Solubilization and Separation of Ethacrynic Acid (EA) Highly Sensitive and EA Less Sensitive Mg$^{2+}$-ATPases in the Rat Brain

Toshizo TANAKA, Chiyoko INAGAKI*, Yukiko KUNUGI* and Shuji TAKAORI
Department of Pharmacology, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, Japan
*Department of Biology, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607, Japan
Accepted November 8, 1986

Abstract—Rat brain microsomal Mg$^{2+}$-ATPases with two distinct activities: ethacrynic acid (EA) highly sensitive and EA less sensitive Mg$^{2+}$-ATPase activities were solubilized by the combined treatment with 10 mM 3-(3-chlolamidopropyl)-dimethylammonio-1-propane-sulfate (CHAPS) and 30 mM octyl-$\beta$-D-glucoside. The solubilized enzymes had properties similar to those of the membrane-bound enzyme in microsomes with respect to the sensitivity to EA and Cl$^-$, although the optimal pH and the affinity to ATP were slightly altered after the solubilization. Fast protein liquid chromatography of the solubilized enzymes on an anion-exchanger (Mono Q) column with a linear NaCl gradient (0–1.0 M) yielded separate peaks for EA highly sensitive and EA less sensitive Mg$^{2+}$-ATPase activities at 0.1 and 0.35 M NaCl, respectively. Polyacrylamide gradient gel electrophoresis of the samples from the peak-fractions of EA highly sensitive and EA less sensitive Mg$^{2+}$-ATPase activities yielded prominent bands at 600 and 70 kDa, respectively. These results indicate that EA highly sensitive Mg$^{2+}$-ATPase is solubilized and separated from EA less sensitive Mg$^{2+}$-ATPase as a large enzyme molecule with anion-sensitive sites.

The occurrence of non-mitochondrial and anion-sensitive Mg$^{2+}$-ATPases has been demonstrated in several animal tissues (1, 2). These enzymes are located in intracellular vesicle membranes such as those of chromaf-fin granules (3), neurohypophysis secretory granules (4) and cholinergic synaptic vesicles (5) or in the plasma membranes of erythrocytes (6) and epithelial cells (7–9); and some of the enzymes coexist with H$^+$-translocating activity and active Cl$^-$ transport activity (3–5, 9). However, little is known about the functions and characteristics of anion-sensitive Mg$^{2+}$-ATPases because of the difficulty in solubilization and purification of the intrinsic membrane proteins.

We previously showed that brain microsomes contain two types of Mg$^{2+}$-ATPases activities: ethacrynic acid (EA) highly sensitive and EA less sensitive Mg$^{2+}$-ATPase activities (10–13). The former activity was mainly located in plasma membrane fractions and was sensitive to anions, especially to Cl$^-$ (a stimulatory anion) and NO$_3^-$ (an inhibitory anion), and the latter was insensitive to the anions and rather widely distributed (12, 13). For further characterization of the Mg$^{2+}$-ATPases, it is necessary to solubilize and separate them from each other. This paper describes the successful solubilization and separation of the two enzymes, and we further analyzed the properties of the solubilized enzymes.

Materials and Methods

Materials: Reagents used were as follows: ethacrynic acid (kindly provided by Merck, Sharp & Dohme Res. Lab.), ouabain (Sigma), ATP-Na$_2$ (Sigma), ethylenediaminetetraacetic acid (EDTA, Sigma), 3-(3-chlolamido-
propyl)-dimethylammonio-1-propane sulfate (CHAPS, Dojindo Lab., Kumamoto), octyl-β-D-glucoside (OcGlu, Dojindo Lab.), 4-morpholine-ethanesulfonic acid (MES, Wako Pure Chem., Osaka) and dithiothreitol (DTT, Wako Pure Chem.). All other reagents were of the highest available purity.

Preparation and solubilization: Wistar rats were used. After exsanguination, the brain was quickly removed and the EDTA-treated microsomes were prepared as described previously (12). The microsomes were suspended in 6 original tissue volumes of 0.25 M sucrose solution containing 1 mM EDTA and 25 mM Tris-MES (pH 7.4). Detergents were added to the suspension of microsomes at 4–10 mg protein/ml with constant stirring. Following a 30-min incubation at 4°C, the mixture were centrifuged at 20,000 x g for 15 min or at 100,000 x g for 60 min. Pellets were resuspended in the solubilization buffer with a hand-held Teflon-glass homogenizer. The supernatants and the suspension of pellets were assayed for ATPase activities and protein contents.

