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Purification of the Microsomal Ca^{2+}-ATPase from Rat Liver

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Summary. The Ca^{2+}-ATPase from rat liver microsomes has been solubilized in Triton X-100 and purified to homogeneity by ficoll-sucrose treatment, column chromatography with agarose-hexane adenosine 5′-triphosphate Type 2, and high pressure liquid chromatography (HPLC). The purified enzyme obtained by this sequential procedure exhibited a 183-fold increase in specific activity. After ficoll-sucrose treatment, the activity of the Ca^{2+}-ATPase was stable for at least two weeks when stored at −70°C. In SDS-polyacrylamide gels, several fractions from HPLC chromatography showed a single band at a position corresponding to a molecular weight of about 107 kDa. This value is consistent with the molecular weight of the phosphoenzyme intermediate of endoplasmic reticulum (ER) Ca^{2+}-ATPase. Further characterization of the ER Ca^{2+}-ATPase was performed by western immunoblots. Antiserum raised against the 100-kDa sarcoplasmic reticulum (SR) Ca^{2+}-ATPase cross-reacted with the purified Ca^{2+}-ATPase from rat liver ER membranes.

Key Words Ca^{2+}-ATPase · endoplasmic reticulum · rat liver · purification

Introduction

The hepatic microsomal Ca^{2+}-ATPase plays a pivotal role in the uptake and storage of Ca^{2+} and therefore is important in regulating Ca^{2+} distribution in the liver. Thus, it plays a role similar to the sarcoplasmic reticulum Ca^{2+}-ATPase (Martonosi, 1984; Inesi, 1985; Fleischer & Inui, 1989). Characteristic with this similarity of function, structural similarities between these enzymes have been described. For example, the microsomal enzyme similarly forms a Ca^{2+}-dependent phosphorylated intermediate (Fleschner, Kraus-Friedman & Wilbert, 1985; Heilman, Spamer & Gerok, 1985). The molecular weight of this phosphorylated protein was around 100 kDa. Additionally, fluorescein-5-isothiocyanate, which binds to the ATP-binding site of the SR[1] enzyme, also binds to the ER enzyme (Mitchinson et al., 1982; Fleschner & Kraus-Friedmann, 1987). Immunological crossreactivity between the SR enzyme and intact microsomal vesicles was also demonstrated (Damiani et al., 1988).

Because of the importance of this enzyme in Ca^{2+} homeostasis (Carafoli, 1987) and its possible involvement in influencing hepatic Ca^{2+} distribution, a process involved in the regulation of hepatic metabolism (Kraus-Friedmann, 1984), it is important to characterize this enzyme. Furthermore, the characterization and elucidation of the regulatory factors involved in its regulation could be determined more clearly using the purified enzyme. We attempted previously to purify the enzyme using a calmodulin-affinity column (Moore & Kraus-Friedmann, 1983). Though calmodulin is clearly present in the hepatic microsomal fraction as well as in the SR (Campbell & MacLennan 1982; Chiesi & Carafoli, 1982; Moore & Kraus-Friedmann, 1983; Gupta, Davis & Kranias, 1988), the purification procedure was not reproducible for the hepatic enzyme. It might be that the enzyme previously purified was due to contamination of the preparation with plasma membrane fraction (Schutze & Soling, 1987). Therefore, different purification methods were implemented. In contrast to the SR enzyme, which comprises almost 90% of the total protein in the microsomal fraction, the ER enzyme comprises about 0.5% or less of the total protein. Because of this difference, purification methods successfully employed for the SR enzyme were unsuccessful for the ER protein (MacLennan, 1970; Van Winkle, Pitts & Entman, 1978). We describe here a purification procedure for hepatic microsomal Ca^{2+}-ATPase

[1] *Abbreviations used:* AGATP, agarose-hexane adenosine 5′-triphosphate; DDT, DL-dithiothreitol; EGTA, ethylene glycol-bis-(β-aminoethly ether) N,N,N′,N′-tetraacetic acid; EDTA, ethylenediamine tetraacetic acid; HEPES, N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; HPLC, high pressure liquid chromatography; SR, sarcoplasmic reticulum; ER, endoplasmic reticulum.

which produced a homogeneous, single protein band in SDS-polyacrylamide gels.

