PURIFICATION OF AN ACTIVATOR OF HUMAN ERYTHROCYTE MEMBRANE (Ca^{2+}+Mg^{2+})ATPase

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Summary: An activator of human erythrocyte membrane (Ca^{2+}+Mg^{2+})ATPase has been isolated in highly purified form from membrane free hemolysate through a combination of carboxymethyl Sephadex chromatography and preparative polyacrylamide gel electrophoresis. This protein was found to have a highly negative charge and migrated as a single fast moving band on analytical polyacrylamide gel electrophoresis. It has a specific activity twelve hundred fold higher than that of the membrane free hemolysate.

Introduction

The occurrence of a soluble activator of (Ca^{2+}+Mg^{2+})ATPase of human erythrocyte membrane has been reported earlier by Bond and Clough (1) and Luthra et al. (2). Its partial purification has been reported earlier from this laboratory (2). The presence of this activator in other mammalian erythrocytes, reticulocytes, and various density (age) erythrocytes was reported (3). This communication describes the purification of this activator, from human erythrocytes, to apparent homogeneity.

MATERIALS

Carboxymethyl Sephadex (C50) was purchased from Pharmacia (Piscataway, New Jersey). Polyacrylamide and N,N'-methylene bisacrylamide were products of Eastman Kodak Co. (Rochester, New York) and both were recrystallized from chloroform and acetone, respectively. Saponin was purchased from Calbiochem (La Jolla, California) and was freed of Ca^{2+} and other contaminating metal ions by passage through AG50-WX8, 200-400 mesh, cation exchanger obtained from BioRad (Richmond, California). Ammonium persulfate, disodium ATP, Tris, histidine and other chemicals were from Sigma Chemical Co. (St. Louis, Missouri). PM10 Diaflo ultrafilters were products of Amicon Corporation (Lexington, Massachusetts).

METHODS

Preparation of hemolysate: Human blood was withdrawn from healthy adult donors and collected in heparinized tubes (Becton-Dickinson). Plasma and buffy coat were removed after centrifugation at 4,000 rpm at 4°C in a Sorvall RC-2B centrifuge using an SS-34 rotor. Packed cells were washed three successive times with a buffer, pH 7.6, containing 155 mM NaCl and 3 mM histidine. After the final wash, cells were resuspended in the same

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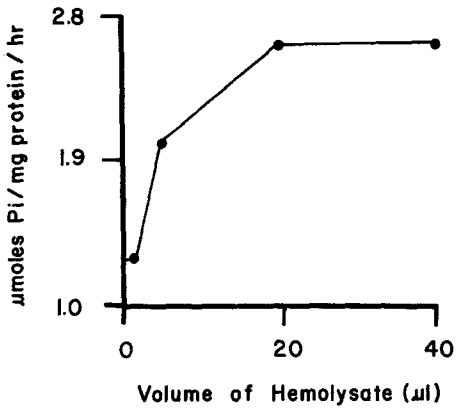

Figure 1. Influence of Human Erythrocyte Hemolysate on Membrane (Ca^{2+}+Mg^{2+}) ATPase

Membrane free hemolysate was isolated and concentrated as described in Methods. Varying amounts of concentrated hemolysate were added to membranes in the (Ca^{2+}+Mg^{2+})ATPase assay mixture and the activity was measured as described in Methods.

buffer to 5% hematocrit and were hemolyzed by addition of saponin at a final concentration of 0.1 mg saponin/ml cell suspension. In order to assure maximum hemolysis, the suspension was incubated at room temperature for 15 minutes and was considered the total hemolysate.

Preparation of membranes: The hemolysate was centrifuged for one hour at 19,000 rpm in a Sorvall RC-2B centrifuge using an SS-34 rotor. The supernatant was removed from the pellet by gentle aspiration and was used as starting material for the purification of (Ca^{2+}+Mg^{2+})ATPase activator. In a typical experiment, a pellet, representing 1 ml packed erythrocytes, was washed three separate times with 19 ml of a solution containing 155 mM NaCl, 3 mM histidine, and 2 mM tetrasodium-EDTA adjusted to pH 7.6 with HCl Excess EDTA was removed by three further washes with buffer containing 155 mM NaCl and 3 mM histidine only. The white, hemoglobin free membranes so obtained were resuspended in 155 mM NaCl, 3 mM histidine buffer, pH 7.6, to a (membrane) protein concentration of 1.4 mg/ml. This preparation was found to have a low Ca^{2+}+Mg^{2+}-ATPase activity. The results in Figure 1 demonstrate that (Ca^{2+}+Mg^{2+})ATPase of these membranes could be stimulated when crude membrane free hemolysate was added during the ATPase assay.

