Characteristics of Ethacrynic Acid Highly Sensitive Mg\textsuperscript{2+}-ATPase in Microsomal Fractions of the Rat Brain: Functional Molecular Size, Inhibition by SITS and Stimulation by CI\textsuperscript{-}

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Abstract—Studies were performed to characterize ethacrynic acid (EA) highly sensitive Mg\textsuperscript{2+}-ATPase isolated from microsomal fractions of the rat brain. The functional molecular sizes of the EA highly sensitive and EA less sensitive Mg\textsuperscript{2+}-ATPases, estimated by a radiation inactivation method, were 480 and 80 kDa, respectively. An anion transport inhibitor, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS) inhibited the EA highly sensitive Mg\textsuperscript{2+}-ATPase activity. The type of inhibition was uncompetitive with respect to ATP, and the inhibition was suppressed by anions such as CI\textsuperscript{-}, Br\textsuperscript{-} and I\textsuperscript{-}. Chloride ions stimulated enzyme activity with an increase in V\textsubscript{max}, but not in K\textsubscript{m}, for ATP. Anions tested also increased the enzyme activity in the following order of decreasing potency: CI\textsuperscript{-}> Br\textsuperscript{-}>CH\textsubscript{3}COO\textsuperscript{-}=I\textsuperscript{-}>SO\textsubscript{4}\textsuperscript{2-}=HCO\textsubscript{3}->SO\textsubscript{3}\textsuperscript{2-}. These results suggest that EA highly sensitive Mg\textsuperscript{2+}-ATPase is a relatively large molecule with anion-sensitive sites that affect the ATP hydrolyzing activity and the SITS binding capacity through anions, with CI\textsuperscript{-} being the most potent.

Since Durbin and Kasbekar (1) first reported that HCO\textsubscript{3}\textsuperscript{-}-stimulated Mg\textsuperscript{2+}-ATPase in the gastric mucosa played a role in anion transport across the cell membrane, non-mitochondrial anion-sensitive Mg\textsuperscript{2+}-ATPase has occupied the predominant focus of attention as a candidate for the anion-translocating system (2, 3). Such enzyme activities have been shown in erythrocytes (4–6), renal and intestinal brush border membranes (7–9), chromaffin granules (10), pituitary secretory granules (11), electric organ synaptic vesicles (12) and gastric pepsinogen granules (13).

We found that ethacrynic acid (EA) highly sensitive Mg\textsuperscript{2+}-ATPase in the rabbit and rat brains possesses a novel anion-sensitive Mg\textsuperscript{2+}-ATPase activity that is located mainly in the plasma membrane (14–16). The properties of the enzyme have been partially described (16), but information is still limited.

A radiation inactivation method has been used to determine the molecular sizes of other membrane-bound enzymes in their intact membrane-bound state (17–19). Therefore, we applied this method to the EA highly sensitive Mg\textsuperscript{2+}-ATPase. Furthermore, inhibition by anion transport inhibitors and stimulation by CI\textsuperscript{-} were analyzed kinetically to characterize the anion sensitive sites of the enzyme.

Materials and Methods

Materials: Ethacrynic acid (kindly provided by Merck, Sharp & Dohme Research Laboratories) was alkalinized to pH 7.4 with Tris for dissolution. Reagents used were as
follows: ouabain, ATP-Na$_2$, ethylenediamine-tetraacetic acid (EDTA, Sigma), 4-morpholine-ethansulfonic acid (MES, Wako Chem., Osaka) and 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS, ICN Nutr. Biochem., Cleveland). The following four purified enzymes were used as standards: horse liver alcohol dehydrogenase (ADH) and yeast ADH were obtained from P.L. Biochemicals Inc., Milwaukee, and rabbit muscle pyruvate kinase and E. coli $\beta$-galactosidase from Sigma.