In the combined detergent-treatment, CHAPS was added to the suspension of microsomes (10 mg protein/ml) to achieve a final CHAPS concentration of 10 mM, and the mixture was centrifuged at 20,000 x g for 15 min. To the resulting supernatant, OcGlu up to 50 mM was added. The mixture was stirred for 30 min at 4°C, and then centrifuged at 10,000 x g for 60 min. The final supernatant was carefully decanted and stored.

Separation of Mg2+-ATPases: Mg2+-ATPases solubilized in the final supernatants were separated using a Fast Protein Liquid Chromatography (FPLC) System (Pharmacia Fine Chem. AB, Uppsala, Sweden) with a 1.0 ml anion exchanger Mono Q column (Type HR5/5, 50×5 mm L.D.). Elution was carried out with NaCl concentration gradients in a buffer containing 20 mM OcGlu, 1 mM EDTA, 1 mM DTT, 10 mM Tris-MES (pH 7.4) and 20% glycerol at a flow rate of 1.0 ml/min at room temperature. The protein concentration of the eluate was monitored spectrophotometrically at 280 nm, or it was determined as described by Bradford (14). For re-chromatography, fractions with the peak-activities of EA highly sensitive and EA less sensitive Mg2+-ATPases were pooled from several runs and diluted to contain 10 mM NaCl with a NaCl-free elution buffer.

Electrophoresis: Protein samples were electrophoresed in Pharmacia polyacrylamide 4/30 slab gradient gels in 90 mM Tris-borate buffer (pH 8.4) with 2.5 mM EDTA, at 100 V for 20–24 hr. The gels were fixed in 10% sulfosalicylic acid for 30 min. Protein was stained overnight with 0.2% Coomassie brilliant blue R-250 in 50% methanol/10% acetic acid. Then, the gels were destained overnight in 30% methanol/10% acetic acid. Molecular weights of proteins were calculated using a High Molecular Weight Calibration Kit Protein Mixture (Pharmacia).

ATPase assay: ATPase activities were determined as reported previously (12, 13). The standard assay was carried out in 0.2 ml of reaction mixture containing 25 mM Tris-MES (pH 7.4), 1 mM EDTA, 6 mM Mg(OH)2-MES, 6 mM ATP-Tris, 1 mM ouabain, 1 mM NaN3 and 20–40 μg enzyme protein in the presence or absence of 0.3 mM EA. The difference between the activities in the presence and absence of EA was designated as EA highly sensitive Mg2+-ATPase activity, and the activity in the presence of EA was denoted as EA less sensitive Mg2+-ATPase activity. The protein concentration was determined by the method of Lowry et al. (15).

Results

Solubilization: Several detergents were tested to solubilize EA highly sensitive and EA less sensitive Mg2+-ATPases in the brain microsomes. The enzymes were not solubilized by Nonidet P-40, deoxycholate or Triton X-100. When CHAPS up to 40 mM was added to the suspension of the microsomes and the mixture was centrifuged at 20,000 x g for 15 min, the activities were recovered mainly in the supernatant (Fig. 1A). However, when the mixture was centrifuged at 100,000 x g for 60 min, both enzyme activities were found mainly in the pellets, and the recovery in the supernatants was only 5–15%. OcGlu was added to the 20,000 x g supernatants of 10 mM CHAPS treated
Fig. 1. Effects of CHAPS (0–40 mM) and octyl-β-D-glucoside (OcGlu: 0–50 mM) on EA highly sensitive (upper panels) and EA less sensitive (lower panels) Mg²⁺-ATPase activities. A: The activities in supernatants (sup: ○) and pellets (ppt: ●) of CHAPS-treated microsomes centrifuged at 20,000 x g for 15 min. B: The activities in sup (○) and ppt (●) of CHAPS (10 mM) plus OcGlu-treated microsomes. Various concentrations of OcGlu were added to the sup obtained from 10 mM CHAPS-treated microsomes after 20,000 x g, 15 min-centrifugation, and the mixtures were re-centrifuged at 100,000 x g for 60 min.