(Ca^{2+}+Mg^{2+})ATPase assay: Fifty µl of the above membrane suspension (70 µg protein) were incubated with and without activator in a total assay volume of 0.60 ml, at a final concentration of 33 mM KCl, 80 mM NaCl, 0.05 mM $CaCl_2$, 80 mM histidine, 3.6 mM $MgCl_2$ and 2.5 mM ATP. The reaction was conducted at 44°C and stopped after 2 hours by the addition of 1 ml of 10% cold trichloroacetic acid. The tubes were vortexed and kept in ice for 15 minutes and then centrifuged at 3,000 rpm in a Sorvall GLC-2B swinging bucket centrifuge. One ml of protein free supernatant was removed for inorganic phosphorus analysis by the method of Fiske and Subbarow (4). (Ca^{2+}+Mg^{2+})ATPase activity was expressed as µmoles Pi released/mg membrane protein/hour. The increase in the enzyme activity due to an activator was measured by the addition of varying amounts of the activator in the assay medium. One unit of an activator was equivalent to a 50% increase in (Ca^{2+}+Mg^{2+})ATPase activity of the above described membranes.

Disc gel electrophoresis: Disc gel electrophoresis of various activator fractions was performed on 58 mm gels made of 7.5% polyacrylamide and 0.25% bisacrylamide. The electrophoresis was conducted between buffer of pH 8.1 and pH 8.9 at constant current of 2 m Amps/gel in a Buchler analytical gel electrophoresis by the method of Ornstein and Davis (5). The gels were stained with Coomassie blue and destained by the method of Fairbanks et al. (6). Unless otherwise stated, protein was measured by the method of Lowry et al. (7).

RESULTS

Purification of An Activator of $(Ca^{2+}+Mg^{2+})$ATPase. A two-step procedure was developed for the purification of an activator of the $(Ca^{2+}+Mg^{2+})$ATPase of human erythrocyte membranes. The details of these steps are as follows:

STEP I: Carboxymethyl Sephadex Column Chromatography

In a typical preparation, washed erythrocytes (50 ml packed cells) were hemolyzed using saponin. The membrane free hemolysate, (approximately 825 ml), was collected as described in the Methods section and then concentrated to 135 ml by ultrafiltration through a PM10 filter under nitrogen pressure. This latter concentrate was dialyzed against 5 liters of 20 mM Tris-maleate buffer, pH 6.7, at $4^{\circ}C$, and then loaded onto a column (2.5 cm x 60 cm) of carboxymethyl Sephadex equilibrated in 20 mM Tris-maleate buffer, pH 6.7, at room temperature. The sample to carboxymethyl Sephadex ratio used here was approximately 10 times greater than that given in the method reported earlier for a small scale partial purification (2). The eluate was collected until the first hemoglobin band had moved close to the bottom of the column. This fraction was usually yellow in color and sometimes contained few white membrane fragments which could be removed by centrifugation at 19,000 rpm for 1 hour. Under these loading conditions, i.e., a concentrated sample on carboxymethyl Sephadex, resolution of the activator from other protein was less than noted before (2) and resulted in a recovery of only 70-80% of the applied activator (Table I). The fraction obtained after carboxymethyl Sephadex chromatography was reduced to 30 ml and dialyzed against 4 liters of 50 mM Tris-HCl, pH 7.6, overnight and designated as the post-CM Sephadex fraction.

TABLE I

Purification of Ca^{2+}+Mg^{2+} ATPase Activator

From Human Erythrocytes

Fraction	Total Protein (mg)	Total Units*	*Units/mg Protein	Fold Purification	% Recovery
Hemolysate	21,732#	325980	15	0	100
Post CM Sephadex	1,565	247270	158	10.5	76
Post Preparative Polyacrylamide Gel Electrophoresis	0.200	3703	18518	1234	1.1

*One unit is equivalent to 50% increase in Ca^{++}-Mg^{++}-ATPase activity of isolated red cell membranes.

#Value equivalent to hemoglobin measured by the method of Kachmar (8). An identical sample gave a value of 104390 when measured by the method of Lowry et al. (7).

