The Mg^{2+} -ATPase of rabbit skeletal-muscle transverse tubule is a highly glycosylated multiple-subunit enzyme

Terence L. KIRLEY

Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati, OH, 45267-0575, U.S.A.

The Mg^{2+} -ATPase present in rabbit skeletal-muscle transverse tubules is an integral membrane enzyme which has been solubilized and purified previously in this laboratory [Kirley (1988) *J. Biol. Chem.* **263**, 12682–12689]. The present study indicates that, in addition to the approx. 100 kDa protein (distinct from the sarcoplasmic-reticulum Ca^{2+} -ATPase) seen previously to co-purify with the Mg^{2+} -ATPase activity, there are also proteins having molecular masses of 160, 70 and 43 kDa. The 70 and 43 kDa glycosylated proteins (50 and 31 kDa after deglycosylation) are difficult to detect by SDS/PAGE before deglycosylation, owing to the broadness of the bands. Additional purification procedures, cross-linking studies and chemical and enzymic deglycosylation studies were undertaken to determine the structure and relationship of these proteins. Both the 97 and 160 kDa proteins were demonstrated to be *N*-glycosylated at multiple sites, the 97 kDa protein being reduced to a peptide core of 84 kDa and the 160 kDa protein to a peptide core of 131 kDa after deglycosylation. Although the Mg^{2+} -ATPase activity is resistant to a number of chemical modification reagents, cross-linking inactivates the enzyme at low concentrations. This inactivation is accompanied by cross-linking of two 97 kDa molecules to one another, suggesting that the 97 kDa protein is involved in ATP hydrolysis. The existence of several proteins along with the inhibition of ATPase activity by cross-linking is consistent with the interpretation of the susceptibility of this enzyme to inactivation by most detergents as being due to the disruption of a protein complex of associated subunits by the inactivating detergents. The 160 kDa glycoprotein can be partially resolved from the Mg^{2+} -ATPase activity, and is identified by its *N*-terminal amino acid sequence as angiotensin-converting enzyme.

INTRODUCTION

The predominant ATPase activity present in purified transverse-tubule membranes is the Mg^{2+} -ATPase. The physiological function of this enzyme and its role in excitation-contraction coupling (if any) are not yet known. The approx. 100 kDa protein component of this enzyme, contrary to a previous report [1], was demonstrated to be structurally distinct from the sarcoplasmic-reticulum Ca^{2+} -ATPase [2]. This finding is consistent with the well-established enzymic differences between the two enzymes. These include ion and nucleotide selectivity, differential inhibition by chemical modification reagents (especially fluorescein isothiocyanate), inhibition by vanadate and inhibition by detergents [3–10]. The Mg^{2+} -ATPase also has a higher turnover rate for hydrolysis of ATP than does either the sarcoplasmic reticulum Ca^{2+} -ATPase or the related (Na,K)-ATPase [2].

In this study, the Mg^{2+} -ATPase was further purified and chemically and enzymically deglycosylated to determine the molecular masses of the protein cores of the co-purifying polypeptides. The effect of cross-linking was examined by measuring the activity remaining and examining the intermolecular protein cross-links formed. The results suggested that the Mg^{2+} -ATPase is a multi-subunit enzyme which is fairly highly glycosylated and contains two 97 kDa protein subunits in close proximity to one another. Angiotensin-converting enzyme (ACE) was found to be co-purifying with both transverse-tubule membranes and the solubilized Mg^{2+} -ATPase.

EXPERIMENTAL

Materials

New Zealand White rabbits were obtained from Clerco (Cin-

cinnati, OH, U.S.A.). All buffer components were obtained from Fisher or Sigma. All electrophoresis chemicals, the Coomassie Blue reagent used to determine transverse-tubule membrane concentrations and the SDS/PAGE standards were from Bio-Rad Laboratories. ATP was purchased from Boehringer-Mannheim, and the colloidal gold solution used to measure solubilized protein concentrations was from Diversified Biotech (Newton Center, MA, U.S.A.). Soluble digitonin was prepared from Sigma digitonin as previously described [2]. Lyssolecithin was from Sigma. Concanavalin A (Con A)-Sephacryl was purchased from Pharmacia. The ion-exchange h.p.l.c. column was from Dionex (Sunnyvale, CA, U.S.A.) [2]. The 30 cm high-performance size-exclusion chromatography (HPSEC) columns (TSK-400) were from Bio-Rad. Trifluoromethanesulphonic acid (TFMSA) and anisole were from Aldrich. Peptide-*N*-glycosidase F (PNGase-F) was from Genzyme. The cross-linking agent 3,3'-dithiobis(sulphosuccinimidylpropionate) (DTSSP) and Sulfo-NHS-Acetate were from Pierce Chemical Co.