**Enzyme preparation:** Brains were removed from Wistar rats and kidneys were removed from guinea pigs after exsanguination. The bloodless tissues were stored at $-70^\circ$C until use. At the time of experiments the tissues were thawed and homogenized in 8 vol. of ice-cold buffer solution containing 0.25 M sucrose, 1 mM EDTA and 12.5 mM Tris-acetate (pH 7.4), and the homogenates were centrifuged (10,000×g, 15 min; 92,000×g, 60 min). The final pellets were suspended in 5 mM EDTA-Tris (pH 7.4) and used as microsomal fractions for radiation inactivation analysis. To obtain EDTA-treated microsomes, the same pellets suspended in 5 mM EDTA-Tris were stirred for 30 min at 4°C, and then centrifuged at 9,000×g for 30 min. The supernatant fraction was brought to a 30% saturation point by the addition of ammonium sulfate, stirred for 30 min at 4°C and centrifuged at 9,000×g for 30 min. The supernatant fraction was brought to a 30% saturation point by the addition of ammonium sulfate, stirred for 30 min at 4°C and then centrifuged at 9,000×g for 30 min. The precipitates were resuspended in 5 mM EDTA-Tris (pH 7.4), dialyzed overnight against the same solution, and then centrifuged at 9,000×g for 30 min. The supernatant fractions were stored at $-70^\circ$C and used as the EDTA-treated microsomes. Further details of the procedures were described previously (16).

**Radiation inactivation procedures:** Radiation inactivation analysis was performed by the modified method of Lo et al. (20). Sucrose, at a final concentration of 0.25 M, was added to the membrane preparations (2–5 mg protein/ml) to protect the enzyme from denaturation during the course of lyophilization. The four purified enzymes of known molecular size were added to the membrane preparations as internal standards. Aliquots of the mixture were placed in Cryo tubes (Nunc) with rubber stoppers, lyophilized and then irradiated in vacuo. Irradiation was carried out using a Van de Graaff accelerator producing a 50 μA beam current of 1.5 MeV. The accelerator was routinely calibrated using a cellulose triacetate dosimeter. During irradiation, the samples were cooled to between $-80$ and $-90^\circ$C using a stream of liquid nitrogen, and were kept at a distance of 10 cm from the beam window. After irradiation, the samples were stored at $-80^\circ$C and resuspended in 5 mM EDTA-Tris (pH 7.4) at the time of enzyme assay. Non-irradiated, lyophilized samples were prepared as controls.

**Assay of enzyme activity:** ATPase activities were determined by spectrophotometric measurement of inorganic phosphate. Mg$^{2+}$-ATPase was assayed in 0.2 ml of either Tris-acetate or Tris-MES buffered medium. When Tris-acetate buffer was used, the medium contained 100 mM Tris-acetate (pH 7.4), 1 mM EDTA, 6 mM magnesium acetate, 6 mM ATP-Na$_2$, 1 mM ouabain and 20–40 μg of microsomal protein. When Tris-MES was used, the medium contained 25 mM Tris-MES (pH 7.4), 1 mM EDTA, 6 mM Mg(OH)$_2$-MES, 6 mM ATP-Tris, 1 mM ouabain and 20–40 μg of microsomal protein. Mg$^{2+}$-ATPase activity found in the presence of 0.3 mM EA was designated as EA less sensitive Mg$^{2+}$-ATPase activity. The difference between the Mg$^{2+}$-ATPase activities in the presence and absence of 0.3 mM EA was labelled as the EA highly sensitive Mg$^{2+}$-ATPase activity. Na$^+$,K$^+$-ATPase activity was measured as ouabain-sensitive ATPase activity using a reaction mixture containing 100 mM Tris-acetate (pH 7.4), 1 mM EDTA, 6 mM magnesium acetate, 6 mM ATP-Na$_2$, 100 mM NaCl, 10 mM KCl and 20–40 μg of microsomal protein with or without 1 mM ouabain. After a 5 min-preincubation at 37°C, the reaction was started by the addition of ATP, sustained for 10–30 min at 37°C, and was stopped by adding 10% trichloroacetic acid. The mixtures were cooled in ice and centrifuged at 3,000×g for 5 min. An aliquot (0.3 ml) of each supernatant was assayed for liberated inorganic phosphate (21).

Horse liver ADH and yeast ADH were assayed by the method of Vallee and Hoch (22). Rabbit muscle pyruvate kinase and
E. coli β-galactosidase were assayed as described by Bücher and Pfeiderer (23) and Lederberg (24), respectively. Protein concentration was determined according to the method of Lowry et al. (25) using bovine serum albumin as a standard.

