Purification of the \((\text{Ca}^{2+}-\text{Mg}^{2+})\)-ATPase from IIuman Erythrocyte Membranes Using a Calmodulin Affinity Column*

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The \((\text{Ca}^{2+}-\text{Mg}^{2+})\)-ATPase from human erythrocyte membranes has been solubilized in Triton X-100 and purified on a calmodulin affinity chromatography column in the presence of phosphatidylserine, to limit the inactivation of the enzyme. The enzyme was purified at least 150 times when compared with the original ghosts and showed a specific activity of 3.8 \(\mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}\). In sodium dodecyl sulfate-polyacrylamide gels, a single major band was visible at a position corresponding to a molecular weight of about 125,000; a minor band (11% of the total protein) was present at a position corresponding to \(M = 205,000\). Upon incubation of the purified preparation with \([\gamma^3\text{P}]\text{ATP}\), both bands were phosphorylated in proportion to their mass, suggesting that both were active forms of purified ATPase.

The \((\text{Ca}^{2+}-\text{Mg}^{2+})\)-ATPase of the erythrocyte membrane is generally accepted as the enzyme responsible for maintaining the concentration of \(\text{Ca}^{2+}\) inside the cell at levels much lower than in the environment (1). Molecular studies of the enzyme have been hindered by the fact that it represents only a minor fraction of the total protein of the membrane and by the fact that, in purification attempts, it co-eluted with Band 3, the most abundant protein component of the erythrocyte membrane. Isolation and purification attempts have nevertheless been made, with the aid of either Triton X-100 or deoxycholate (2-5), and have yielded partially purified soluble fractions of the specific activity of which was 20 to 150 times higher than in the starting membrane material. The specific activities reported for purified preparations ranged from 0.15 to 3.1 \(\mu\text{mol}\cdot \text{mg}^{-1} \cdot \text{min}^{-1}\), as compared with the usual value of 0.005 to 0.05 for whole membranes. The most highly purified form previously reported contained three proteins in approximately equal amounts; only one of these proteins was phosphorylated (4). Studies on the partially purified soluble enzyme have indicated that it has a molecular weight of between 125,000 and 150,000 (2, 4) and have established a specific requirement for acidic phospholipids (6). Starting from partially purified preparations, it has also been possible to reconstitute the (\(\text{Ca}^{2+}-\text{Mg}^{2+}\))-ATPase activity (2) and the transport of \(\text{Ca}^{2+}\) (5) in artificial phospholipid vesicles.

Calmodulin, the ubiquitous modulator of \(\text{Ca}^{2+}\) dependent functions, has been shown (7-9) to activate both the \(\text{Ca}^{2+}-\text{Mg}^{2+}\)-ATPase and ATP-dependent \(\text{Ca}^{2+}\) transport in whole erythrocyte membranes. The ability to interact with calmodulin is retained by the solubilized \((\text{Ca}^{2+}-\text{Mg}^{2+})\)-ATPase (10). This ability has been exploited in the present work. An affinity chromatography column containing calmodulin has been prepared, and the \(\text{Ca}^{2+}\)-dependent formation of a calmodulin-ATPase complex in the column has been used to separate the \((\text{Ca}^{2+}-\text{Mg}^{2+})\)-ATPase from the other membrane proteins present in the ghost solubilize. The specific activity of the enzyme purified in this way is at least 150 times higher than that of the original ghost membranes, and it consists primarily of a single phosphorylatable protein.

**MATERIALS AND METHODS**

All reagents were of the highest purity available. Phosphatidylserine and lecithin were grade I, obtained from Lipid Products, South Nutfield, Surrey. CNBr-activated Sepharose 4B was obtained from Pharmacia, Uppsala, Sweden. \([\gamma^3\text{P}]\text{ATP}\) was obtained from the Radiochemical Centre, Amersham, England. All steps of the purification were carried out at \(4^\circ\text{C}\) and all buffers were adjusted to the specified pH at \(4^\circ\text{C}\), unless otherwise specified.

**Preparation of Human Red Cell Membranes Deficient in Calmodulin**

Recently outdated blood obtained from the blood bank in a citrate/glucose buffer was centrifuged at 5800 \(\times g\) and washed twice in 5 volumes of \(130 \text{mM KCl, } 20 \text{mM Tris-Cl, pH 7.4.}\) The white cells were removed. Packed washed red blood cells were lysed in 5 volumes of 1 mM EDTA, 1 to 10 mM Tris-Cl, pH 7.4, and centrifuged 10 min at \(18,000 \times g\). The ghosts were washed five times in the hemolysis buffer and 4 times in 10 mM Tris-Cl, pH 7.4. The \((\text{Ca}^{2+}-\text{Mg}^{2+})\)-ATPase activity of the ghosts (0.4 \(\mu\text{mol ATP split/mg/h}\) at \(37^\circ\text{C}\)) was activated up to four times by addition of 1 \(\mu\text{g}\) of calmodulin/mg of ghost protein. The ghosts (2-5 mg/ml) were stored at \(-80^\circ\text{C}\).

