain. Lactate dehydrogenase and 5'-nucleotidase activities were determined as described previously (13). Acetylcholinesterase activity was measured by the method of Ellman et al. (15), as modified by Saborido et al. (13). We have also assayed (16, 17) for angiotensin converting enzyme (ACE), since it is present in the TT preparations (3) and has a known membrane orientation (active site on the outside (18, 19)), and the substrate used (hijelly-His-Leu) is reported to be membrane impermeable (20).

RESULTS

Further purification schemes have been devised in a continuing effort to purify and characterize the Mg\(^{2+}\)-ATPase from rabbit skeletal muscle in order to extend earlier results (2, 3). Transverse tubules were solubilized and glycoproteins isolated using two different purification schemes (Fig. 1). Both procedures, solubilization with lysolecithin and ConA purification (two preparations) or solubilization with digitonin and WGA isolation of glycoproteins (10 preparations, done in the presence of proteolytic inhibitors (9)) gave identical end results for the purified Mg\(^{2+}\)-ATPase.

The sugar-eluted TT glycoproteins were concentrated and applied to either a Propac PA1 9 × 250 mm (for WGA-isolated proteins) or a Propac PA1 4 × 250 mm (for ConA-isolated proteins) HPIEC column under low pH (7.1) conditions. The elution and results were as described previously (9). Two fractions were assayed for inorganic phosphate, and the two most active fractions from two column runs (3292 \(\mu\)mol of P\(_{i}\)/h total activity)(data not shown) were concentrated, buffer-exchanged, and loaded onto the Propac PA1 4 × 250-mm HPIEC column under high pH (9.1) conditions. Fractions (1 ml) were collected and assayed for activity and protein concentration. The peak of ATPase activity occurred in fraction 16a as indicated by its specific activity (75,700 \(\mu\)mol of ATP hydrolyzed per mg of protein/h). Under the high pH conditions, the peak of Mg\(^{2+}\)-ATPase activity now eluted at approximately 80 mM NaCl. Fractions around the peak of inorganic phosphate activity were analyzed by SDS-PAGE gradient gel (5–15% separating/4% stacking gel) electrophoresis. These fractions are represented in Fig. 2 as lanes 1–7. Tubes 16a and 17a had the highest specific activity and the least amount of contaminating proteins (according to the SDS-PAGE results in Fig. 2) and were combined, concentrated, and desialylated for the next stage of purification. It should be noted that fractions 18a-20a contained the majority of the 160-kDa protein, identified previously as angiotensin converting enzyme (3), and the 100-kDa protein which was previously observed to co-purify with the Mg\(^{2+}\)-ATPase complex (3). It is evident from Fig. 2 (see arrows, fractions 18a-20a) that these proteins are still present in intact form and therefore proteolysis is not the explanation for the absence of these proteins in the fractions containing the highest Mg\(^{2+}\)-ATPase activity. Thus, HPIEC purification under high pH conditions resulted in resolution between the Mg\(^{2+}\)-ATPase activity and the 100-kDa protein. Therefore, the 100-kDa protein is not part of the Mg\(^{2+}\)-ATPase complex.

The separation of the peak Mg\(^{2+}\)-ATPase activity from the 160-kDa (ACE) protein, as well as other proteins (Fig. 2, lanes 1–7), by the Propac PA1 anion exchanger under high pH conditions indicated that further purification could be
achieved by changing the charge on the protein. Another source of considerable charge heterogeneity among nonrelated glycoproteins can be attributed to the presence of terminal N-acetyllactosamine (sialic) acid residues on the complex glycan chains. The 2-h treatment of the Mg²⁺-ATPase with neuraminidase changed its retention time considerably after reionjection on the HPIEC column. After loading the desialylated Mg²⁺-ATPase under the high pH conditions (the desialylated Mg²⁺-ATPase no longer bound to the same column at pH 7.1), the peak of Mg²⁺-ATPase activity now eluted at approximately 40 mM NaCl, indicating the loss of a substantial proportion of the negative charge on the protein. Since neither incubating the Mg²⁺-ATPase in 0.1 mM sodium acetate, pH 5.0, for 2 h at 37 °C or desialylating resulted in a significant change in specific activity (results not shown), the 5-fold increase seen in the specific activity after desialylation and the second pH 9.1 HPIEC purification is due to the further removal of contaminating proteins (see Fig. 2). The purity of the Mg²⁺-ATPase after desialylation and elution from the HPIEC was assessed by SDS-PAGE gradient gel electrophoresis (5–15% separating/4% stacking gel) (Fig. 2, lanes 8–13). Fractions 12b and 13b were combined, concentrated, and buffer-exchanged for running on a native gel.