Transverse-tubule membrane isolation

Transverse-tubule membranes were isolated from approx. 200 g of rabbit back and hind-limb muscle by a previously described modification (ref. [2], unloaded preparation) of the method of Roseblatt *et al.* [11]. A typical preparation from one rabbit (200 g of starting muscle) resulted in 9–16 mg of transverse-tubule membranes with a Mg^{2+} -ATPase specific activity of approx. 400–500 μ mol of ATP hydrolysed/h per mg of protein at 37 °C. The transverse-tubule ATPase activities were measured spectrophotometrically by using the linked enzyme assay system [12] as described previously [2]. Briefly, this assay involves the use of pyruvate kinase and lactate dehydrogenase to couple the ATP hydrolysis to the oxidation of NADH, which is measured as a

Abbreviations used: Mg^{2+} -ATPase, millimolar- Mg^{2+} - or $-Ca^{2+}$ -activated transverse-tubule ATPase; TFMSA, trifluoromethanesulphonic acid; PNGase-F, peptide-*N*-glycosidase F; DTSSP, 3,3'-dithiobis(sulphosuccinimidylpropionate); Con A, concanavalin A lectin; HPSEC, high-performance size-exclusion gel chromatography; DTT, dithiothreitol; ACE, angiotensin-converting enzyme.

decrease in absorbance at 340 nm. Residual Ca^{2+} -ATPase activity (presumably contaminating sarcoplasmic reticulum) in these preparations varied slightly, but was typically 5% of the Mg^{2+} -ATPase activity. The protein concentration of the transverse-tubule preparations was measured either as described by Lowry *et al.* [13] or by the NaOH modification of the Bio-Rad Coomassie Blue dye-binding assay as described by Stoscheck [14]. The use of this modification allowed the use of this simpler total protein assay for membrane protein preparations and gave results nearly identical with those obtained by the method of Lowry *et al.* [13]. BSA was used as the standard protein for all protein-determination methods.

Solubilization and purification of the transverse-tubule Mg^{2+} -ATPase

Transverse-tubule membranes were solubilized and purified basically as described previously [2], with the following modifications. First, lysolecithin was used as the solubilization detergent in place of digitonin. All subsequent purification steps were carried out in 20 mM-Mops, pH 7.4, containing 5 mM- MgCl_2 and 0.1% digitonin (buffer A). Typically, solubilization was carried out for 5 min at 22 °C at a protein concentration of 1.0 mg of transverse tubules/ml and a lysolecithin concentration of 3.0 mg/ml. After centrifugation at 100000 *g* for 1 h, the supernatant fraction was diluted to yield a lysolecithin concentration of 0.5 mg/ml or less (usually 0.3 mg/ml), and the protein was incubated with Con A-Sepharose. Second, the final purification step of native gel electrophoresis was replaced by h.p.l.c. size-exclusion chromatography (Bio-Rad TSK-400 300 mm column, run at 0.5 ml/min in buffer A) in order to generate soluble purified Mg^{2+} -ATPase free from the gel matrix. Total protein concentration of the purified Mg^{2+} -ATPase was determined by using colloidal gold basically as described by Stoscheck [15] and heating the sample at 60 °C for 10 min after addition of the reagent.

Gel electrophoresis

SDS/PAGE was performed as described by Laemmli [16]. Typically, 0.75 mm-thick minigels consisting of a 4% (w/v) stacking gel and a 5–15% gradient separating gel were used. Analytical gels were silver-stained as described by Ansorge [17].

Native gel electrophoresis was carried out using a 6% (w/v) acrylamide gel and the buffers described by Laemmli [16], except that there was no SDS in any buffer or gel, and 0.1% digitonin was included in the gel. Native gels were cut vertically and a small section was stained for ATPase activity, and the area of the remaining gel corresponding to the activity was electroeluted as described previously [2]. In some experiments, the area of the gel corresponding to the Mg^{2+} -ATPase activity was cut out, incubated in SDS/PAGE sample buffer, and the gel slice loaded directly into the well of a second SDS/polyacrylamide gel in an attempt to eliminate the potential problem of differential recovery of different-size proteins by the technique of electroelution.

