

Partial purification and characterization of the human erythrocyte Mg^{2+} -ATPase

A candidate aminophospholipid translocase

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A Mg^{2+} -ATPase-enriched fraction was obtained from solubilized human erythrocyte membranes by ammonium sulphate precipitation and anion-exchange chromatography. The solubilized enzyme, of apparent molecular weight 120 kDa, requires phosphatidylserine to be fully active. Phosphatidylethanolamine but not other anionic phospholipids can only partially restore the activity. The Mg -ATPase has a low affinity for Mg^{2+} -ATP and is inhibited by fluoride, vanadate, vanadyl and calcium ions. From these characteristics, we infer that this Mg^{2+} -ATPase is the same protein as the aminophospholipid translocase which regulates the membrane phospholipid transverse distribution in human erythrocytes by actively transporting aminophospholipids from the outer to the inner monolayer.

Mg-ATPase; Aminophospholipid translocase; Phosphatidylserine; Erythrocyte

1. INTRODUCTION

The human erythrocyte membrane and other plasma membranes contain an aminophospholipid translocase which actively transports phosphatidylserine (PS) and phosphatidylethanolamine (PE) from the outer to the inner monolayer [1,2]. This transport requires the hydrolysis of cytosolic ATP [1,3,4], the presence of magnesium [5] and is inhibited by vanadate [1,5]; it is therefore a vanadate sensitive Mg -ATPase. As a consequence of the action of the aminophospholipid translocase, phospholipids are symmetrically distributed over the two membrane halves, PS and PE being located principally in the inner leaflet, while phosphatidylcholine (PC) and sphingomyelin are the main components of the outer leaflet [6]. The kinetic properties of the aminophospholipid translocase are well established, but the protein has not yet been isolated and characterized. Using either photoactivable phospholipid analogues or SH-group reagents, Schroit and collaborators proposed a 32 kDa protein may be involved in translocase action [7,8]. On the basis of the existence of a PS transport activity in chromaffin granules

[9], we proposed that the translocase would be similar to the 116 kDa-ATPase II present in this intracellular organelle membrane.

In the human erythrocyte membrane, 5 different ATPases have been reported; the Ca^{2+} -stimulated Mg^{2+} -ATPase, the $(\text{Na}^+, \text{K}^+)$ -stimulated Mg^{2+} -ATPase, two vanadate-insensitive Mg^{2+} -ATPases of molecular weight 65 and 85 kDa which appear to be the glutathion transporters [10] and a vanadate-sensitive Mg^{2+} -ATPase of molecular weight 120–150 kDa whose function is unknown [11,12]. In this paper we report that the translocation of PS and PE by the aminophospholipid translocase in red cells and the hydrolysis of ATP by the solubilized Mg^{2+} -ATPase of molecular weight 120 kDa exhibit precisely the same properties (lipid, cation and nucleotide specificity, inhibition spectrum).

2. MATERIALS AND METHODS

2.1. Preparation of the ATPase-enriched fraction

Human blood was obtained from the Fondation Nationale de Transfusion Sanguine. All steps were carried out at 4°C. Erythrocytes (200 ml) were washed 4 × in isotonic saline buffer and hemolyzed in 20 vols of 0.1 mM EGTA, 5 mM phosphate buffer, pH 8.0. Membranes were pelleted at $23\,000 \times g$ for 40 min and solubilized by 1% C_{12}E_9 from a 10% stock solution. After 20 min solid ammonium sulphate was added to give 45% saturation. The cloudy suspension was stirred 20 min, then centrifuged 20 min at 20 000 rpm in a Beckman SW27 rotor. The floating precipitate was carefully removed, resuspended in 200 ml of 0.1 mM EGTA, 2 mM DTE, 0.02% sodium azide, 10 mM Tris-HCl buffer, pH 7.4 (buffer A) and brought to 45% saturation in ammonium sulphate. After centrifugation as

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Abbreviations: PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PA, phosphatidic acid; DPG, di-phosphatidylglycerol; C_{12}E_9 , polyoxyethylene 9 lauryl ether; EGTA, ethyleneglycol bis(β -aminoethylether)- N,N,N',N' -tetraacetic acid; DTE, di-thio erythritol; EDTA, ethylene diamine tetraacetic acid