Materials and Methods

Materials

Ficoll (Type 400) was obtained from Sigma. Triton X-100 was obtained from Pierce as Surfact-Amps X-100. AGATP Type 2 (the linkage is through the N^6 amino group of adenine ring) affinity resin was obtained from Pharmacia LKB Biotechnology.

Isolation of Microsomes

The microsomal fraction was prepared from the livers of male, fed, Sprague-Dawley rats (250–300 g) essentially as previously described (Fleschner et al., 1985), except that the homogenization medium was modified to contain 250 mM sucrose, 10 mM HEPES, 5 mM DTT, 0.02% NaN_3, 0.3 mM PMSF, 1 mM benzamidine, 0.1 mg/ml bacitracin, 0.1 mg/ml aprotinin, 300 kallikrein-inhibitory units/ml, 10 μg/ml leupeptin, 10 μg/ml antipain, pH 7.2.

Purification of Ca^{2+}ATPase

Freshly prepared microsomes (300–350 mg protein) were solubilized for 30 min at 4°C in 30–35 ml medium containing 0.4% (vol/vol) Triton X-100 (0.4 mg of detergent/mg of protein), 20% (vol/vol) glycerol, 120 mM KCl, 20 mM HEPES, 5 mM $CaCl_2$, 0.5 mM ATP, 0.02% NaN_3, 5 mM DTT, pH 7.0, and the proteinase inhibitors mentioned above. The detergent was added drop by drop to the slowly stirred protein solution. After solubilization, sonicated phosphatidylcholine was added (final concentration 0.1 mg/ml) and the suspension was centrifuged at 105,000 × *g* for 45 min. The solubilized fraction was mixed with an equal volume of a ficoll-sucrose solution containing 11% (wt/vol) ficoll-400, 11% (wt/vol) sucrose, 10% (vol/vol) glycerol, 5 mM DTT, 120 mM KCl, 20 mM HEPES, 0.5 mM ATP, 5 mM $CaCl_2$, pH 7.0, and the proteinase inhibitors mentioned above, and centrifuged at 43,000 × *g* for 30 min.

The supernatant was diluted with 3 volumes of the medium containing 10% (vol/vol) glycerol, 5 mM DTT, phosphatidylcholine (0.1 mg/ml), 120 mM KCl, 20 mM HEPES, 5 mM $CaCl_2$, 0.5 mM ATP, pH 7.0, and proteinase inhibitors mentioned above, and centrifuged for 1 hr at 120,000 × *g* to collect the pellet. The pellet is the ficoll-sucrose pellet. This pellet can be stored at −70°C until use.

The ficoll-sucrose pellet (10–20 mg protein) was resuspended in approximately 2 ml of 0.2% (vol/vol) Triton X-100, 10 mM HEPES, 5 mM DTT, 20% (vol/vol) glycerol, 5 mM $CaCl_2$, pH 7.2, and applied to an AGATP Type 2 column (0.7 × 12 cm) equilibrated with 10 mM HEPES, 5 mM DTT, 20% (vol/vol) glycerol, 5 mM $CaCl_2$, 0.05% (vol/vol) Triton X-100, pH 7.2. The column was washed with 25 ml of this buffer and then eluted with 15 ml of the above buffer plus 0.5 M KCl and 5 mM ATP at a flow rate of 4 ml/hr. Two ml fractions were collected. Protein concentrations and Ca^{2+}-ATPase activity of each eluant fraction were determined, and those fractions containing Ca^{2+}-ATPase activity were pooled and dialyzed against 4 liters of 10 mM Tris-HCl, 0.5 mM EDTA, pH 7.2, overnight. The total volume of the dialyzed sample was 4 ml. Three ml (3 mg total protein) of the dialyzed solution was applied to a Pharmacia Mono Q HR5/5 HPLC column, previously equilibrated in buffer A (10 mM Tris-HCl, 0.5 mM EDTA, pH 7.2). Protein was eluted from the column with a linear gradient of 100% buffer A/0% buffer B (buffer A + 2 M NaCl) to 70% buffer A/30% buffer B in 80 min at a flow rate of one ml/min, using a Dionex BioLC system. One ml fractions were collected, and the absorbance of the eluent was monitored at 280 nm. Protein concentrations and Ca^{2+}-ATPase activities (in the presence of 1 mg/ml asolectin) were also measured. Fractions containing Ca^{2+}-ATPase activity were analyzed by SDS gel electrophoresis to assess protein purity, and those containing pure Ca^{2+}-ATPase were lyophilized. All steps of the purification were carried out at 4°C except for the HPLC procedure.