**Solubilization of Ghosts in Triton X-100**

Ghosts, 4 mg/ml in 10 mM KCl, 10 mM Tris-Cl, 0.05 mM CaCl\(_2\), pH 7.4, were solubilized with 1 mg of Triton X-100/mg of protein at 5°C for 10 min, and were then centrifuged at 100,000 \(\times g\) for 35 min at \(2^\circ\text{C}\). The supernatant, about 0.5 mg of phosphatidylserine/ml was added. This mixture was concentrated to about one-fifth of the original volume in an Amicon (Lexington, Mass.) ultrafiltration chamber with a XM-100 A filter (exclusion limit, 100,000 daltons).

**Coupling of Calmodulin to Sepharose 4B**

CNBr-activated Sepharose 4B was swollen for 2 h at room temperature with gentle mixing. The resin was then washed with 10 bed volumes of the buffer used for the original suspension, and was finally reconditioned in 2 bed volumes of 0.5 mM aminothiol, pH 8.2, at room temperature. The mixture was again incubated for 16 h at 4°C with gentle mixing. The resin was then washed with several bed volumes of H\(_2\)O, alternating with 130 mM KCl, 10 mM Tris-Cl, pH 7.4.

**Affinity Chromatography Column**

To the concentrated Triton X-100 solubilize of red cell membranes, CaCl\(_2\) was added to a final concentration of 100 \(\mu\text{M}\). The solubilize was then applied to a

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1. The abbreviations used are: CNBr, cyanogen bromide; EGTA, ethylene glycol bis(\(\beta\)-aminoethyl ether)N,N',N'-tetraacetic acid; HEPES, N-(2-hydroxyethyl)-\(N\)-pipеразинейансульфоновская кислота; SDS, sodium dodecyl sulfate.
Purification of (Ca\(^{2+}\)·Mg\(^{2+}\))·ATPase from Erythrocyte Membranes

Sepharose 4B calmodulin column, which was equilibrated in Buffer A (130 mM KCl, 10 mM Hepes, pH 7.4, 0.5% Triton X-100, 0.05\% phosphatidylserine, 100 \(\mu \)M CaCl\(_2\), 20 \(\mu \)M phenylmethylsulfonyl fluoride). Transmittance at 280 nm was recorded by a LKD Uvicord. The column was washed with 2 to 3 bed volumes of Buffer A, until no further protein and no Ca\(^{2+}\)·Mg\(^{2+}\)·ATPase activity was eluted. Then the buffer was changed to Buffer B (same composition as Buffer A, but 5 mM EDTA instead of 100 \(\mu \)M CaCl\(_2\)). To the fractions eluted by EDTA, CaCl\(_2\) was added to a final concentration of 6 \(\mu \)M. All fractions were assayed immediately after column chromatography.

(Ca\(^{2+}\)·Mg\(^{2+}\))·ATPase assay—The reaction was followed spectrophotometrically in a mixture containing 50 \(\mu \)M CaCl\(_2\), 120 \(\mu \)M KCl, 30 \(\mu \)M Hepes, pH 7.4, 0.5 \(\mu \)M MgCl\(_2\), 0.3 \(\mu \)M ATP, 0.2 \(\mu \)M NADH, 0.5 \(\mu \)M phosphoenolpyruvate, 1 IU of pyruvate kinase/ml, and 1 IU of lactate dehydrogenase/ml, at 37°C. Twenty microliters of the sample to be assayed for ATPase activity were added to start the reaction, which was carried out in a final volume of 1 ml. The difference in absorbance between 366 and 550 nm was plotted versus time using a dual wavelength spectrophotometer, during the first 4 min after the addition of the enzyme. Then, EDTA was added to a final concentration of 2 mM, and monitoring was continued for 4 additional min. The (Ca\(^{2+}\)·Mg\(^{2+}\))·ATPase activity was calculated from the slope of the plot, with the activity in the presence of EDTA subtracted from that in its absence.

Protein determinations—To avoid interference by Triton X-100 and Hepes, the protein was precipitated using deoxycholate and trichloroacetic acid, and the protein content was then determined.