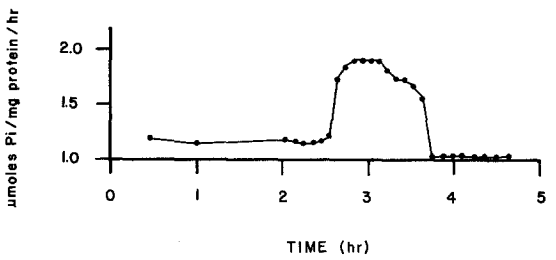

Figure 2. Elution Profile of $(Ca^{2+}+Mg^{2+})$ATPase Activator on Preparative Polyacrylamide Gel Electrophoresis

Electrophoresis was conducted as described in the text. Aliquots of 0.1 ml were withdrawn from each eluate (vol. 6 ml), added into the $(Ca^{2+}+Mg^{2+})$ATPase assay mixture and activity was measured as described in Methods.

STEP II: Preparative Polyacrylamide Gel Electrophoresis

The polyacrylamide gel used here was composed of 7.5% acrylamide and 0.25% bisacrylamide. The gel mixture (80 ml) containing appropriate catalysts (5) was funneled into a Buchler preparative polyacrylamide gel electrophoresis apparatus model Poly-Prep 200. The gel was allowed to set for one hour and then the top was filled with upper buffer containing 0.052 M Tris-0.052 M glycine, pH 8.9. The bottom buffer used was 0.1 M Tris-HCl, pH 8.1. The entire apparatus was kept at 6^{o} and the gel was pre-run for 8 hours at a constant current of 50 m Amps with continuous flow of upper, lower and elution buffers. Elution buffer used was a four-times diluted mixture of the lower buffer. The post-CM Sephadex fraction was mixed with pure sucrose to a final concentration of 20% and then was gently layered on top of the gel. Electrophoresis was conducted at a constant current of 50 m Amps. Fractions were collected at 3 minute intervals. Aliquots were taken from each fraction for assay $(Ca^{2+}+Mg^{2+})$ATPase activator and for checking electrophoretic purity by disc gel electrophoresis. The activator was eluted from the gel within 3 to 4 hours of electrophoresis, suggesting its highly negative character. Usually only the front running portion of the activator peak contained a single protein band, while the slower running fraction contained some minor protein contaminants. Further