N-Terminal sequence analysis

The N-terminal sequences of the proteins were obtained after Western blotting of purified Mg^{2+} -ATPase as described by Matsudaira [18]. The stained excised poly(vinylidene difluoride) membrane containing the protein was then sequenced directly in a 2090E Porton protein sequencer in the Protein Chemistry Facility of the Department of Pharmacology and Cell Biophysics at the University of Cincinnati Medical Center.

Cross-linking and chemical modification of Mg^{2+} -ATPase

Cross-linking was performed at 22 °C in buffer A with various concentrations of DTSSP. Samples of the reaction mixture were taken at various times and diluted to stop the cross-linking

reaction. For the measurement of Mg^{2+} -ATPase activity, the samples were diluted into assay buffer (10 mM-Tris/maleate, pH 7.0, 100 mM-KCl, 5 mM- MgCl_2 , 1 mM-EGTA, 0.1% digitonin) containing 5 mM-lysine to stop the reaction and placed on ice until assayed for the release of P_i (ATPase) by the method of Fiske & Subbarow [19]. For determination of protein cross-linking, lysine was added to a final concentration of 5 mM to stop the reaction, followed by addition of a final concentration of 2 mM-N-ethylmaleimide to avoid the potential problem of disulphide interchange. After 30 min at 22 °C, an equal volume of SDS/PAGE sample buffer containing 8 M-urea and no reducing agent was added, and the sample heated at 60 °C for 10 min before SDS/PAGE. Some samples were electrophoresed in the absence of reducing agents, then reduced and re-electrophoresed as described by Adachi *et al.* [20] in order to determine which proteins had been cross-linked to one another and subsequently broken apart by reduction of the disulphide bond contained in the cross-linking agent (DTSSP).

Enzymic and chemical deglycosylation of purified Mg^{2+} -ATPase

The purified enzyme in buffer A was first precipitated with 4 vol. of acetone as described [21]. For chemical deglycosylation, the precipitated protein was cooled on ice and 5 μl of anisole added. Then, 45 μl of TFMSA was added and the sample vortex-mixed and incubated on ice for 2 h as described by Horvath *et al.* [22] (a modification of the method of Edge *et al.* [23]). In our laboratory, it was observed that, after opening the sealed ampoule, the TFMSA could be stored in a glass vial with a Teflon cap at 4 °C for several weeks with no apparent loss of potency. Also, it was not necessary to flush the reaction with nitrogen. The difficult part of this procedure is the extraction of the final acetone-wash solution without loss of the (very soft) precipitated protein pellet. This was done carefully with a 500 μl Hamilton syringe. The final pellet was allowed to dry in air and dissolved in SDS/PAGE buffer and heated for 10 min at 60 °C before electrophoresis.

For enzymic deglycosylation, the acetone-precipitated proteins were dissolved in a small volume (typically 25 μl) of 0.2% SDS, bath-sonicated for 5 min and heated for 10 min at 60 °C to denature the protein. Then water and buffer were added to give final concentrations of 0.1% SDS, 0.6% octyl glucoside, 10 mM-EDTA, 0.5% 2-mercaptoethanol and 20 mM-sodium phosphate, pH 7.3. Typically 1 μl of a 5-fold dilution of PNGase-F was added (0.05 unit of PNGase-F) to start the deglycosylation. Samples were then taken as a function of time at 22 °C, diluted with SDS/PAGE sample buffer, and analysed by SDS/PAGE. In other experiments, 0.5 unit of PNGase-F was added to the active purified Mg^{2+} -ATPase (in buffer A) and incubated for various times at 4, 22 or 37 °C to measure the effect of deglycosylation on Mg^{2+} -ATPase activity. The PNGase-F enzyme purchased from Genzyme was reproducibly of good specific activity and was used in all the studies reported here.