The final step in the purification of the Mg²⁺-ATPase was running the desialylated protein on a native gel. It appeared from the minimal retention to the HPIEC column that the Mg²⁺-ATPase had very little negative charge after desialylation under these pH conditions. Since the pH (8.8) of the running buffer for the native gel would give a similar charge to the Mg²⁺-ATPase after desialylation and elution from the HPIEC was assessed by SDS-PAGE gradient gel electrophoresis (5–15% separating/4% stacking gel) (Fig. 2, lanes 8–13). Fractions 12b and 13b were combined, concentrated, and buffer-exchanged for running on a native gel.

The diffuse Mg²⁺-ATPase band was treated with PNGase-F for 24 h. The deglycosylated Mg²⁺-ATPase was run on a thick (1.5 mm) 5–15% gradient gel and stained with Coomassie Blue (Fig. 3) to determine the protein composition after deglycosylation. Two bands resulted from the Mg²⁺-ATPase treated with PNGase-F, a major band at approximately 52 kDa and a minor band slightly higher (Fig. 3, lane 2; large and small arrows, respectively). Lane 4 of Fig. 3 contains the densitometric scan of the deglycosylated Mg²⁺-ATPase in lane 2. The major peak which corresponds to the 52-kDa protein is the Mg²⁺-ATPase, whereas the next largest peak in terms of quantity at approximately 35 kDa (indicated by an asterisk) is the deglycosylating enzyme PNGase-F, since its electrophoretic migration is equivalent to the PNGase-F blank in lane 3. The minor band appearing as a shoulder of the 52-kDa protein in the densitometric scan is believed to be a minor population of the Mg²⁺-ATPase that was not fully deglycosylated. The deglycosylation experiment indicated that the Mg²⁺-ATPase is N-glycosylated at multiple sites and has a protein core molecular mass of 52 kDa.

An estimation of the size of the digitonin-solubilized active Mg²⁺-ATPase complex was evaluated by HPLC size exclusion chromatography. Standard curves were generated (Fig. 4) using the standards bovine serum albumin (monomer and dimer), ovalbumin, α₂-macroglobulin, and thyroglobulin all run in 20 mM MOPS, pH 7.1, containing 5 mM MgCl₂ and 0.1% digitonin (±0.2 mM NaCl). The retention time for the Mg²⁺-ATPase was determined from fractionating impure solubilized Mg²⁺-ATPase and determining the retention time of the ATPase activity. Using the standard curves in Fig. 4 and the measured retention times for the Mg²⁺-ATPase, an estimated molecular size of 122 kDa for the Mg²⁺-ATPase was determined. When the same sizing experiments were repeated in the same buffer containing 0.2 mM NaCl (to inhibit nonideal charge interactions), an apparent molecular size of 109 kDa was obtained (Fig. 4). In addition, the apparent molecular size for the desialylated Mg²⁺-ATPase complex run in the same buffer containing 0.2 mM NaCl was estimated to be 148 kDa.

The possibility that the Mg²⁺-ATPase is an ecto-enzyme was evaluated as described previously (13). The experimental data in Table II were designed to elucidate the sidedness and sealedness of the TT membrane preparation. Since it is known that ACE, acetylcholinesterase, and 5'-nucleotidase are all oriented with their respective active sites on the outside of the cell, the data in Table II indicate that approximately 65% of our vesicles were sealed-right-side-out (RSO), ~15% were leaky (L), leaving ~20% sealed and inside-out (ISO). These data are in general agreement with that of Saborido et al. (13).