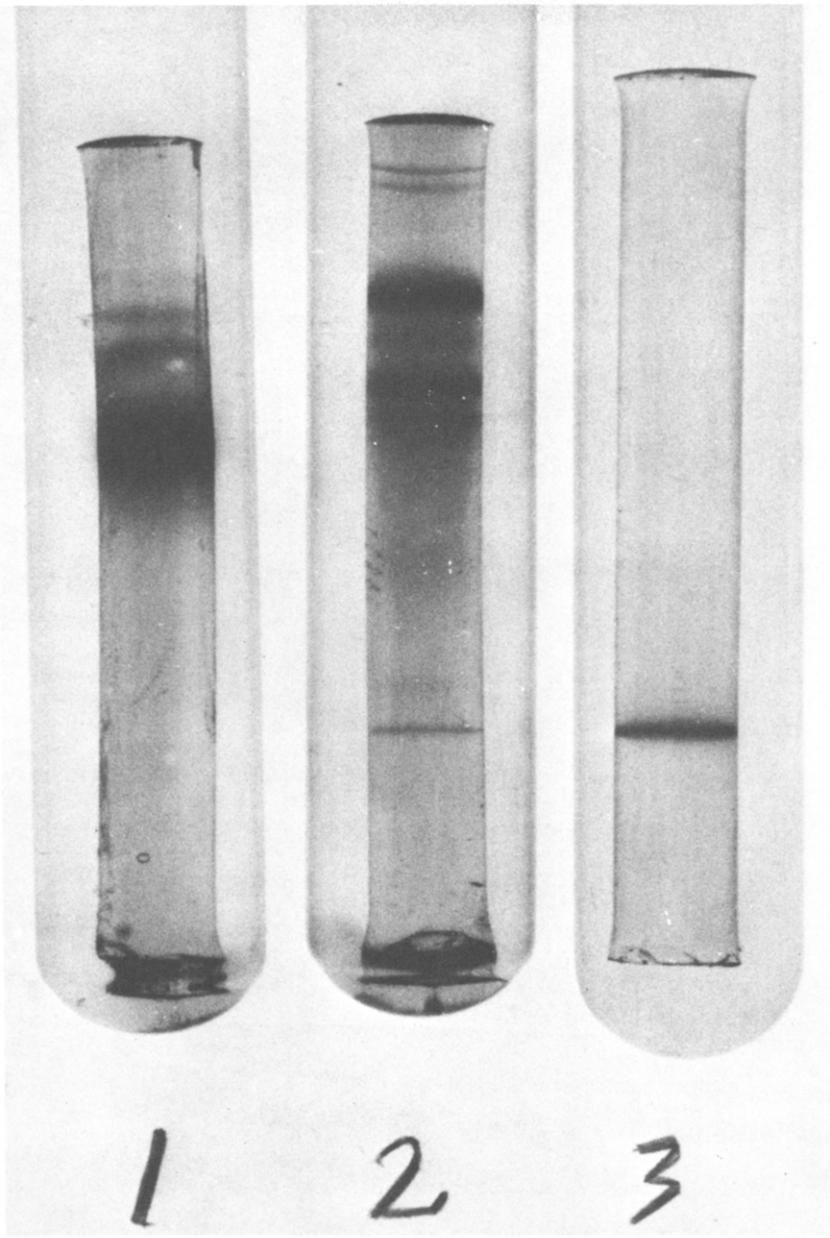

Figure 3. Gel Electrophoretic Pattern of Activator at Various Stages of Purification

Samples at various steps of purification were subjected to analytical disc gel electrophoresis as described in Methods. 1 = membrane free hemolysate, 2 = post-CM Sephadex fraction and 3 = pure activator after preparative gel electrophoresis.

purification of the slightly contaminated activator was achieved by subjecting it to a second preparative polyacrylamide gel electrophoresis under conditions described above. A typical profile of the latter run is shown in Figure 2. After testing the purity of each eluate by disc gel electrophoresis the fractions were combined. The polyacrylamide electrophoretic pattern of the pooled fractions showed only a single band with very high mobility towards the positive charge. Figure 3 illustrates the electrophoretic patterns of the activator at various stages of its purification. It should be noted that the activator band in the membrane free hemolysate was usually not visible.

In order to establish that the $(Ca^{2+}+Mg^{2+})$ATPase activator was in fact the single band visualized on gel, the following experiment was conducted. Two analytical gels on the purified activator were run simultaneously with one gel stained with Coomassie blue and the other frozen and sliced into 2 mm sections. These slices were incubated separately at room temperature in the ATPase assay medium without added membranes. Then, 50 µl of the membrane suspension (70 µg protein) were added to the tubes containing various slices and assayed for ATPase activity at 44^{o}. As can be seen in Figure 4, the largest stimulatory effect on membrane $(Ca^{2+}+M^{2+})$ATPase activity was observed with slices corresponding to the region where a single Coomassie blue staining band was seen.

Table I summarizes the recoveries and fold purification of the activator in a typical experiment. The maximum recovery of pure activator from 50 ml pooled erythrocytes after two preparative gel electrophoresis runs ranged from 200 to 300 µg. The specific activity of this material was 1200 fold higher than the total membrane free hemolysate activity. On the other hand total units recovered as purified activator were only 2-3% of the starting material. The largest loss of activator units was encountered at the electrophoresis step (Table I). The influence of electrophoresis or the stability of the activator when it is in the highly purified form was not

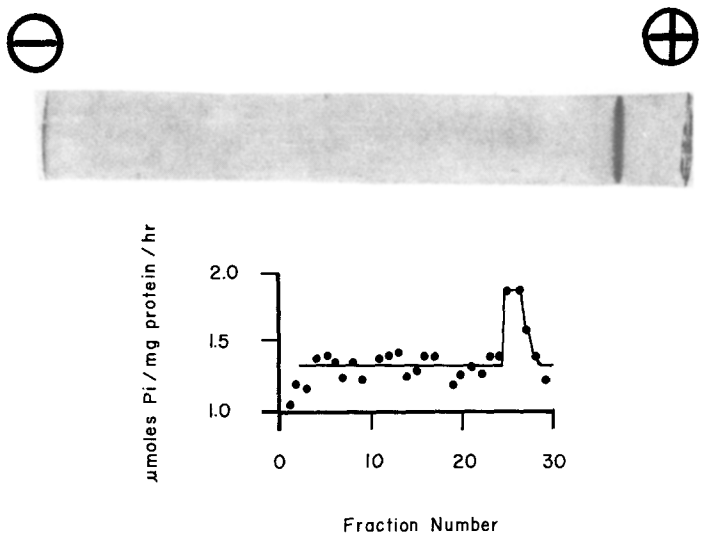

Figure 4. Location of $(Ca^{2+}+Mg^{2+})$ATPase Activator on Disc Gel after Electrophoresis