Results

Estimation of molecular size: The radiation inactivation method was used to determine the molecular sizes of EA highly sensitive and EA less sensitive Mg$^{2+}$-ATPases in the rat brain. The inactivation of Na$^+,K^+$-ATPases in the same preparations and in the guinea pig kidneys were also measured, as internal standards of intrinsic membrane proteins. As shown in Fig. 1, the activities of the four ATPases declined with the increase in total radiation dose (Mrad) following single-exponential lines, which indicated that the enzymatically active unit of each enzyme had a single class of molecular size. The sensitivity of the ATPases to EA and ouabain did not change during lyophilization or irradiation (data not shown). Sensitivity to the radiation was expressed by the D$_{37}$ value, i.e. the dose required to achieve 37% survival, as described by Kepner and Macey (17). The D$_{37}$ values for EA highly sensitive and EA less sensitive Mg$^{2+}$-ATPases and Na$^+,K^+$-ATPases in the rat brain and guinea pig kidney were 5.3, 19.9, 10.3 and 7.7, respectively (Fig. 1). The D$_{37}$ values for the four standard enzymes were as follows: liver ADH, 19.3 Mrad; yeast ADH, 12.3 Mrad; pyruvate kinase, 10.4 Mrad; β-galactosidase, 5.5 Mrad. From these four D$_{37}$ values, the inactivation ratio relative to β-galactosidase (I$_o$) and molecular size of each enzyme (Mx) with respect to the size of β-galactosidase (Mx/Mo, Mo: 464 kDa) were calculated, and I$_o$ of each enzyme was plotted against Mx/Mo (Fig. 2). The plots gave a good linear fit. The I$_o$ values for the four ATPases were also plotted on the least-squares slope of the line, and then the Mx/Mo of each ATPase was obtained. The molecular sizes of EA highly sensitive and EA less sensitive Mg$^{2+}$-ATPases were 480 and 80 kDa, respectively (Table 1); those of Na$^+,K^+$-ATPases in the rat brain and guinea pig kidney

![Fig. 1. Radiation dose-dependent inactivation of EA highly sensitive (○) and EA less sensitive (△) Mg$^{2+}$-ATPases in the rat brain and Na$^+,K^+$-ATPases in the rat brain (▲) and guinea pig (G.P.) kidney (△). Each point represents the mean value of 3–6 determinations, and each line was fitted by the least squares method.](image)

![Fig. 2. Determination of molecular sizes of EA highly sensitive (●) and EA less sensitive (○) Mg$^{2+}$-ATPases in the rat brain and Na$^+,K^+$-ATPases in the rat brain (▲) and guinea pig kidney (△). Ordinate (I$_o$): inactivation ratio relative to β-galactosidase (G). Abscissa (Mx/Mo): ratio of molecular size of each enzyme (Mx) to size of β-galactosidase (Mx: 464 kDa). The calibration line is plotted by using I$_o$ values of four standard enzymes of known molecular sizes: horse liver ADH (H: 84 kDa), yeast ADH (Y: 160 kDa), rabbit muscle pyruvate kinase (P: 224 kDa) and β-galactosidase. The least-squares slope of the line is 0.88. The abbreviations for the ATPases are the same as in Fig. 1.](image)
Table 1. Molecular sizes of Mg^{2+}-ATPases and Na^+,K^+-ATPases determined by radiation inactivation

| Enzymes                     | D_{37} (Mrad) | I_{0}   | M_{X}/M_{464} | M_{X} (kDa) |
|-----------------------------|---------------|---------|---------------|-------------|
| Mg^{2+}-ATPases (rat brain) |               |         |               |             |
| EA highly sensitive         | 5.3           | 1.041   | 1.034         | 480         |
| EA less sensitive           | 19.9          | 0.277   | 0.172         | 80          |
| Na^+,K^+-ATPases            |               |         |               |             |
| Rat brain                   | 10.3          | 0.532   | 0.517         | 240         |
| Guinea pig kidney           | 7.7           | 0.715   | 0.647         | 300         |

D_{37} value (Mrad): the dose required to achieve 37% survival. See the value on the abscissa in Fig. 1. 
I_{0}: inactivation ratio relative to β-galactosidase. This value corresponds to the reciprocal of the D_{37} value of each enzyme compared to the value (5.5 Mrad) of β-galactosidase. M_{X}/M_{464}: molecular size of each enzyme (M_{X}) compared to that of β-galactosidase (M_{464}: 464 kDa). See Fig. 2.

were 240 and 300 kDa, respectively, under the same experimental conditions.