Protein Determination

Protein concentrations were determined using the Pierce Coomassie protein assay reagent employing the Bradford (1976) method, or with the Pierce BCA protein assay reagent (Smith et al., 1985).

SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (7.5% gels) was performed in a slab gel apparatus utilizing the discontinuous system described by Laemmli (1970). Proteins were denatured by boiling for 4 min in a buffer containing 62.5 mM Tris-Cl, pH 6.8, 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 5% (vol/vol) 2-mercaptoethanol, and 0.0025% (wt/vol) bromophenol blue. Proteins were stained with Coomassie brilliant blue.

Ca^{2+}-ATPase Assay

Microsomal or purified protein was incubated for 10 min at 37°C in the standard assay medium (final volume 500 μl) which contained 100 mM KCl, 20 mM HEPES/KOH, pH 7.4, 1 mM ouabain, 2 mM EGTA, 1 mM NaN_3, 5 mM ATP, 5 mM Mg^{2+} and 1 mg/ml asolectin with or without 2 mM Ca^{2+} (to produce free Ca^{2+} and Mg^{2+} concentrations of 0.1 and 2.5 mM, respectively). One μM of the ionophore A23187 was also included in the reaction solution when the unsolubilized microsomal fraction was used. The reaction was initiated by the addition of protein and terminated by the addition of 0.5 ml of ice-cold 16% (wt/vol) trichloroacetic acid. The protein-free supernatant was assayed for P_i using the colorimetric method described by Lanzetta et al. (1979).

Preparation of Antiserum Against SR Ca^{2+}-ATPase

Partially purified rabbit SR Ca^{2+}-ATPase was subjected to 7.5% SDS-polyacrylamide gel electrophoresis. The Ca^{2+}-ATPase protein band, identified by KCl staining (Hager & Burgess, 1980), was excised, suspended (by fragmentation) in 25 mM HEPES/KOH, pH 7.5, and extracted overnight on a rotor at 4°C. The resulting protein solution was separated from the gel by centrifugation at 500 × *g* for 5 min and concentrated using a 30 kDa molecular weight cut-off Amicon microconcentrator and washed several times with PBS. The final concentrate was sent to Bethyl Lab. (Montgomery, TX) as antigen for antibody production. The

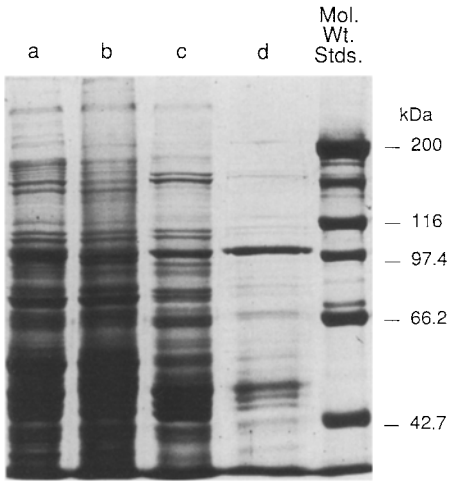

Fig. 1. SDS-Polyacrylamide gel electrophoresis of fractions obtained during ER Ca^{2+}-ATPase purification. Lane *a*, microsomes (25 μg); lane *b*, Triton X-100-solubilized fraction (25 μg); lane *c*, ficoll-sucrose pellet (15 μg); lane *d*, AGATP chromatography peak II (8 μg); right lane, mol wt standards

antibodies were raised in adult hens by intramuscular injections, at three-week intervals, of 100 μg of antigen, emulsified with an equal volume of complete Freund's adjuvant (for first injection) or with an equal volume of incomplete Freund's adjuvant (for subsequent injections). After two booster injections, the animal was bled 49 days from the beginning of immunization to obtain antiserum.

Western Blots and Immunostaining

The SR Ca^{2+}-ATPase or ER Ca^{2+}-ATPase were applied to 7.5% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose paper. For immunostaining, the blots were incubated for 1 hr in PBS, pH 7.5, with 0.05% (vol/vol) Tween-20 (TPBS) and 2% (vol/vol) fetal calf serum. The blots were repeatedly washed in TPBS buffer and then incubated with antiserum (diluted 1/100) in TPBS 1 hr with gentle agitation. All incubations were carried out at room temperature. After washing three times with TPBS, the blots were incubated for 1 hr in a 10 μg/ml solution of biotinylated anti-chicken IgG in TPBS. The blots were again washed three times with TPBS and transferred to the Vectastain ABC reagent. The blots were incubated in the reagent solution for 1 hr. After washing with PBS, the blots were developed using 4-chloro-1-naphthol and H_2O_2 as substrate.