RESULTS

A final step in a previous purification of the Mg^{2+} -ATPase was native gel electrophoresis [2]. Difficulties have been encountered in subsequently freeing the active enzyme from this gel, and consequently an alternative strategy has been devised. Partially purified Mg^{2+} -ATPase (solubilized, eluted from Con A-Sepharose, and further purified by high-performance anion-exchange chromatography [2]) was concentrated with Centricon 30 micro-concentrators and purified by HPSEC by injection on to a TSK-400 (Bio-Rad) column equilibrated in buffer A. Assay of each 1 min (0.5 ml) fraction revealed the elution position of the Mg^{2+} -ATPase (results not shown). The fractions containing

Fig. 1. HPSEC repurification of the transverse-tubule Mg^{2+} -ATPase

The Bio-Rad TSK 400 column was equilibrated and eluted at 0.5 ml/min with buffer A. Fractions were taken based on the A_{280} as indicated (a) and equal volumes of each fraction were assayed for ATPase activity (b). Fractions 7–11 were pooled and used for subsequent experiments. (c) SDS/PAGE analysis of aliquots of the numbered fractions from (a) was performed on a 5–15% gradient acrylamide gel [16] with a 4% stacking gel and silver-stained. The peak eluted at ~22 min (no. 13 in a) is due to excess digitonin micelles.

the majority of the activity were pooled from two such experiments, concentrated and re-injected on the same column, and the resulting chromatogram is shown in Fig. 1(a). Samples of each fraction were taken and analysed for ATPase activity (Fig. 1b) and for protein content by SDS/PAGE (Fig. 1c). In addition to the heavily stained 160 and 97 kDa proteins, diffuse protein bands at approx. 110–125, 67–74 and 43–46 kDa copurify with the Mg^{2+} -ATPase activity. Fractions 4–6 contain impurities, as evidenced by a much lower specific activity and a visible shoulder on the 280 nm peak (Fig. 1a). This shoulder was evident as a discrete 280 nm peak on the first HPSEC run (results not shown). Fractions 7–11 (nearly identical specific activities and protein composition; Fig. 1c) were pooled for the subsequent experiments. The specific activity measured for the pooled fractions was 56 mmol of ATP hydrolysed/h per mg of protein. This measured specific activity is typical of several experiments using this purification scheme. This value is significantly (5–10-fold) higher than that obtained previously (6–12 mmol/h per mg [2]). The resulting purified preparation was used in the

Fig. 2. SDS/PAGE analysis of purified Mg^{2+} -ATPase, time-course of PNGase-F deglycosylation, TFMSA deglycosylation and DTSSP cross-linking of purified Mg^{2+} -ATPase

Silver-stained 4%–15% Laemmli gel. Lane 1, Bio-Rad high-plus low-molecular-mass standards; lane 2, purified transverse-tubule Mg^{2+} -ATPase; lanes 3–8 and 11, time course of deglycosylation of purified transverse-tubule Mg^{2+} -ATPase by PNGase-F at 22 °C after 0, 5, 10, 20, 30, 60 and 120 min of treatment (left to right); lane 9, TFMSA-treated purified Mg^{2+} -ATPase; lane 10, Bio-Rad high-molecular-mass standards; lane 12, unreduced purified transverse-tubule Mg^{2+} -ATPase; lane 13, DTSSP-treated unreduced transverse-tubule Mg^{2+} -ATPase (the cross-linked band is indicated by two asterisks); lane 14, unreduced control transverse-tubule Mg^{2+} -ATPase (put through same procedure as sample in lane 13, leaving out the DTSSP cross-linking reagent).

deglycosylation and cross-linking studies described below. Also, in order to determine if any further purification could be obtained by native gel electrophoresis, a sample of the purified preparation was concentrated and run on a native gel. The activity was detected on a part of the native gel as described previously [2], and the remainder of the gel was carefully sliced to cut out the regions above and below the activity, as well as in the active region. All the gel slices containing activity also had the same protein composition as each other and the original HPSEC-purified material (except for the 110–125 kDa glycoproteins, which were removed) as evaluated by SDS/PAGE (i.e. prominent 160 and 97 kDa proteins; results not shown).