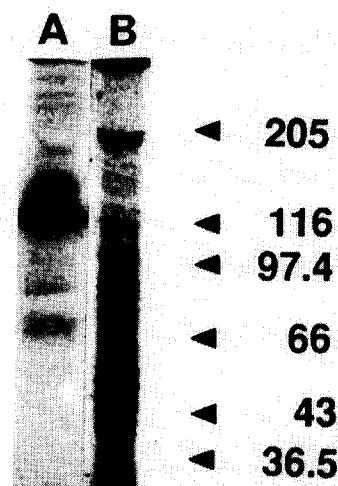


Fig. 1. Autoradiogram (A) and gel electrophoresis (B) of the fraction eluted from a DEAE column by 0.25 M NaCl. Electrophoresis was carried out with a 7.5% acrylamide slab gel as described in Materials and Methods. Arrowheads refer to migration standard proteins of known molecular mass (kDa).

above, this procedure was repeated once. The final, hemoglobin-free, floating precipitate was resuspended in 100 ml of buffer A and centrifuged for 1 h at 40 000 rpm in a Ti45 rotor (Beckman). The supernatant was mixed vol:vol with a 50% polyethylene glycol ($M_w \approx 4000$) solution in water, stirred for 20 min and centrifuged for 1 h at 20 000 rpm in a SW27 rotor. The pellet was resuspended in 30 ml of buffer A supplemented with 20% glycerol and 0.1% $C_{12}E_9$ (buffer B) and then applied on a fast-flow DEAE (Pharmacia) column (total volume 50 ml), equilibrated in the same buffer, and run at a rate of 5 ml/h. The activity was retained on the column and eluted by buffer B + 250 mM NaCl.

2.2. ATPase assay

An aliquot of the active fraction was eluted from DEAE preincubated at 37°C in a medium containing 0.1% $C_{12}E_9$, 2 mM DTE, 0.2 mM EGTA, 2 mM $MgCl_2$, 20 mM Tris, pH 7.4 buffer and varying amounts of phospholipids from a 25 mM solution in 2% $C_{12}E_9$. When

the ATP concentration to be used was greater than 1 mM, $MgCl_2$ was added in order to reach a final Mg^{2+} concentration twice that of ATP. Effect of Ca^{2+} ions on the activity was assayed in an incubation medium where EGTA was omitted. Dependence on Mg^{2+} was assayed in a medium devoid of $MgCl_2$ and containing 1 mM EDTA. After a 10 min-preincubation, kinetic studies were initiated by addition of ATP from a concentrated stock solution and followed by taking aliquots at given times. The amount of ATP hydrolyzed was assayed as the amount of Pi liberated determined by colorimetry [13].

2.3. Electrophoresis and autoradiography

Aliquots of active fraction were incubated as above to the exception of the addition of ATP- $\gamma^{35}S$ (Amersham) instead of ATP in the incubation. After a 5 min-incubation, samples were denatured at room temperature and electrophoresed (Phast-System, Pharmacia) on SDS-slab gels according to Laemmli [14]. After Coomassie blue staining and drying, the gel was autoradiographed with Hyperfilm- β max (Amersham).

2.4. Miscellaneous

When the activity was assayed according to the pH of the incubation medium, the following buffering systems were used at 20 mM: Mes (pH range 5.5–7.0), Hepes (pH range 6.5–8.0) and Tris (pH range 7.4–8.5). Chemicals were generally obtained from Sigma. PS was from bovine brain; lyso-PS was produced from PS by phospholipase A2 hydrolysis followed by purification on silica gel. PC, PA and PE were from egg yolk. DPG was from bovine heart. All the incubations were carried out at 37°C.

3. RESULTS

3.1. Partial purification

The electrophoretic pattern of a denatured fraction showed that several proteins were present in the active material eluted from DEAE column at 250 mM NaCl. Autoradiography of an aliquot reacted with ATP- $\gamma^{35}S$ revealed that the major labeled band comigrated with β -galactosidase (used as a marker) and thus, had an apparent molecular weight of about 116–120 kDa (Fig. 1). On the basis of specific activity in the presence of PS (see below), this fraction was purified approximately 100-fold when compared to the activity in the erythrocyte membrane (Table I).

