Reconstitution of ATP-dependent aminophospholipid translocation in proteoliposomes

(Shape change/Mg\textsuperscript{2+}ATPase/flipase)

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ABSTRACT In addition to ion-pumping ATPases, most plasma membranes of animal cells contain a Mg\textsuperscript{2+}ATPase activity, the function of which is unknown. This enzyme, of apparent molecular mass 110 kDa, was purified from human erythrocyte membranes by a series of column chromatographic procedures after solubilization in Triton X-100. When reincorporated into artificial bilayers formed from phosphatidylcholine, it was able to transport a spin-labeled phosphatidylserine analogue from the inner to the outer membrane leaflet. Mg\textsuperscript{2+}ATP provided Mg\textsuperscript{2+}ATP was present in the incubation mixture. The ATP-dependent transport of the phosphatidylethanolamine analogue required the presence of an anionic phospholipid (e.g., phosphatidylinositol) in the outer membrane leaflet. In contrast the transmembrane distribution of spin-labeled phosphatidylcholine was unaffected in the same experimental conditions. This transmembrane movement of aminophospholipid analogues was inhibited by treatment of the proteoliposomes with a sulfhydryl reagent. We conclude that the Mg\textsuperscript{2+}ATPase is sufficient for the biochemical expression of the aminophospholipid translocase activity, which is responsible for the inward transport of phosphatidylserine and phosphatidylethanolamine within the erythrocyte membrane. The presence of this transport activity in many animal cell plasma membranes provides a function for the Mg\textsuperscript{2+}ATPase borne by these membranes.

The rapid passage of aminophospholipids, phosphatidylserine (PS) and phosphatidylethanolamine (PE), from the outer to the inner leaflet of the human erythrocyte membrane is an ATP-dependent process that involves the activity of a Mg\textsuperscript{2+} ATPase (1, 2). This selective transport is certainly the major cause of the asymmetrical transmembrane distribution of phospholipids in the human erythrocyte and in the plasma membranes of animal cells. A similar selective transport of aminophospholipids was found in chromaffin granules (3), organelles derived from the Golgi complex of chromaffin cells. In 1989, it was proposed (3) that a 110- to 120-kDa polypeptide was the major component of the phospholipid transport system of eukaryotes and since then attempts have been made to purify the “aminophospholipid translocase” from human erythrocytes (4, 5).

In parallel, a characteristic of the erythrocyte membrane that is dependent upon hydrolyzable ATP is the membrane shape change (6–8). ATP-depleted erythrocytes are echinocytic but if ATP is regenerated they recover their normal discocyte shape and become stomatocytic if the level of cytosolic ATP becomes very high. Similarly, white ghosts, which are echinocytic, change their shape to discocytes and then to stomatocytes upon addition of Mg\textsuperscript{2+}ATP (9, 10). It was postulated that this phenomenon was driven by a shapechange ATPase (11, 12). In fact, the ATP-dependent aminophospholipid translocation and the echinocytoto-stomatocyte conversion share some common features: an apparent ATP specificity in the millimolar range, a sensitivity to phosphate analogues, and a preference for Mg\textsuperscript{2+} or Mn\textsuperscript{2+} over Co\textsuperscript{2+} as an ATP cofactor (11–14). An erythrocyte Mg\textsuperscript{2+}ATPase with an apparent molecular mass of 110 kDa and with these characteristics has been purified to homogeneity. Its ATPase activity in detergent was sensitive to the presence of PS and to a lesser extent of PE. To further the evidence that this Mg\textsuperscript{2+}ATPase can be identified as the aminophospholipid translocase of erythrocytes, we have reconstituted this purified ATPase in artificial bilayers and tested the transmembrane diffusion of spin-labeled phospholipids in the reconstituted vesicles in the presence or absence of Mg\textsuperscript{2+}ATP. We found an ATP-dependent transport of aminophospholipids in these vesicles.