To demonstrate the glycosylation states of the proteins purifying with the Mg^{2+} -ATPase activity, the HPSEC-purified material was deglycosylated. The 110–125 kDa glycoprotein, which is not a part of the Mg^{2+} -ATPase, is deglycosylated to a core molecular mass of 101 kDa (Fig. 2, lane 11). By monitoring the protein molecular masses as a function of time of incubation with PNGase-F, it was apparent that the two major silver-staining protein components present (97 kDa and 160 kDa, see Fig. 2) are both *N*-glycosylated, and yield protein cores of 84 and 131 kDa respectively after complete enzymic deglycosylation (Fig. 2, lane 11). Despite numerous attempts, it was not possible to determine the exact number of glycosylation sites from this type of experiment, owing to the small molecular-mass change on removal of a single glycan chain and the inherent broadness of glycoprotein bands on SDS/polyacrylamide gels. However, it appears that both proteins (160 and 97 kDa) are glycosylated at at least three sites, because of the presence of at least two intermediate-molecular-mass forms observed in Fig. 2, lanes 3–8. The molecular mass of the approx. 100 kDa protein, reported to be 105 kDa in the previous work from this laboratory [2], was re-evaluated and determined to be 97 kDa before deglycosylation. In fact, if a small amount of the Bio-Rad high-molecular-mass

Fig. 3. Effect of DTSSP cross-linking and Sulfo-NHS-Acetate chemical modification on vesicular transverse-tubule Mg^{2+} -ATPase

The concentration of transverse-tubule membranes was 0.1 mg/ml in buffer A (without digitonin) at 22 °C. At zero time, either 100 μ M-DTSSP (●) or 200 μ M-Sulfo-NHS-Acetate (○) was added and samples taken for assay at the indicated times. The curves are double-exponential decay fits to the data.

Fig. 4. Effect of DTSSP cross-linking and DTT reversal of cross-linking on solubilized transverse-tubule Mg^{2+} -ATPase

Solubilized and partially purified (by Con A-Sepharose) transverse-tubule Mg^{2+} -ATPase at 0.06 mg/ml in buffer A was treated with 150 μ M-DTSSP at 22 °C. Samples for assay were taken at the indicated times. The data from two experiments are shown. In one experiment (●), DTT was added to a final concentration of 25 mM at the time indicated. In another experiment (○), DTT was added to a final concentration of 15 mM at the time indicated. The continuous curve is a fit to a double-exponential decay process for the data from the first experiment (●) (excluding the time points after the addition of DTT).

standards are co-electrophoresed with the purified Mg^{2+} -ATPase, it could be demonstrated that this 97 kDa Mg^{2+} -ATPase protein exactly co-migrates with the molecular-mass standard of 97.4 kDa (rabbit muscle phosphorylase *b*), which should allow easier identification of this Mg^{2+} -ATPase protein by other laboratories, since the molecular mass of this Mg^{2+} -ATPase protein has been disputed [24]. The 97 kDa transverse-tubule Mg^{2+} -ATPase subunit protein, however, is not phosphorylase *b*, since it has different enzymic properties [2–10] and structural properties (by CNBr peptide mapping [2]). All molecular-mass assignments were made by non-linear curve fitting of the plot of relative mobility versus molecular mass, by using the molecular masses of the Bio-Rad standards given in Fig. 2. The prominence of the 160 kDa protein was unexpected on the basis of previous results, but was very reproducible.

The purified preparation was also chemically deglycosylated by using TFMSA, and the results are also shown in Fig. 2 (lane 9). The molecular masses of the chemically deglycosylated proteins were typically either identical with or slightly higher

than the PNGase-F-treated proteins (compare lanes 9 and 11 in Fig. 2); this is due to the fact that PNGase-F will remove all *N*-linked carbohydrates and convert the glycan-attached asparagine into an aspartic acid [25], whereas TFMSA cleaves *O*- and *N*-linked carbohydrates but leaves the bond between *N*-acetylglucosamine and the asparagine intact [23]. These results are consistent with the conclusion that these proteins contain little, if any, *O*-linked glycosylation.

The effect of the cleavable homobifunctional lysine-reactive cross-linking reagent DTSSP was measured on the Mg^{2+} -ATPase activity of vesicular transverse tubules, solubilized and partially purified Mg^{2+} -ATPase and the HPSEC-purified enzyme. In all cases the activity was inhibited by relatively low concentrations of cross-linker (100–150 μ M), and the kinetics of inhibition could be fitted by a double-exponential decay process. Results obtained with 0.1 mg of transverse-tubule membranes/ml and either 100 μ M-DTSSP or 200 μ M-Sulfo-NHS-Acetate (not a cross-linking agent, but very similar in structure and reactivity to one-half of the DTSSP molecule) in buffer A containing no digitonin are shown in Fig. 3.