SDS-electrophoresis—the system described by Ronner (3) was used. Aliquots of the fractions of the calmodulin-Sepharose 4B column eluted by EDTA were precipitated with 10% trichloroacetic acid, washed once with 5% trichloroacetic acid, and one time with H\(_2\)O. The pellet was then resuspended in a buffer containing 60 \(\mu \)M Tris-HCl, pH 6.8, 2% SDS, 4 mM urea, and 0.001% bromphenol blue and 3% mercaptoethanol (v/v). This mixture was heated for 4 min in a boiling waterbath and then was applied to 8% polyacrylamide gels.

Formation of a [\(^{32}\)P]Phosphoprotein—the incubation medium contained 130 mM KCl, 20 mM Tris, pH 7.4 (at 20°C), 12.5 \(\mu \)M CaCl\(_2\), 0.5 \(\mu \)M MgCl\(_2\), 0.05 \(\mu \)M [\(^{32}\)P]ATP (specific activity 5 aCi/nmol), 10 to 20 \(\mu \)g of isolated enzyme/ml and 50 \(\mu \)l of CaCl\(_2\), or 500 \(\mu \)M EDTA. The reaction (at 0°C) was started by the addition of the radioactive ATP and stopped after 15 s by the addition of 1 volume of ice cold 10% trichloroacetic acid, 0.1 mM ATP, 0.8 mM phosphoric acid. After centrifugation for 5 min at 1300 \(\times g\) at 4°C, the pellet was washed with 2 volumes of 5% trichloroacetic acid, 0.1 mM ATP, 1 mM phosphoric acid, then with 2 volumes of H\(_2\)O, and was resuspended for gel electrophoresis in 0.3 ml of buffer containing 100 mM sodium phosphate, pH 6.0, 1% SDS, bromphenol blue (as a tracking dye), and 10% glycerol. SDS-polyacrylamide gel electrophoresis of the [\(^{32}\)P]-labeled enzyme on 5% gels of pH 6.0 was done as described (3). The gels were then frozen on dry ice, and pairs of 1-mm slices were incubated overnight with shaking in 1 ml of 0.5% SDS, 10 mM Tris-Cl, pH 9, at 40°C. After addition of 10 ml of Insta-Gel (Packard) the vials were counted in a Nuclear Chicago scintillation counter.

RESULTS

Isolation Procedure—The results of a typical isolation experiment are shown in Fig. 1 and Table I. The supernatant of Triton X-100-solubilized ghosts contained 21% of the total ghost protein and 27% of the final (Ca\(^{2+}\)·Mg\(^{2+}\))·ATPase activity of ghosts. Such a stimulation of the ATPase was always observed when calmodulin-deficient ghosts were solubilized by Triton X-100. When ghosts from which calmodulin had not been removed were solubilized, the ATPase activity was depressed instead (3).

During the concentration of the Triton X-100 solubilize, no (Ca\(^{2+}\)·Mg\(^{2+}\))·ATPase activity was lost. After loading on a 4-ml Sepharose 4B calmodulin column, 16% of the (Ca\(^{2+}\)·Mg\(^{2+}\))·ATPase activity and 94% of the protein applied to the column, was eluted by the Ca\(^{2+}\)-containing buffer. Most of the total ATPase activity, together with less than 6% of the total protein remained bound to the affinity column. Appropriate controls showed that these values did not depend on the amount of material applied to the column. When the Ca\(^{2+}\) in the elution buffer was replaced by 5 mM EDTA, a small peak of material absorbing at 280 nm was eluted, coincident with about 19% of the total (Ca\(^{2+}\)·Mg\(^{2+}\))·ATPase activity loaded on the column.

In erythrocyte ghost membranes, a certain amount of Mg\(^{2+}\)-dependent and Ca\(^{2+}\)-independent ATPase activity is always observed. In the experiments described here, this Mg\(^{2+}\)·ATPase was about 5% of the (Ca\(^{2+}\)·Mg\(^{2+}\))·ATPase. In the concentrated supernatant, the Mg\(^{2+}\)·ATPase was 3.4% and in the purified enzyme, 5.2% of the (Ca\(^{2+}\)·Mg\(^{2+}\))·ATPase. It may be significant that the activity eluted from the column in the presence of Ca\(^{2+}\) contained a higher percentage of Mg\(^{2+}\)·ATPase activity: 17.6%.

When the isolation was carried out as described above, but in the absence of phosphatidyserine in the buffers and in the supernatant, the yield of activity in the peak eluted by EDTA was only 5%. However, this enzyme preparation could be stimulated up to four times by the addition of 100 \(\mu \)g of phosphatidyserine during the assay. When lecithin instead of phosphatidyserine was present during the isolation procedure, only 4.4% of the activity was eluted by EDTA.