MATERIALS AND METHODS

Mg\textsuperscript{2+}ATPase Isolation. Human erythrocytes were obtained from the New South Wales Blood Transfusion Service (Sydney) or from the Fondation Nationale de Transfusion Sanguine (Paris). Washed erythrocytes were lysed in 10 vol of ice-cold buffer (2 mM EDTA/10 mM Tris-HCl, pH 8 at 4°C). Membranes were washed four times in this buffer and collected by centrifugation (16,000 × g, 30 min). EDTA was removed by three further washes in 10 mM Tris-HCl (pH 7.4 at 4°C). Hemoglobin-free membranes were solubilized with 0.5% Triton X-100. The soluble extract was loaded onto a 10-ml calmodulin-agarose column (Sigma) equilibrated with solubilization buffer [0.5% Triton X-100/2 mM CaCl\textsubscript{2}/5 mM MgCl\textsubscript{2}/20 mM Hepes/10% (vol/vol) glycerol/2 mM dithiothreitol]. The flow-through from the calmodulin column was directed onto a 50-ml heparinagarose column (Sigma) equilibrated with solubilization buffer excluding Ca\textsuperscript{2+}. The heparin column was washed with 50 ml of buffer C (0.2% Triton X-100/2 mM dithiothreitol/10% glycerol/5 mM MgCl\textsubscript{2}/20 mM Hepes). The ATPase activity was eluted in buffer C with 200 ml of a KCl gradient from 50 to 250 mM. ATPase activity was measured colorimetrically as described (13). Active fractions were pooled and dialyzed against two changes of 100 vol of buffer C to reduce the salt concentration. The dialysate was loaded onto a 30-ml DEAE-Sepharose 6B fast flow column (Pharmacia) equilibrated with buffer C. The bound ATPase activity was eluted in buffer C with a 200-ml KCl gradient from 70 to 200 mM. Fifty 4-ml fractions were collected and.

Abbreviations: PC, phosphatidylincholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC*, 1-palmitoyl, 2-(4-doxyl)pentanoyl-sn-glycero(3)phosphocholine; PS*, 1-palmitoyl, 2-(4-doxyl)pentanoyl-sn-glycero(3)phosphoserine; PE*, 1-palmitoyl, 2-(4-doxyl)pentanoyl-sn-glycero(3)phosphoethanolamine.

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assayed for calcium-independent ouabain-insensitive Mg\textsuperscript{2+} ATPase activity. Protein concentration was determined using the method of Bradford (16). Polyacrylamide gel electrophoresis (a 10% running gel with a 4% stacking gel) was carried out by the method of Laemmli (17).

**Proteoliposomes.** Five μmoles of egg yolk phosphatidylcholine (PC) was mixed with 0.05 μmol of the desired spin-labeled phospholipid analogue, all dissolved in chloroform; in some instances, 0.05–0.1 μmol of phosphatidylinositol (PI) was added. The lipid solution was dried under reduced pressure and the film was resuspended by vigorous vortex mixing in 0.5 ml of 150 mM KCl/5 mM MgCl\textsubscript{2}/0.2 mM EGTA/20 mM Hepes, pH 7.45 (buffer A). To these multilayered liposomes were added 10 μg of purified protein in 0.5 ml and 10 μg of Triton X-100. After 2 min of magnetic stirring, 300 μg of Bio-Beads SM-2 (Bio-Rad), previously washed in methanol and water, was added and the stirring was continued for 1 hr. Formation of sealed liposomes was indicated by the resistance of 50% of the spin-labeled analogues to ascorbate reduction (see below).

**Assay of Spin-Labeled Phospholipid Asymmetry.** At various times, an aliquot was taken from the proteoliposome suspension and transferred to an ESR cell to record the spectrum. To another aliquot was added buffered (pH 7.4) sodium ascorbate (30 mM, final concentration) to reduce the spin probes (18). Immediately, the ESR spectrum was recorded on a Bruker (Wissembourg, France) model ER 200 D spectrometer and its decrease in intensity was recorded with time. Reduction kinetics are in fact biphasic with a rapid component corresponding to the reduction of the freely accessible analogue in the outer leaflet. The second slow component corresponds to the reduction of the probe situated in the inner leaflet and the extrapolation to time zero corresponds to the fraction of the analogue in the intravesicular leaflet. For each liposome suspension, an experiment was carried out in the absence of ATP and another experiment was carried out in the presence of 2 mM ATP from a concentrated stock solution. In some experiments, proteoliposomes were incubated for 20 min with 1 mM N-ethylmaleimide prior to ATP addition. All incubations were carried out at room temperature.