DISCUSSION

Many additional strategies for purifying the transverse tubule Mg²⁺-ATPase in addition to those described here and

### Table II

Properties of rabbit transverse tubule membrane purified from skeletal muscle

| Measured parameter | Saponin | +Saponin unmasking agent |
|--------------------|---------|-------------------------|
| Percent of total population |

| Class | Measured parameter | L | RSO | +Saponin | Unmasking agent |
|-------|--------------------|---|-----|----------|-----------------|
| Mg-ATPase activity | 404 | 515 | L + RSO | 79 |
| 5'-Nucleotidase activity | 2.46 | 3.12 | L + RSO | 79 |
| Acetylcholinesterase activity | 8.74 | 12.4 | L + RSO | 70 |
| ACE activity | 3.17 | 4.01 | L + RSO | 79 |
| Lactate dehydrogenase activity | 24.2 | 213.8 | L | 11 |
| (Na,K)-ATPase activity | 3.2 | 17.6 | L | 18 |
in the previous studies (2, 3) were evaluated without success. However, in this work, the somewhat unusual charge characteristics of the Mg\(^{2+}\)-ATPase were utilized to allow purification of the active enzyme to homogeneity for the first time. At pH 7.1 the protein has a net negative charge which does not distinguish it from many other proteins. The apparent pI (determined by chromatofocusing experiments, data not shown) of the sialylated enzyme is approximately 5. However, unlike many proteins, the net negative charge on the Mg\(^{2+}\)-ATPase does not increase very much going from pH 7.1 to 9.1, allowing substantial purification and removal of the majority of the 160-kDa protein (earlier identified as angiotensin converting enzyme (ACE) (3)) and the 100-kDa protein implicated by several groups (4, 6), including our own (2, 3), as being part of the Mg\(^{2+}\)-ATPase. Neither the 160- nor the 100-kDa proteins were significantly proteolyzed in the purification process, since they are still present in fractions 18a through 20a after the first pH 9.1 chromatography (see the arrows in Fig 2). The vast majority of the remaining protein impurities are removed by the same chromatography after neuraminidase treatment, although in some preparations there are significantly more 35- and 40-kDa protein impurities than are shown in Fig. 2 (fractions 12b and 13b). However, native gel electrophoresis reproducibly removes these lower molecular mass impurities. Furthermore, the specific activity reported for fractions 12b and 13b (Fig. 2) was shown not to be due to the neuraminidase treatment required for the purification of the Mg\(^{2+}\)-ATPase. The highest specific activity measured for the purified Mg\(^{2+}\)-ATPase (396,000 μmol/mg/h, fraction 12b in Fig. 2) is approximately eight times that of the highest previously reported (56,000 (3)) and approximately 40 times that of the original purification from this laboratory (2). It is not possible to measure the specific activity of the native gel-purified protein, since it is electroduted in the presence of SDS (due to its low negative charge), but we would estimate that the specific activity of the completely pure Mg\(^{2+}\)-ATPase is greater than 400,000 μmol/mg/h. This value is 200-400 times that of the purified (Na,K)-ATPase. The activity of this enzyme may be regulated by phorbol esters and diacylglycerols as has been recently proposed (22).

The fact that the glycosylated 67-kDa Mg\(^{2+}\)-ATPase is a very diffuse hard to visualize band on SDS-PAGE and appears to be present in very small quantities would account for the lack of identification of this protein by other workers in the past. However, our laboratory did observe this protein co-purifying with the ATPase activity in a recent previous work (the “70-kDa protein” (3)), but the purification obtained in that work was not sufficient to ascribe the Mg\(^{2+}\)-ATPase activity to any single protein band on an SDS-PAGE gel.