Results and Discussion

The results of a typical isolation experiment are presented in Figs. 1–4 and the Table. In Fig. 1 the proteins present in the whole microsomal fraction, the solubilized fraction, the ficoll-sucrose pellet, and the fraction eluted from the ATP-affinity column are shown. A gradual enrichment in a protein band between the 97 and 116 kDa mol wt markers is evident. The biochemical correlates of the purification process are presented in the Table. Following solubilization of the enzyme by treatment with Triton X-100 and sedimentation in ficoll-sucrose, an increase in the specific activity of the enzyme is evident. The Ca^{2+}-ATPase activity in the fricoll-sucrose pellet was stable at −70°C for at least 2 weeks (*data not shown*). Subsequent binding to and elution of the enzyme from the AGATP type 2 affinity chromatography column resulted in an additional increase in specific activity. These results suggest that the resin was capable of interacting in a specific manner with the Ca^{2+}-ATPase, as expected. Approximately 50% of the original activity was recovered from the affinity column (peak II) by elution with 0.5 M KCl and 5 mM ATP (Fig. 2). Though the specific activity of the Ca^{2+}-ATPase in peak II was 50-fold higher than in the microsomal fraction, SDS-gel electrophoresis showed that peak II still contained numerous proteins in addition to the Ca^{2+}-ATPase (Fig. 1*d*). Therefore, in order to obtain further purification, the peak II fractions containing the

Table. Purification of Ca^{2+}-ATPase from rat liver microsomes

Fraction	Total protein (mg)	Total activity (μmol/ mg-min)	Yield (%)	Specific activity (μmol/ mg-min)	Purification
Microsomes	324	22.68	100	0.07	1
Ficoll-sucrose pellet	14.6	14.75	65.0	1.01	14.4
AGATP Type 2	3.4	12.48	55.0	3.67	52.4
HPLC	0.05	0.64	2.8	12.84	183.4

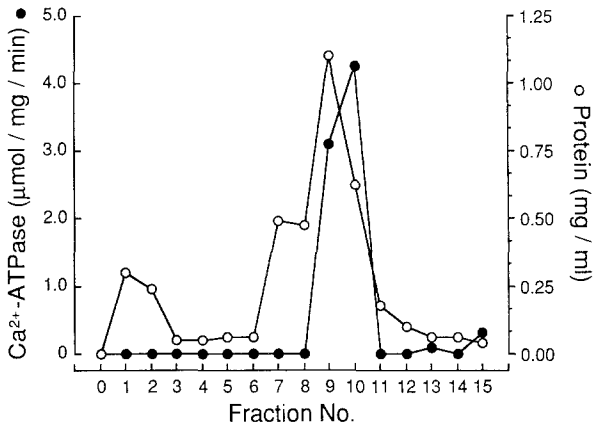

Fig. 2. Purification of Ca^{2+}-ATPase by chromatography on a AGATP Type 2 column. The ficoll-sucrose pellet was applied to an AGATP Type 2 column and washed and eluted as described in Materials and Methods. Fractions of peak II (nos. 9, 10) were pooled

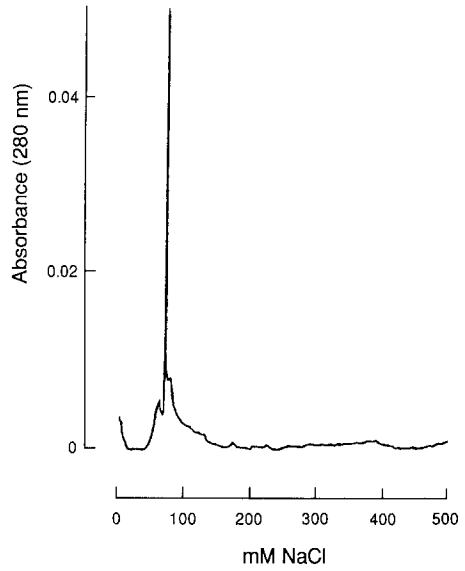

Fig. 3. HPLC purification of ER Ca^{2+}-ATPase. ER Ca^{2+}-ATPase (AGATP chromatography peak II) was applied to an HPLC column and eluted with a linear gradient of NaCl as described in Materials and Methods. The absorbance of the eluant at 280 nm is shown. The purified ER enzyme eluted as a sharp peak at approximately 75 mM NaCl under these conditions