50 µg of pure activator obtained after preparative polyacrylamide gel electrophoresis was subjected to analytical disc gel electrophoresis as described in Methods. One gel was stained with Coomassie blue and other gel was frozen and sliced. Slices were eluted with ATPase assay medium and the activity of $(Ca^{2+}+Mg^{2+})$ATPase activator was measured as described in Methods. Membrane $(Ca^{2+}+Mg^{2+})$ATPase activity, without any exposure to polyacrylamide, was 0.9 µmole Pi/mg protein/hour.

investigated. This pure activator, however, was able to saturate the $(Ca^{2+}+Mg^{2+})$ATPase activity of 70 µg protein equivalent membranes when present in concentration of as little as 400 ng/0.6 ml of ATPase assay medium.

DISCUSSION

This communication describes for the first time the purification to apparent homogeneity of a protein capable of activating the $Ca^{2+}+Mg^{2+}$-ATPase activity of human erythrocyte membrane. This purified activator was obtained by a simple two step procedure in which a carboxymethyl Sephadex chromatography was used first, and then preparative polyacrylamide gel electrophoresis. This protein migrated as a single, Coomassie blue staining band on analytical disc gel electrophoresis with an exceptionally high mobility towards the cathode. This result obviously suggested a negatively charged

molecule and this idea was consistent with the observations that the activator did not bind to CM-Sephadex at pH 6.7 but did bind to DEAE-cellulose (DE-52) at pH 6.7. However, the activator could only be eluted from the latter column at high concentrations of salt, i.e., 0.5 M Tris-maleate buffer, pH 6.7 (unpublished experiments). Even though DEAE-cellulose chromatography provided a high degree of purification of the Ca^{2+}+Mg^{2+}-ATPase activator, the preparation still contained three other minor, contaminating proteins as judged by polyacrylamide gel electrophoresis. Recently, Jarrett and Penniston (9) briefly described similar observations and found that the activator bound tightly to DEAE-cellulose, eluted only in a high salt concentration and showed four bands on SDS gel electrophoresis. Consequently, the application of a preparative gel electrophoretic separation step provided the desired purification of this activator in our study.

Preliminary observations (unpublished) on the amino acid composition of the purified activator revealed a high content of glutamic acid (17%) and aspartic acid (14) and relatively low levels of lysine (4%) and arginine (5%). This again is consistent with the highly acidic nature of the activator as noted above. Other preliminary studies have shown that the minimal molecular weight of the purified activator was approximately 16,000 as judged by SDS gel electrophoresis (unpublished).

The role of this Ca^{2+}+Mg^{2+}-ATPase activator in the regulation of ATP-ase activity and hence potentially in controlling the Ca^{2+} flux in the erythrocytes is under active investigation.

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REFERENCES

1. Bond, G. H. and Clough, D. L. (1973) Biochim. Biophys. Acta 323, 592-599.
2. Luthra, M. G., Hildenbrandt, G. R., and Hanahan, D. J. (1976) Biochim. Biophys. Acta 491, 164-179.
3. Luthra, M. G., Hildenbrandt, G. R., Kim, H. D., and Hanahan, D. J. (1976) Biochim. Biophys. Acta 491, 180-186.

4. Fiske, H. and Subbarow, J. (1925) J. Biol. Chem. 66, 375-400.
5. Ornstein, L. and Davis, B. J. (1964) Ann. N. Y. Acad. Sci. 121, 404-427.
6. Fairbanks, H., Steck, A., and Wallach, E. E. (1971) Biochemistry 10, 2606-2617.
7. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
8. Kachmar, J. F. (1970) in Fundamentals of Clinical Chemistry (Tietz, N., ed.) pp. 268-269, W. B. Saunders, Philadelphia, Pa.
9. Jarrett, H. W. and Penniston, J. T. (1977) Fed. Proc. 36, 642.