Properties of solubilized Mg²⁺-ATPase: Microsomes, with EA highly sensitive and EA less sensitive Mg²⁺-ATPase activities of 3.0 and 7.1 μmol Pi/mg protein/hr, respectively, were solubilized using 10 mM CHAPS and 30 mM OcGlu. With the resulting preparation, the sensitivity to EA, stimulation by Cl⁻, pH dependency and affinity to ATP were examined. As presented in the Eadie-Scatchard plots of Fig. 2A, solubilized Mg²⁺-ATPases showed the activities with different sensitivities to EA, as observed in the activities of the original microsomes, with deflecting points between EA concentrations of 0.3 and 0.4 mM. Using 0.3 mM EA, specific activities of the solubilized EA highly sensitive and EA less sensitive Mg²⁺-ATPases were determined to be 4.6 and 1.6 μmol Pi/mg protein/hr, respectively.

Solubilized EA highly sensitive Mg²⁺-ATPase was stimulated by Cl⁻ with the half maximum stimulation points over 30 mM to a level 2.5 times higher than the control (Fig. 2B). In contrast, EA less sensitive Mg²⁺-ATPase was not affected by Cl⁻.

The maximum activity of solubilized EA highly sensitive Mg²⁺-ATPase was observed in the pH range of 6.6–7.0 (Fig. 3A). EA less sensitive Mg²⁺-ATPase activity was higher in the lower pH range (pH 6.2–7.0). From Lineweaver-Burk plots of the reciprocal of reaction velocity against the reciprocal of ATP concentration (Fig. 3B), Kₘ values for ATP of the solubilized EA highly sensitive and EA less sensitive Mg²⁺-ATPases were estimated to be 0.29 and 1.3 mM, respectively.
Fig. 5. Elution patterns in re-chromatography of Fractions I (A) and II (B). In diagram A, elution with a linear NaCl gradient (0–0.2 M) yielded a sharp protein peak with EA less sensitive Mg\textsuperscript{2+}-ATPase activity (○), but not the EA highly sensitive one (●). A sample containing 2 mg of protein was applied. In diagram B, elution with a linear NaCl gradient (0.2–0.45 M) yielded a protein peak with EA highly sensitive Mg\textsuperscript{2+}-ATPase activity (●), but not the EA less sensitive one (○). A sample containing 1 mg of protein was applied. Front peaks and the following high level absorbance at 280 nm are due to the mechanical absorption (See Text).

protein bands with a concentrated one near the 67 kDa protein band. On the other hand, fractions of EA highly sensitive Mg\textsuperscript{2+}-ATPase after FPLC (Fraction II, lanes 8 and 9) produced a prominent band below the 669 kDa protein band. With the five standard proteins of known molecular weight (Lane 1), the distance of each band from the baseline (Rf) was plotted against the molecular weight (Fig. 6B). The plots showed a good linear fit. From the Rf values for the concentrated bands in the FPLC eluates, the molecular weights of the proteins concentrated in the fractions of EA less sensitive and EA highly sensitive Mg\textsuperscript{2+}-ATPase were estimated to be approximately 70 and 600 kDa, respectively.

Discussion

As far as we know, brain microsomal Mg\textsuperscript{2+}-ATPases were first solubilized in this study. Bile salts such as cholate and deoxycholate have been used in the solubilization of ion translocating ATPases (1); however, these anionic detergents are known to interfere with the adsorption of enzyme proteins by the anion exchanger column. In the present study, two non-ionic detergents, CHAPS...
and OcGlu, were found to be suitable for the solubilization of Mg\textsuperscript{2+}-ATPases in the brain microsomes. The combined use of these detergents favored the effective solubilization and subsequent anion exchanger column chromatography of the enzymes. The superiority of the detergent combination has been reported in the solubilization of ATPases from renal brush-border membranes (16) and thermophilic cyanobacteria (17).