Cross-linking results obtained by treating solubilized and partially purified Mg^{2+} -ATPase (0.06 mg/ml) in buffer A with 163 μ M-DTSSP are shown in Fig. 4; combined results of two experiments are shown. In addition to the reproducibility of the cross-linking-induced inactivation, Fig. 4 also shows that the DTSSP-induced inactivation can be partially reversed by reductive cleavage of the disulphide-containing cross-linking molecule (and therefore the cross-link) by DTT, again indicating that the inhibition is at least partially due to cross-linking, and not just chemical modification. Control experiments demonstrated that under identical experimental conditions, 25 mM-DTT had no effect on activity of the native complex (results not shown). It appears that the longer the time of cross-linking, the less reactivation possible on reduction of the cross-links formed or, alternatively, the more resistant to reduction the cross-links become.

To obtain information as to which proteins were being intermolecularly cross-linked to one another during inactivation, samples of the HPSEC-purified Mg^{2+} -ATPase were incubated in the presence or absence of 100 μ M- or 150 μ M-DTSSP in buffer A for 10 min. Samples were then taken for ATPase measurement and non-reducing SDS/PAGE analysis. For the sample shown in Fig. 2 (lane 13), the activity of the cross-linked protein was approx. 14% of the control activity. The reproducible result was that the staining intensity of the 97 kDa protein was reduced and a band at approx. 199 kDa appeared (Fig. 2, lane 13). The fact that this approx. 200 kDa band was derived from the intermolecular cross-linking of two 97 kDa subunits was confirmed by performing two-dimensional SDS/PAGE (non-reducing followed by reducing conditions [20]). The 200 kDa protein disappeared on reduction (and therefore cleavage of the disulphide-containing cross-link) and was replaced by the 97 kDa protein (results not shown). No other proteins were intermolecularly cross-linked under conditions leading to loss of Mg^{2+} -ATPase activity in any of several experiments performed. It is noteworthy that, if samples are heated at only 37 °C for 10 min in non-reducing SDS/PAGE sample buffer before electrophoresis, then a diffuse band at about 200 kDa is present in both the control and DTSSP-treated samples (results not shown). This phenomenon is consistent with the postulate that two 97 kDa proteins are closely associated with each other, and, even in the presence of SDS, are only dissociated by heating above 37 °C.

N-Terminal protein sequencing has been carried out on the 160 kDa (131 kDa after deglycosylation) as well as the 97 kDa (84 kDa after deglycosylation) and 70 kDa (50 kDa after

deglycosylation) proteins. The *N*-terminal sequence obtained for the 160 kDa protein (Thr-Leu-Asp-Pro-Gly-Leu-Leu-Pro-Gly-Asp-Phe-Ala-Ala-Asp-Glu*-Ala-Gly-Ala-Arg-Leu-Phe-Ala) identified that protein as ACE. The sequence found was identical with that reported for rabbit pulmonary ACE, except for the glutamate residue indicated by an asterisk, which was reported to be asparagine by Iwata *et al.* [26]. Sequencing of the 97 kDa protein resulted in two *N*-terminal sequences, suggesting two proteins of the same molecular mass [close inspection of Fig. 2, lane 11 (PNGase-F-deglycosylated Mg^{2+} -ATPase) reveals a closely spaced protein doublet at 84 kDa]. Sequencing of the 70 kDa protein resulted in the sequence Ala-Lys-Lys-Val-Leu-Pro-Leu-Leu-Leu-Pro-Pro-Leu-Val-Xaa-Ala-Ala-Leu-Gly-Leu-Ala-Xaa-Phe, which does not have significant sequence identity with any protein in the current Swiss protein data bank.