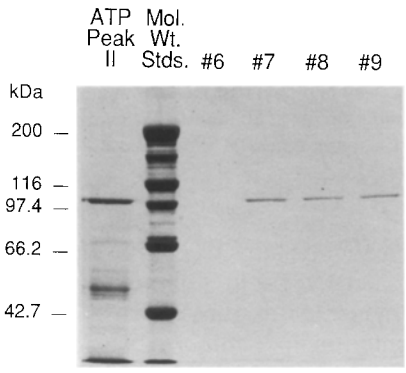

Fig. 4. SDS-polyacrylamide gel electrophoresis of purified Ca^{2+}-ATPase. Lane 1, AGATP chromatography peak II (6 μg); lane 2, mol wt standards; lane 3–6, HPLC fractions #6–9. Approximately 1.8–2.8 μg protein were loaded in each lane

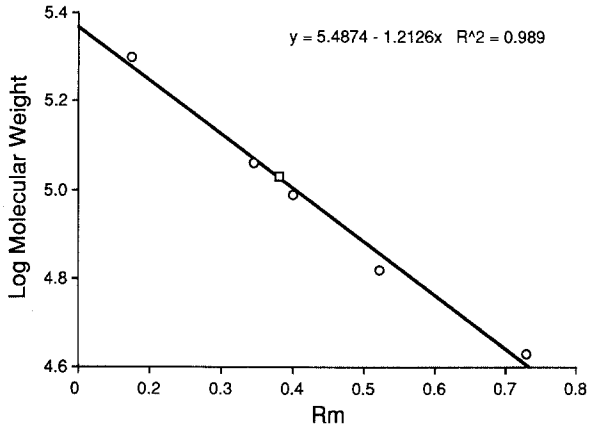

Fig. 5. Molecular weight determination of denatured Ca^{2+}-ATPase (□) by SDS-polyacrylamide gel electrophoresis. Standards (○) and their M_r values (daltons) were, from top to bottom: myosin, 200,000; β-galactosidase, 116,250; phosphorylase *b*, 97,400; bovine serum albumin, 66,200; and ovalbumin, 42,700

highest enzymatic activity were applied to an HPLC column. Several fractions eluted from the HPLC column contained homogeneous protein as demonstrated by the presence of a single band upon SDS-polyacrylamide gel electrophoresis (Figs. 3 and 4). These fractions demonstrated Ca^{2+}-ATPase activity, but the total activity was less than that observed in the previous samples (Table). Thus, while the protein obtained was the purified enzyme, some loss of activity occurred during this step of the purification procedure. However, the specific activity and enrichment of the enzyme is very high throughout the procedure; the HPLC-purified Ca^{2+}-ATPase demonstrated 183-fold increase in enzymatic activity compared with microsomes. We found that the presence of protease inhibitors, and high concentrations of glycerol, DTT, and Ca^{2+}, were important in preserving activity.

The molecular weight of the purified protein was determined by comparing its migration in SDS-polyacrylamide gels with the migration of known molecular weight standards. Comparison of the rates of migration during SDS-gel electrophoresis with appropriate standards gave an M_r value for the hepatic microsomal Ca^{2+}-ATPase of 107 kDa for the denatured enzyme (Fig. 5). This value is consistent with the molecular weight of the phosphoenzyme intermediate of ER Ca^{2+}-ATPase (Fleschner et al., 1985).

In order to further characterize the enzyme, its interaction with antiserum against the 100-kDa SR Ca^{2+}-ATPase was examined by western immunoblots. Antibodies produced against the SR Ca^{2+}-ATPase cross-reacted with the purified ER enzyme (*results not shown*). This implies that the SR and ER enzymes share antigenic determinants. Similar observations were made using intact endoplasmic reticulum membranes by Damiani et al. (1988).

In summary, we present data demonstrating for the first time the purification to homogeneity of the hepatic microsomal Ca^{2+}-ATPase. Because the purified protein possesses high specific activity, this preparation will be useful in future studies concerning the structural characterization of the enzyme and its mechanism of action.

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