**Inhibition by SITS:** As previously reported (16), SITS inhibits EA highly sensitive Mg^{2+}-ATPase with a K_i at 8 μM, after 5 min-preincubation and 10 min-incubation in the presence of the inhibitor. Reversibility of the SITS-induced inhibition was investigated by using the time dependency of the inhibition and by dilution. Figure 3 shows the time course of inactivation of EA highly sensitive Mg^{2+}-ATPase induced by 5 or 10 μM SITS. Both inactivation curves reached equilibrium in 5 min, suggesting that irreversible binding of SITS was unlikely. In a dilution method, the enzyme was incubated with 10 or 100 μM SITS in the reaction mixture without ATP at 37°C for 15 min, then the mixture was diluted 1:10 with the ATP-free reaction mixture. After letting the mixture stand at 0°C for 60 min, the enzyme was assayed for the ATPase activity at a 100 times lower concentration of the inhibitor. Parallel treatment and assay of the enzyme without SITS was done as a control. After dilution, the enzyme which had been preincubated with either 10 or 100 μM SITS recovered 100 or 85% of the control activity, respectively. These data suggest that SITS acts as a reversible inhibitor.

SITS inhibition was measured at various concentrations of ATP. Dixon plots (26) of the data yielded a set of parallel lines (Fig. 4A), suggesting that the type of inhibition by SITS is uncompetitive with respect to ATP.

The inhibitory effect of SITS was examined at different concentrations of NaCl. The slope of Dixon plots of the inhibition decreased with increasing concentrations of NaCl ranging from 12.5-50 mEq, and the inhibition appeared to be competitive with respect to NaCl (Fig. 4B). A similar inhibitory effect of SITS was observed when Na^+ was replaced with K^+ or Li^+ (Fig. 5A). When Cl^- was replaced with other halide ions, Br^- showed a similar inhibitory effect compared with Cl^- (Fig. 5B). I^- had a greater suppressive effect on the SITS inhibition, but F^- appeared to augment it.

**Stimulation by Cl^-:** Effects of NaCl on EA highly sensitive Mg^{2+}-ATPase activity were tested in Tris-MES buffers as well as in Tris-acetate buffers (Fig. 6). The enzyme activity assayed in Tris-acetate buffer was stimulated...
Fig. 4. Dixon plots of the inhibition by SITS in different concentrations of ATP (A) and NaCl (B). The reciprocal of EA highly sensitive Mg$^{2+}$-ATPase activity is plotted against the doses of SITS (0–10 μM). In diagram A, the ratio of ATP to Mg is 1:1. In diagram B, the concentration of ATP is 3 mM. Tris-acetate buffered mixtures were used for ATPase assays.

Fig. 5. Modified Dixon plots of the inhibition by SITS in the presence of monovalent cation-Cl salts (A: NaCl, KCl and LiCl) and Na-halide salts (B: NaF, NaCl, NaBr and NaI). The reciprocal of the ratio of EA highly sensitive Mg$^{2+}$-ATPase activity in the presence (vi) and absence (v) of SITS is plotted against the doses of SITS (0–10 μM). The final concentration of each salt was 50 mEq. Tris-acetate buffered mixtures were used for ATPase assays.

by concentrations of NaCl over 20 mEq up to a level 28% greater than in the absence of NaCl. In Tris-MES buffer, the activity in the absence of NaCl was about 60% of that in Tris-acetate buffer. This activity, however, was also increased by addition of NaCl up to the same maximum levels of Cl$^{-}$ stimulation observed in Tris-acetate buffer. To determine the effect of NaCl on the apparent Michaelis constant ($K_m$) and $V_{max}$ for ATP-Mg, the enzyme activities were assayed at various concentrations of ATP-Mg (1:1) in the presence and absence of NaCl. Lineweaver-Burk plots of the data revealed a $K_m$ of 0.7 mM and $V_{max}$ of 6.49 μmol Pi/mg protein/hr in the absence of NaCl (Fig. 7). In the presence of 20 mEq NaCl, an increase in $V_{max}$ (9.83 μmol Pi/mg protein/hr) was observed, but the $K_m$ value was not altered.