DISCUSSION

The purpose of this study was to define further the structure of the skeletal-muscle transverse-tubule Mg^{2+} -ATPase. None of the proteins in the purified preparation were linked by intermolecular disulphide bonds, since SDS/PAGE analysis in the presence and absence of reducing agents yielded identical protein patterns [see control samples, Fig. 2, lanes 2 (reduced) and 12 (non-reduced)]. Surprisingly, it was found that, in addition to the 97 kDa protein seen in previous work [2], there was also a prominent 160 kDa protein present. In the present study, by direct application of the native gel slices containing the Mg^{2+} -ATPase activity to an SDS/polyacrylamide gel (without intermediate electroelution), this protein was reproducibly observed, indicating that it did indeed co-purify with the 97 kDa protein and the Mg^{2+} -ATPase activity in the native gel and must have been either degraded or poorly recovered in the electroelution of the native gel reported previously [2]. This 160 kDa protein (131 kDa after deglycosylation) was determined to be ACE by *N*-terminal sequencing. What, if any, physiological role this ACE enzyme performs in transverse-tubule membranes is not known at present.

There are several other glycoproteins present in the purified enzyme preparation which are only apparent on darkly stained gels or after deglycosylation of the purified Mg^{2+} -ATPase (Figs. 1 and 2). Deglycosylation experiments were not performed in the previous purification work [2], explaining why these putative subunits were not reported, since they migrate as poorly staining diffuse bands on SDS/PAGE before deglycosylation. This complex of proteins proposed is more similar to the multiple-subunit coated-vesicle (H^+)-ATPase [2] and the F_1F_0 (H^+)-ATPases than to the E_1/E_2 class of ion-transporting ATPases [e.g. the Ca^{2+} -, (H,K)- and (Na,K)-ATPases], which are composed of only one or two subunits.

All the major proteins co-purifying with the Mg^{2+} -ATPase are *N*-glycosylated, with little, if any, *O*-glycosylation (Fig. 2). Although not shown in Fig. 2, incubation with more PNGase-F for longer times (approx. 24 h) at higher temperatures (37 °C) did not result in further decrease in apparent molecular mass of these subunits, indicating that the enzymic deglycosylation was complete. Chemical deglycosylation with TFMSA also gave nearly identical results, again indicating completeness of the enzymic deglycosylation and the presence of little *O*-glycosylation. The agreement between the enzymic and chemical deglycosylation results suggested that non-specific (i.e. proteolytic or acidic) cleavage of the peptide chain did not occur, as does in fact the expected deglycosylated molecular mass of 131 kDa obtained for the ACE protein. In addition, control deglycosylation experiments using lamb kidney (Na,K)-ATPase showed that the methodologies used in this work gave the

expected results (i.e. no change in the non-glycosylated α subunit, whereas the β subunit was deglycosylated to its core polypeptide molecular mass of 35 kDa [27]). Numerous attempts were made to measure the effect of PNGase-F treatment (deglycosylation) on the active, solubilized and purified Mg^{2+} -ATPase activity. Even after overnight incubations in buffer A at 37 °C with large amounts of PNGase-F, there was little, if any, effect on activity compared with the control. Unfortunately, little deglycosylation occurred under these conditions, as monitored by SDS/PAGE (results not shown). The 97 kDa protein was not deglycosylated at all, and a very small decrease in the apparent molecular mass of the 160 kDa ACE protein suggested that a single glycan chain may have been removed from that protein. Therefore the question of what effect deglycosylation has on Mg^{2+} -ATPase activity may not be able to be answered by using this methodology, since it appears that the conditions necessary to deglycosylate the proteins will also inactivate the Mg^{2+} -ATPase enzyme.