The significance of the 70-kDa protein, and its N terminus was not readily apparent when it was reported previously (3). However, since the Mg\(^{2+}\)-ATPase has been purified to homogeneity in this report, the unique N-terminal sequence obtained for the 67-kDa protein demonstrates that the Mg\(^{2+}\)-ATPase is not closely related to any previously sequenced family of ATPases. There are some interesting similarities between this enzyme and the rat liver ecto-ATPase described and cloned by Lin and co-workers (23, 24). These similarities include ion and nucleotide triphosphate specificities, molecular masses before and after deglycosylation (70 and 57 kDa, respectively, for the rat liver ecto-ATPase), resistance to inactivation by proteases, and lack of a good specific inhibitor. However, unlike the rat liver ecto-ATPase, the TT Mg\(^{2+}\)-ATPase does not hydrolyze ADP significantly (ADP hydrolyzed at only 3% of the rate of ATP (results not shown), see also Ref. 4), is inhibited by the detergent used to solubilize the rat ecto ATPase (C\textsubscript{24}E\textsubscript{6}), and has a distinct N-terminal sequence which does not have significant homology with the rat ecto-ATPase sequence. However, recently Saborido et al. (13) have indicated that the Mg\(^{2+}\)-ATPase from both chicken and rabbit skeletal muscle is an ecto-enzyme, a conclusion which is in direct contrast with other reports (2, 25-27). Our data (Table II) from measuring the activities of enzymes of known membrane topology to determine the sidedness and sealedness of our TT vesicles is consistent with the results of Saborido et al. (13), which suggested that the Mg\(^{2+}\)-ATPase is an ecto-enzyme. Also, our data indicate that the angiotensin converting enzyme present in the TT preparations is an integral membrane protein, rather than a trapped soluble serum protein. The reason for the discrepancy concerning the sidedness of the TT membranes between earlier data (2, 25-27) and the data reported here and also by Saborido et al. (13) is not clear. Our experimental approach to this question was not as comprehensive as that just reported (13), but the agreement between our results and those of Saborido et al. (13) is excellent. The explanation for no effect of concanavalin A on rabbit TT Mg\(^{2+}\)-ATPase reported earlier (2) must not be lack of accessibility of the glycan chains due to the glycosylation sites being inaccessible (inside sealed vesicles), as was postulated earlier (2). The fact that the Mg\(^{2+}\)-ATPase is an ecto-enzyme might explain why no one has been successful in finding a function such as ion transport or kinase activity. The rat liver ecto-ATPase has been postulated to be involved in the degradation of extracellular ATP to terminate the response to ATP by P2-purinergic receptors or to function in concert with 5’-nucleotidase in the conversion of ATP to adenosine, which has many known direct and indirect effects (24). Moreover, very recently Lin et al. (28) showed that the rat liver ecto-ATPase is identical to the cell adhesion molecule responsible for rat hepatocyte aggregation and is a member of the immunoglobulin supergene family. Whether or not the TT Mg\(^{2+}\)-ATPase serves any of these functions is unknown at present, but the similarities between it and the liver ecto-ATPase are strong enough to suggest further work along these lines.

The apparent molecular size of the digitonin-solubilized active Mg\(^{2+}\)-ATPase (122 and 168 kDa in the absence and presence of 0.2 m NaCl, respectively, and 148 kDa after desialylation) suggests that it is a dimer of two identical 67-kDa subunits in its active form (theoretical molecular size of 134 kDa). The postulate of multiple subunits is consistent with the inactivation observed when many detergents are used to solubilize the activity (2, 4) and with the inhibition seen when treating both the vesicular enzyme and the solubilized enzyme with cross-linking reagents (3). However, any data obtained on sizing of solubilized membrane glycoproteins using soluble nonglycosylated proteins as standards is inherently subject to question because of the known anomalous behavior of glycoproteins and membrane proteins in size exclusion chromatography. Unfortunately, despite repeated attempts, no conditions could be found which allowed deglycosylation with the retention of Mg\(^{2+}\)-ATPase activity, due to the incompatability of the Mg\(^{2+}\)-ATPase with the detergents needed to allow complete deglycosylation. The technique of radiation inactivation analysis applied to both the vesicular and purified ATPase may answer this question more convincingly in the future.

The Mg\(^{2+}\)-ATPase derived from chicken skeletal muscle TT membranes has some striking biochemical differences with the rabbit enzyme, including very different modulation by substrate (ATP) and lectins (2, 29). A monoclonal antibody obtained from the laboratory of Dr. R. Sabbadini (San Diego...
State University, San Diego, CA) which inhibited the chicken TT Mg$^{2+}$-ATPase and labeled a glycoprotein of 85 kDa on a Western blot was evaluated in our laboratory. Although we reproduced their inhibition and western blot results using chicken TT membranes, we were unable to demonstrate any effect of the antibody on the activity of the vesicular or solubilized rabbit Mg$^{2+}$-ATPase (0% inhibition under the same conditions where the chicken TT Mg$^{2+}$-ATPase was 85% inhibited), and we could not demonstrate any cross-reactivity on a Western blot of either rabbit TT or purified Mg$^{2+}$-ATPase (results not shown). Therefore, there is likely to be significant species (and tissue) differences among “MP-ATPases,” and there may even be differences in the sidedness of different enzymes (endo- versus ecto-enzymes), and the answer to the degree of relatedness of all of the MP-ATPases reported in the literature will await purification, cloning, and antibody cross-reactivity studies.

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