The effects of chloride salts of monovalent
cations and sodium salts of several anions on EA highly sensitive Mg\textsuperscript{2+}-ATPase activity was examined in Tris-MES buffer. As shown in Table 2, chloride salts of K\textsuperscript{+}, Li\textsuperscript{+} and choline all stimulated enzyme activity to similar extents as did NaCl. On the other hand, the sodium salts of anions other than Cl\textsuperscript{−} stimulated enzyme activity to lesser extents in order of decreasing potency: Cl\textsuperscript{−} > Br\textsuperscript{−} > CH\textsubscript{3}COO\textsuperscript{−} = I\textsuperscript{−} > SO\textsubscript{4}\textsuperscript{2−} > HCO\textsubscript{3}− > SO\textsubscript{3}^{2−}. Sodium salts of SCN\textsuperscript{−} and F\textsuperscript{−} had no significant effect on enzyme activity. In contrast, sodium salts of OCN\textsuperscript{−} and NO\textsubscript{3}− inhibited enzyme activity. Residual EA less sensitive Mg\textsuperscript{2+}-ATPase activity was unchanged by these anions.

**Discussion**

Radiation inactivation analysis has been found to be the most suitable method to estimate the molecular sizes of membrane bound enzymes and receptors in situ without

![Fig. 6. Effects of Cl\textsuperscript{−} (added as NaCl) on EA highly sensitive Mg\textsuperscript{2+}-ATPase activity in Tris-acetate (●) and Tris-MES (○) buffered mixtures. The assay conditions are described in "Materials and Methods".](image)

![Fig. 7. Lineweaver-Burk plots of EA highly sensitive Mg\textsuperscript{2+}-ATPase activity against the concentration of ATP in Tris-MES buffered mixtures with (●) and without (○) 20 mEq NaCl. The ratio of ATP to Mg is 1:1.](image)

**Table 2. Effects of salts on ethacrylic acid highly sensitive Mg\textsuperscript{2+}-ATPase activity**

| Salts         | ATPase activity (µmol Pi/mg protein/hr) | Ratio |
|---------------|----------------------------------------|-------|
| No addition   | 5.69±0.08                              | 1.00  |
| NaCl          | 9.67±0.04                              | 1.70  |
| KCl           | 9.67±0.02                              | 1.70  |
| LiCl          | 9.64±0.08                              | 1.69  |
| Choline Cl    | 9.59±0.05                              | 1.68  |
| NaBr          | 9.28±0.05                              | 1.63  |
| NaCH\textsubscript{3}COO | 8.23±0.03                        | 1.46  |
| NaI           | 8.27±0.16                              | 1.45  |
| Na\textsubscript{2}SO\textsubscript{4} | 8.00±0.05                        | 1.40  |
| NaHCO\textsubscript{3} | 8.00±0.03                        | 1.40  |
| Na\textsubscript{2}SO\textsubscript{3} | 7.27±0.22                        | 1.28  |
| NaSCN         | 5.61±0.09                              | 0.99  |
| NaF           | 5.55±0.08                              | 0.97  |
| NaOCN         | 5.01±0.17                              | 0.88  |
| NaNO\textsubscript{3} | 3.68±0.20                        | 0.65  |

Assay was performed in Tris-MES buffered mixture and each salt was tested at 10 mEq. The values are expressed as the mean±S.E. (n=3).
puriﬁcation (17–20). To calculate the target size from the D37 value, Kepner and Macey (17) employed an empirical equation with a correction factor of 6.4 × 10^{11}. However, since the D37 value is affected by experimental conditions such as temperature and dryness during the irradiation (18, 27), four pure enzymes of known molecular sizes were used in the present study, as previously suggested and described by Lo et al. (20). The D37 values for the pure enzymes used herein were about 1/2 of the values reported by them (20). The discrepancy in the values is probably due to the difference in temperature during irradiation, i.e. we used −80°C but they employed 30°C, and is represented by the D37(T)/D37(+30) ratio in the radiation inactivation (28). Parallel analysis of membrane-bound Na+,K+-ATPases from the rat brain and guinea pig kidney yielded the molecular sizes of 240 and 300 kDa, respectively. These data are consistent with the values for Na+,K+-ATPase reported using radiation inactivation analysis (17, 29–31).