Characterization of the Isolated Enzyme—Protein determinations of the material eluted by EDTA gave a total of about 0.09% of the ghost protein used as starting material. The specific activity of the most active fraction was determined to be 3.8 \(\mu \)mol/mg/min, at 37°C and in the presence of 50 \(\mu \)M Ca\(^{2+}\). However, the purified enzyme could not be

| Fraction | Total protein | Protein yield | Total ATPase activity | Specific ATPase activity |
|----------|--------------|--------------|----------------------|-------------------------|
| Ghosts   | 156          | 100          | 1.05                 | 0.007                   |
| Triton X-100-solubilized ghosts | 32 | 21 | 2.92 | 0.091 |
| Column  | 30 | 19 | 0.48 | 0.016 |
| Peak eluted with Ca\(^{2+}\) buffer | 0.15 | 0.095 | 0.57 | 3.8 |
| Peak eluted with EDTA buffer | | | |

Fig. 1. Affinity chromatography of Triton X-100-solubilized ghosts on a Sepharose 4B calmodulin column (see "Materials and Methods" for details). The (Ca\(^{2+}\)·Mg\(^{2+}\))·ATPase activity (*) was monitored as described under "Materials and Methods," using 20 \(\mu \)l of the column eluate. ---, the transmittance at 280 nm. The column was first washed with Buffer A, containing 100 \(\mu \)M Ca\(^{2+}\). At the point indicated by the arrow, the buffer was changed to Buffer B, containing 5 mM EDTA.
stimulated by added calmodulin. The reasons for the absence of stimulation are currently being investigated. One possibility that is being explored is that some calmodulin might elute with the enzyme (14), another that the large amount of Triton, relative to the small amount of enzyme protein, could interfere with the activation process.

Because the original ghosts were capable of being stimulated by calmodulin, and because the activity of the solubilized ghosts decreased with time, it was difficult to choose a proper reference point for calculation of the degree of purification. A minimal degree of purification of 147-fold was calculated by comparing the specific activity of the purified protein with the calmodulin-stimulated activity of the ghosts. A degree of purification of 570-fold was calculated by direct comparison of the specific activity eluted from the column with that of the original ghosts without calmodulin. The range of 150- to 570-fold purification probably underestimates the purity of the enzyme, since the presence of inactive forms in the purified enzyme is probable. The solubilized enzyme is known to lose activity when kept on ice, even in the presence of phosphatidylserine.

The percentage of total ghost protein recovered in the fraction eluted by EDTA indicates a value for the total ATPase content between the 0.02% obtained by [32P]ATP labeling of ghosts (15) and the value of 0.21% obtained by kinetic titration (16) or of 0.35% by calmodulin binding (10). The value obtained from [32P]ATP labeling may be too low, due to lability of the phosphoprotein. The higher values may be more nearly correct, with the recovery of ATPase protein being lowered by losses during purification.

Fig. 2 shows an SDS-polyacrylamide gel with 10 μg of the isolated enzyme, stained with Coomassie blue. One major band with an apparent mass of 125,000 ± 3,500 daltons (determined on two different enzyme preparations isolated from two different ghost preparations, by comparison with protein standards) appears. This mass corresponds to the value of 125,000 daltons for the [γ-32P]ATP-labeled phosphoprotein in ghosts determined by Ronner (3), and is not far from the values of 145,000 given by Knauf et al. (15) and by Wolf et al. (4).

In addition, a small protein peak of 205,000 ± 8,800 daltons appears and, in the region of 90,000 daltons, a faint band can be detected. This peak probably represents some residual Band 3; in the experiment shown, it represented ~6% of the total protein (according to the intensity of the Coomassie stain). The 205,000-dalton peak (~11% of the total protein) could be detected in all preparations isolated so far, in varying amounts (10 to 50% of the total protein). This band could represent an impurity (e.g. spectrin) not removed during isolation, or some aggregated Band 3, but it could also represent a dimer of the 125,000-dalton protein not dissociated completely in SDS.

The purified enzyme preparation was labeled with [γ-32P]ATP in the presence of (Ca2+ and Mg2+) and electrophoresed on 5% polyacrylamide gels, in a phosphate buffer-SDS system. A control without radioactive ATP was run. [γ-32P]ATP labeled the 125,000-dalton protein (RF = 0.25) in the presence of 50 μM Ca2+ and 12 μM Mg2+ (for details, see "Materials and Methods"). B, this diagram represents a similar experiment as described under A, but the phosphorylation was carried out in the presence of 500 μM EGTA, 12 μM Mg2+, and no Ca2+. In the same experiment, enzyme was treated as described under A, but with cold ATP, and after electrophoresis, the gel was stained with Coomassie blue. A photograph of this gel is shown below the diagrams. Twenty micrograms of enzyme was applied to all gels.