As previously described (10, 12), Mg\textsuperscript{2+}-ATPases in the microsomes were inhibited by EA biphasically with a deflecting point at 0.3–0.4 mM. In the present study, the solubilized Mg\textsuperscript{2+}-ATPases exhibited these properties with respect to the sensitivity to EA. These results suggest that EA highly sensitive Mg\textsuperscript{2+}-ATPase is solubilized with the intact EA sensitive sites. Stimulation by Cl\textsuperscript{-} was observed only with EA highly sensitive Mg\textsuperscript{2+}-ATPase in the solubilized enzymes, as well as in the microsomes (12, 13). The extent of stimulation by Cl\textsuperscript{-} of the solubilized enzyme was comparable to that of the enzyme in the microsomes. However, the concentration of Cl\textsuperscript{-} required for the half maximum stimulation in the former enzyme (over 30 mM) was higher than that in the latter enzyme (5 mM). Thus, the procedures for solubilization are considered to reduce the affinity of the enzyme to Cl\textsuperscript{-}. The activity of EA highly sensitive Mg\textsuperscript{2+}-ATPase in the microsomes showed the highest value at pH 7.4, and EA less sensitive Mg\textsuperscript{2+}-ATPase showed the highest activity at pH 6.2–6.6. Furthermore, the K\textsubscript{m} value for ATP of EA highly sensitive Mg\textsuperscript{2+}-ATPase was slightly lower in the solubilized enzyme (0.29 mM) than that in the microsomes (0.77 mM), while the K\textsubscript{m} values of EA less sensitive Mg\textsuperscript{2+}-ATPase did not change by the solubilization (1.3 mM in the solubilized enzyme and 1.6 mM in the microsomes). The solubilization-induced changes in the enzymatic parameters are probably due to factors influencing the enzyme such as adjacent lipid and/or enzyme conformation.

Separation of the solubilized Mg\textsuperscript{2+}-ATPases was performed using the FPLC with a Mono Q column. In the preliminary experiments (18), we employed a DEAE column for separation of the enzymes. The
solubilized Mg\textsuperscript{2+}-ATPases were adsorbed by the column and recovered in the eluates; however, separation of the peaks of enzyme activities was insufficient. In the present work, rapid and good separation of the peaks of two Mg\textsuperscript{2+}-ATPase activities was accomplished by the new chromatography system. Thus, the non-mitochondrial and anion-sensitive Mg\textsuperscript{2+}-ATPase in the brain was separated from the anion-insensitive enzyme, suggesting that these Mg\textsuperscript{2+}-ATPases behave as distinct enzymes in the neuronal plasma membranes. As mentioned above, the specific activity of EA highly sensitive Mg\textsuperscript{2+}-ATPase, after re-chromatography was reduced to one fourth that in the crude microsomes. Specific activity of EA less sensitive Mg\textsuperscript{2+}-ATPase after re-chromatography was slightly increased to about 1.25-fold that in the crude microsomes. Both enzymes, especially EA highly sensitive Mg\textsuperscript{2+}-ATPase seemed to be unstable after re-chromatography.

Polyacrylamide gel electrophoresis of the peak fraction of EA highly sensitive or EA less sensitive Mg\textsuperscript{2+}-ATPase yielded a concentrated protein band at approximately 600 or 70 kDa, respectively. In our previous study using the radiation inactivation method (13), functional molecular sizes of EA highly sensitive and EA less sensitive Mg\textsuperscript{2+}-ATPases in the microsomes were estimated to be 480 and 80 kDa, respectively. Taking these molecular sizes into account, the 600 kDa protein concentrated in the EA highly sensitive Mg\textsuperscript{2+}-ATPase fraction may be the complex of functional protein and accessory structures, while the 70 kDa protein concentrated in the EA less sensitive Mg\textsuperscript{2+}-ATPase fraction probably represents the functional unit of EA less sensitive Mg\textsuperscript{2+}-ATPase.

Two distinct Mg\textsuperscript{2+}-ATPases with different functions were demonstrated in the adrenal chromaffin granules (19, 20), that is a 460 kDa protein with H\textsuperscript{+}-translocating activity and a 14.1 kDa protein whose function is unknown. Separation and characterization of the neuronal plasma membrane Mg\textsuperscript{2+}-ATPases will also contribute to studies on neuronal membrane functions, including the anion-dependent process.

Acknowledgement: The authors are grateful to Merck, Sharp & Dohme Research Laboratories (West Point, PA, U.S.A.) for the gift of ethacrynic acid. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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