Under these experimental conditions, the molecular sizes of EA highly sensitive and EA less sensitive Mg2+-ATPases were 480 and 80 kDa, respectively. These results indicate that the EA highly sensitive Mg2+-ATPase is distinct from EA less sensitive Mg2+-ATPase and Na+,K+-ATPase in the rat brain. A similarly large molecular size was reported for H+,K+-ATPase from the porcine gastric mucosa (31). The molecular size of EA less sensitive Mg2+-ATPase was relatively small, and was comparable to that of red cell alkaline phosphatase (70 kDa) measured in the absence of K+ (17).

It has been reported that stilbene derivatives such as SITS, which are amino reactive inhibitors of anion transport, are bound reversibly or irreversibly to the transport system (32–35). In the present study, SITS inhibited the EA highly sensitive Mg2+-ATPase activity by reversibly binding to it (Fig. 3). In addition, an uncompetitive type of inhibition by SITS was observed with respect to ATP (Fig. 4A). Therefore, SITS may preferentially bind to the enzyme-ATP complex.

Sodium chloride at concentrations over 12.5 mEq suppressed the SITS-induced inhibition of EA highly sensitive Mg2+-ATPase activity (Fig. 4B). Since a competitive type of inhibition was found in the Dixon plots, NaCl and SITS may compete for a common binding site that affects the ATPase activity. In addition, the suppressive effect on the SITS-induced inhibition was dependent upon the anions tested, but not upon the monovalent cations (Fig. 5). Suppression by the anions occurred, in order of decreasing potency: I−>Br−>Cl−, but F− enhanced the SITS-induced inhibition. A similar order has been reported in the case of binding of the halide ions to human erythrocytes (36). Levine et al. (37) proposed that the observed halide sequences inversely correlate with the hydration energy of the anions. Thus, the suppressive effects of anions on the SITS inhibition may be mediated by anion binding with free energies comparable to the ion’s hydration energies.

EA highly sensitive Mg2+-ATPase activity was stimulated by several anions in Tris-MES buffer, in the following order of decreasing potency: Cl−>Br−>CH3COO−= I−>SO42−>HCO3−>SO32−, and the activity was stimulated to a similar extent by chloride salts of Na+, K+, Li+ and choline. H+,K+-ATPase is preferentially stimulated by K+, but not by Na+ (38, 39). Therefore, EA highly sensitive Mg2+-ATPase is an enzyme with activity different from that of H+,K+-ATPase. The use of MES as a buffer anion with a less stimulatory effect on EA highly sensitive Mg2+-ATPase enabled us to detect the stimulatory effects of several anions. Mitochondrial Mg2+-ATPase was reportedly stimulated by SO42− and HCO3−, but not by Cl− (40–42). Non-mitochondrial anion-sensitive Mg2+-ATPases in several tissues (4–13) were also stimulated to a greater extent by SO42− and HCO3− than Cl−. Thus, the marked stimulation by Cl− rather than HCO3− and SO42− represents a characteristic of EA highly sensitive Mg2+-ATPase. Chloride ion had no effect on the K_m for ATP during the Cl−-induced stimulation of EA highly sensitive Mg2+-ATPase activity (Fig. 7), suggesting that the affinity of the enzyme to ATP is not changed by the anion. During the HCO3−-induced stimulation of Mg2+-ATPases, the anion stimulated the enzyme with (11, 12,
41, 42) or without (9) changes in $K_m$ value for ATP.

From these results, it is suggested that EA highly sensitive Mg$^{2+}$-ATPase is a large molecule with a functional molecular size of 480 kDa, and that SITS and Cl$^-$ (as the most potent anion) compete with the anion-sensitive sites that regulate ATP hydrolyzing activity of the enzyme.

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