![Fig. 2. Electrophoresis of the isolated (Ca2+-Mg2+)-ATPase on 8% SDS-polyacrylamide gels, as described under "Materials and Methods". The gel was loaded with 10 μg of ATPase and stained with Coomassie blue. TD, position of the tracking dye, bromphenol blue.](http://www.jbc.org/)

![Fig. 3. Demonstration of the phosphorylated intermediate of the isolated (Ca2+-Mg2+)-ATPase by labeling with [γ-32P]ATP. (The same enzyme preparation was used as in Fig. 2.) A, distribution of 32P radioactivity in a 5% SDS-polyacrylamide gel after formation of the phosphorylated intermediate by 2 μM [γ-32P]ATP in the presence of 50 μM Ca2+ and 12 μM Mg2+ (for details, see "Materials and Methods"). B, this diagram represents a similar experiment as described under A, but the phosphorylation was carried out in the presence of 500 μM EGTA, 12 μM Mg2+, and no Ca2+. In the same experiment, enzyme was treated as described under A, but with cold ATP, and after electrophoresis, the gel was stained with Coomassie blue. A photograph of this gel is shown below the diagrams. Twenty micrograms of enzyme was applied to all gels.)](http://www.jbc.org/)
labeled by \([\gamma\text{-}^{32}\text{P}]\text{ATP}\) in the presence of \(\text{Ca}^{2+}\) and \(\text{Mg}^{2+}\), and that the 205,000-dalton protein may indeed represent a dimer of this protein not dissociated in SDS. This experiment also shows that the 205,000-dalton protein is not spectrin, because spectrin would not be labeled by \([\gamma\text{-}^{32}\text{P}]\text{ATP}\) under these conditions (e.g. only 2 \(\mu\text{M}\) ATP, and in the presence of \(\text{Ca}^{2+}\)) (17).

**DISCUSSION**

Unlike the \(\text{Ca}^{2+}\) transporting ATPase of sarcoplasmic reticulum, the \((\text{Ca}^{2+}-\text{Mg}^{2+})\)-ATPase of erythrocytes has some unfavorable properties which have so far limited the success of the studies aimed at resolving it molecularly in a functionally active state. It is contained in the membrane in extremely low amounts, it is co-purified with Band 3, a protein which is many times more abundant than the \((\text{Ca}^{2+}-\text{Mg}^{2+})\)-ATPase, and it is rather unstable in the solubilized state. The procedure described in the present paper eliminates these drawbacks, and permits the extraction and purification from the membrane of an active enzyme. The specific, and \(\text{Ca}^{2+}\)-dependent, association of the detergent-solubilized ATPase with the column-bound calmodulin eliminates in a single step the contaminating proteins, while the presence of acidic phospholipids throughout the entire solubilization and purification limits the inactivation of the enzyme. The purified enzyme contains a single major phosphorylatable protein which gave an apparent molecular weight of 125,000. A minor phosphorylatable protein was also present at a position corresponding to a molecular weight of 205,000. The first band corresponds to the size already indicated by others for the \((\text{Ca}^{2+}-\text{Mg}^{2+})\)-ATPase. The identity of the slower band is not certain. However, since aggregation of proteins in SDS-polyacrylamide gels has been reliably reported (18), it may well represent an aggregated form of the monomeric enzyme. Although its apparent molecular weight is slightly low for a dimer, its \(\text{Ca}^{2+}\)-dependent phosphorylation suggests the possibility that it is indeed an aggregate of the main band. Further investigation will be necessary to determine definitely the identity of the larger protein.

The purest previously reported \((\text{Ca}^{2+}-\text{Mg}^{2+})\)-ATPase contained three polypeptide chains in approximately equal amounts; only one of these chains was phosphorylatable (4). That study reported that attempts to separate the phosphorylatable protein from the others resulted in loss of activity. The present study demonstrates that affinity chromatography can separate an active phosphorylatable protein from other membrane components.

A recent report of higher specific activity of a \((\text{Ca}^{2+}-\text{Mg}^{2+})\)-ATPase (19) from porcine erythrocytes presented no evidence of purity. Since porcine erythrocyte membranes have a much higher specific activity of \((\text{Ca}^{2+}-\text{Mg}^{2+})\)-ATPase than does human, it is not possible to compare the purity of this preparation with others, based on specific activity alone.

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