Table I
Typical Mg^{2+} -ATPase activity in various fractions

| Fraction | Specific activity (nmol/min/ mg prot) | Protein content (mg) | Total activity (nmol/min) | Yield (%) | Purification factor |
|--|---|-------------------------|------------------------------|--------------|---------------------|
| Erythrocyte membrane (white ghost) | 7.8 | 600 | 4 680 | 100 | 1 |
| Pellet (polyethylene glycol) | 74.4 | 58.2 | 4 330 | 92.5 | 9.5 |
| DEAE-fraction (eluted by 0.25 M NaCl) | 786.7 | 5.0 | 3 930 | 84.0 | 100.9 |

The activity was determined in the presence of 1 mM Mg -ATP and defined as the vanadate-sensitive ATPase activity expressed in presence of ouabain and EGTA. For the solubilized fractions, the incubation medium contained PS

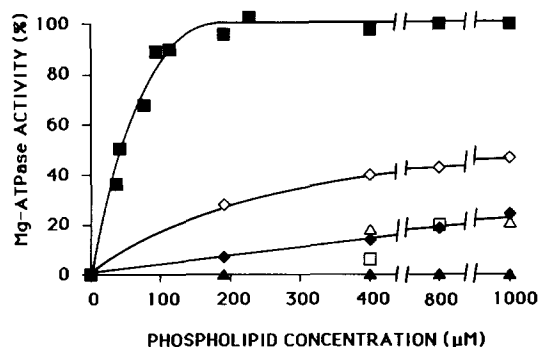


Fig. 2. Mg-ATPase activity found in incubations containing various amounts of exogenous phospholipids. 100% activity was taken as the maximum hydrolysis rate obtained with PS. Incubations were carried out at 37°C in the presence of 1 mM ATP. Lipids are PS (■), PE (◇), lyso PS (△), PA (◆), DPG (□) and PC (▲). Lipid concentration is expressed as phosphate equivalent.

3.2. Phospholipid-dependence of Mg^{2+} -ATPase activity

If phospholipids were absent from the incubation medium, no ATP hydrolysis was detected. Reactivation of the ATPase was strongly dependent on the nature of the phospholipid introduced in the medium (Fig. 2). Addition of PS to the incubation restored ATPase activity in an apparently saturable manner, the activity being maximal at approximately 200 μ M PS and half-maximum activity was obtained with ca. 40 μ M PS. The role of PS could not be mimicked neither by its lyso counterpart nor by other anionic phospholipids such as PA or DPG. As to zwitterionic phospholipids, while PC was unable to reactivate the activity, PE was partially active since 1 mM PE sustained about 50% of the maximal activity recovered by PS. All the forthcoming experiments were carried out with 400 or 800 μ M PS present in the incubation medium.

3.3. Nucleotide specificity

The ATPase was saturable with regards to the ATP concentration. Apparent K_m determined with different enzyme preparations was in the range 220–260 μ M. The following phosphorylated compounds, at 1 mM, gave $58.6 \pm 7.3\%$ (deoxy-ATP), $5.8 \pm 2.5\%$ (GTP), $3.4 \pm 3.0\%$ (CTP) and 0% (ADP) of the activity obtained with 1 mM ATP. The specificity, if any, of the divalent cation chelated with ATP and recognized by the ATPase was also studied. If the medium did not contain cations (i.e. in presence of EDTA), no hydrolysis of ATP occurred. 2 mM Mn^{2+} sustained only $15 \pm 5\%$ of the activity exhibited in presence of 2 mM Mg^{2+} . Neither Co^{2+} nor Ca^{2+} were effective. On the other hand, in incubations containing 2 mM Mg^{2+} , Ca^{2+} ions inhibited the hydrolysis by approximately 50% at 0.1 mM and by 70% at 1 mM.

3.4. Inhibition by phosphate analogues

In order to assess the formation of a phosphorylated intermediate during the reaction, inhibition of the

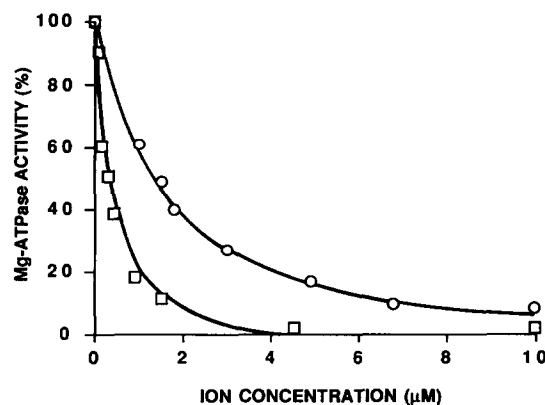


Fig. 3. Mg-ATPase activity in presence of various amounts of vanadate (VO_4^{3-}) ions (○), or vanadyl (VO^{2+}) ions (□), in the incubation medium. 100% activity refers to the activity obtained, in the absence of either vanadium compound, with 1 mM ATP and 400 μ M PS.