The Mg^{2+} -ATPase activity in all forms (transverse-tubule vesicles, solubilized and purified) was inhibitable by relatively low concentrations of DTSSP cross-linker. This suggests that solubilization in lysolecithin and purification in digitonin do not significantly change the interaction of the putative subunits. Although it is not known how much of the inhibition is due to intramolecular cross-linking, it is interesting that the inactivation is accompanied by intermolecular cross-linking of two 97 kDa subunits, and that none of the other co-purifying proteins were intermolecularly cross-linked under conditions leading to inactivation of the enzyme. This not only demonstrates that the 97 kDa subunit(s) is(are) likely to exist as a closely associated dimer or higher-order complex in the active enzyme complex, but also suggests that they may be involved in ATP hydrolysis. The intermolecular cross-linking must occur on the cytoplasmic side of the transverse-tubule membrane, since DTSSP is membrane-impermeant and the transverse-tubule membrane preparation used is known to be sealed, luminal side in (cytoplasmic side exposed [2,3]). Interestingly, Beeler *et al.* [5] reported that cross-linking rat skeletal-muscle transverse-tubule vesicles with glutaraldehyde prevented the ATP-dependent inactivation of the Mg^{2+} -ATPase seen in the rat skeletal-muscle Mg^{2+} -ATPase enzyme (this phenomenon of ATP-dependent inactivation is not seen in the rabbit enzyme used in this study), and also decreased the sensitivity of the rat Mg^{2+} -ATPase to inactivation by detergents. Those data are consistent with a model suggested by the studies reported here of the transverse-tubule Mg^{2+} -ATPase as a multi-subunit enzyme which is inactivated by disruption of subunit associations induced by a variety of detergents. Unfortunately, the solution as to which subunit(s) is(are) involved in ATP hydrolysis has thus far not been obtained by the more direct approach of using affinity-labelled analogues of ATP to label the ATP-binding subunit. For example, in this laboratory, fluorosulphonylbenzoyl-adenosine, pyridoxal phosphate and 5'-diphospho-5'-adenosine (the last two compounds were utilized successfully in this laboratory to inactivate and label the putative ATP-binding site in the (Na,K)-ATPase [28]) do not inactivate the Mg^{2+} -ATPase under the appropriate conditions.

Although the data presented in this paper do not conclusively demonstrate that the proteins co-purifying with the Mg^{2+} -ATPase are truly subunits of that enzyme, the following model is consistent with the data obtained to date. The 160 kDa ACE protein, although it co-purifies with the Mg^{2+} -ATPase activity even in a native gel, is not part of the enzyme complex, since it is partially separated from the Mg^{2+} -ATPase activity by using two size-exclusion TSK-400 columns in series. Thus the peaks of activity for ACE and Mg^{2+} -ATPase activities, although overlapping considerably, are not superimposable, with the ACE activity being eluted just before the Mg^{2+} -ATPase activity (results

not shown). Whether the ACE is truly a component of transverse-tubule membranes or is simply adsorbed or trapped during vesiculation of the membranes requires investigation. If the ACE is indeed a component of the transverse-tubule membranes, then the physiological ramifications of this finding may be important.

It can also be demonstrated that the profiles of the minor proteins at 110–125 kDa do not exactly match the profile of the Mg^{2+} -ATPase activity in the same two TSK-400 columns (Figs. 1 and 2) and do not co-purify on a native gel (results not shown). However, it appears that glycoproteins of approx. 70 and 43 kDa do correlate with the Mg^{2+} -ATPase activity (Fig. 1). These proteins are difficult to see before deglycosylation, which explains why they were not reported in an earlier study from this laboratory [2] or in a more recent confirmation of this laboratory's earlier results [29]. The fact that there are evidently some impurities in the present Mg^{2+} -ATPase preparation (notably the 160 kDa ACE protein) means that the true specific activity of the pure Mg^{2+} -ATPase must be significantly higher than the very high value reported here (approx. 56 mmol of ATP hydrolysed/h per mg) and is probably at least 100 mmol of ATP hydrolysed/h per mg. The highest specific activity measured to date in this laboratory is 74 mmol/h per mg. The Mg^{2+} -ATPase is an extremely 'fast' enzyme, and undoubtedly this high enzymic rate is important in its (as yet undefined) physiological function.

In general, it is very difficult to prove that proteins are subunits without some way to label them specifically. However, it is easy to prove that proteins are not subunits if a means of separating them from the activity of interest is found. The 97, 70 and 43 kDa proteins reported in this work co-purify with the Mg^{2+} -ATPase activity throughout multiple separation techniques. They are all transverse-tubule membrane glycoproteins which bind to Con A and wheat-germ agglutinin lectins, are co-eluted on ion-exchange and size-exclusion h.p.l.c. columns, and co-electrophorese during native gel electrophoresis. Numerous other separation techniques have failed to provide further purification. Therefore it is tentatively concluded that these proteins are subunits of the transverse-tubule Mg^{2+} -ATPase. However, it is possible that one or more of the proteins could be impurities or degradation products. The question of subunits can be resolved by raising antibodies to all the proteins involved and exploring whether or not antibodies raised against one protein can be used to immunoprecipitate and/or immunopurify the other putative subunits. The definitive answers concerning subunit composition will await these results as well as sequencing and reconstitution of this enzyme system.

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