ATPase activity by VO_4^{3-} or AlF_4^- ions was studied. Fig. 3 shows that vanadate was a potent inhibitor of the activity, the half-inhibition taking place at approximately 1.5 μ M. Aluminum tetra-fluoride also inhibited the ATPase, ca. 12 μ M being required to get a 50% inhibition. We also studied the effect of another vanadium-containing ion, vanadyl (VO^{2+}). This ion exhibited a strong inhibitory effect on the ATPase whose activity was half-inhibited by 0.30–0.35 μ M VO^{2+} and completely abolished at 4 μ M ion (Fig. 3).

3.5. Other properties

As could be expected from an ATPase activity, the erythrocyte Mg^{2+} -ATPase was sensitive to N-ethyl maleimide. The pH dependence of the activity was assayed between pH 5.5 and pH 8.5. The activity was maximum between pH 7.0 and 8.0 and decreased when the pH was adjusted to be out of this range. The presence of 0.5 mM ouabain did not affect the level of activity detected, showing that no (Na^+ , K^+)ATPase was present.

4. DISCUSSION

The human erythrocyte membrane contains a vanadate and fluoride-sensitive Mg^{2+} -ATPase, a minor component present at 100 to 400 copies per cell [11,12] which requires the presence of PS in the medium to totally express its activity. This PS specificity is different from a general requirement of anionic phospholipids, which is found with other enzymes such as the (Na^+ , K^+)ATPase [15], as other negatively charged phospholipids, were less than one-tenth efficient as PS was. On the other hand, PE allowed the protein to express some activity. This remarkably specific lipid specificity expressed by the Mg^{2+} -ATPase parallels the substrate specificity of the aminophospholipid translocase [2,16]: the enzyme transports PS and PE, but the apparent affinity of PS is one order of magnitude

higher than that of PE. Besides, lyso PS is not a good substrate and is transported very inefficiently, and PC is not recognized at all by the carrier. Yet there are other properties common to both the Mg-ATPase and the translocase activities. Both enzymes recognize a Mg-ATP complex as there is neither ATP hydrolysis nor aminophospholipid transport [5] in the absence of Mg^{2+} . Other divalent cations were almost ineffective in this regard. Again in parallel to Mg-ATPase, GTP was not a substrate of the translocase (G.M., unpublished observations). These two activities were also inhibited by vanadate, vanadyl and calcium ions [5]. However, it is difficult to compare the dose-response curves obtained for the ATPase and the translocase, as one was assayed using a solubilized, semi-purified membrane protein and the other was studied using intact cells. We hypothesized that, in the human erythrocyte membrane, aminophospholipid translocase and Mg-ATPase are properties of the same protein. This is similar to the situation encountered in bovine chromaffin granules where we argued for the identity of the translocase and of a Mg^{2+} -ATPase found in these membranes and called ATPase II [9]. This ATPase [17] and that found in the erythrocyte share several properties related to substrate specificity, inhibition by vanadate, fluoride and by calcium ions. However, ATPase II appears to be less specific for the cation associated with ATP. A Mg^{2+} -ATPase with similar properties exists also in clathrin-coated vesicles [18]. A striking common feature is the PS requirement for a full activity expressed by the erythrocyte (this work), the coated vesicle [18] or the chromaffin granule Mg-ATPase (G.M., unpublished data). Labeling by ATP- $[\gamma\text{-}^{35}\text{S}]$ confirmed that the apparent molecular weight of the erythrocyte Mg-ATPase, determined by electrophoresis under denaturing and reducing conditions, is in the 115–120 kDa range [11]. The labeling of that band is abolished under conditions where the ATPase is inactive, i.e. in the absence of Mg^{2+} , or after addition of vanadate or NEM (not shown). This molecular weight is that of the Mg-ATPase from chromaffin granules [17] and coated vesicles [18]. Thus far, no one was able to identify a role for this ouabain-insensitive Mg-ATPase present in the erythrocyte membrane and in the plasma membrane

from many other eukaryotic cells. We propose that this PS stimulated Mg-ATPase is the aminophospholipid translocase, or a part of the complex which is responsible for the asymmetric transverse distribution of the membrane phospholipids.

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