**Miscellaneous.** Egg yolk PC and PE, bovine brain PS and PI, and all the chemicals were purchased from Sigma. Spin-labeled phospholipid analogues were synthesized as described (19). The ones used in these studies were 1-palmitoyl, 2-(4-doxy)octanoyl-sn-glycero-3-phosphocholine (PC*), -serine (PS*), and -ethanolamine (PE*).

**RESULTS**

Polyacrylamide gel electrophoresis under denaturing conditions (SDS) of the fractions with peak Mg\textsuperscript{2+} ATPase activity eluted from the DEAE column showed a single polypeptide of apparent molecular mass 110 kDa (Fig. 1). More details on this purification will be given elsewhere (M.E.A., unpublished results). This fraction is representative of the samples reconstituted for translocation studies.

Formation of sealed proteoliposomes was revealed by the inaccessibility of half of the probes to ascorbate reduction in the absence of ATP in the incubation medium. The exact amount was independent of the nature of the analogue used and from the bilayer composition. However, it varied slightly, from 50 to 55%, from one preparation to another. The amount of analogue in the outer monolayer decreased progressively with increasing time of incubation with BioBeads, indicating the ability of the beads to extract some of the paramagnetic molecules more readily than they extract phospholipids (20). The time required to chemically reduce the outwardly exposed nitrooxides, however, depended on the lipid composition, as the presence of anionic species, such as PI, slightly slowed the reduction kinetics (Figs. 2 and 3).

Addition of ATP to the incubation medium had various consequences depending on the phospholipid analogue. When studying PS* behavior in PC (Fig. 2) or PC/PI bilayers (Fig. 3), the presence of Mg\textsuperscript{2+} ATP induced a change in the analogue distribution: 15–32% (mean, 20.0%; SEM, 2.7%) of the PS* molecule was originally present in the intravesicular leaflet were moved to the outer layer, as determined in more than 13 experiments. This new transmembrane distribution occurred within 10 min after addition of ATP and did not change significantly for an extra 30-min period. For two experiments, pretreatment of the reconstituted membranes with 1 mM N-ethylmaleimide inhibited the ATP-induced PS* redistribution by 70% and 100%. As a control, liposomes were prepared by the detergent removal procedure but in the absence of purified protein; PS* distribution in these pure lipid bilayers was insensitive to the addition of Mg\textsuperscript{2+} ATP (data not shown).

Comparable results were obtained when proteoliposomes contained PE*, only if 1 or 2% PI was incorporated in the bilayer (Fig. 3). However, it is more difficult to quantify the amount reoriented, which was less than 10%. If the bilayer did not contain PI (i.e., if the only lipid besides PE* was PC), we were unable to detect any rearrangement after addition of Mg\textsuperscript{2+} ATP. The requirement of anionic phospholipids to ensure a transport activity was established with PS* in PC and PC/PI proteoliposomes, to which was added 1% defatted bovine serum albumin prior to ATP addition. The albumin extracted the PS* molecules located in the outer leaflet. After this treatment, only the PC/PI proteoliposomes exhibited a transport activity.

The distribution of PC* embedded in proteoliposomes containing only PC or PC plus 1% PI or 1% PS did not change further on addition of ATP (Figs. 2 and 3) over incubation times of 1 hr or more.

![Fig. 1. Gel electrophoresis of an active fraction of Mg\textsuperscript{2+} ATPase (arrowhead) eluted from the DEAE column (lane a). Electrophoresis was carried out on a 10% polyacrylamide slab gel that was stained with Coomassie blue. Lane b shows the molecular mass markers. The ATPase fraction shown is representative of the samples reconstituted for translocase studies.](image-url)
Fig. 2. Kinetics of ascorbate reduction of PS* (A) or PC* (B) in PC bilayers. The remaining oxidized analogue was assayed by measuring the low-field line height (plotted in a logarithmic scale) as a function of time after ascorbate addition. The fast component corresponds to the reduction of the analogue in the outer leaflet and the slow component corresponds to the reduction of the analogue in the intravesicular leaflet. Extrapolation of the second kinetics to time zero gives the fraction of analogue localized in the inner leaflet. Incubations have been carried out for 10 min in the absence (open symbols) or in the presence (solid symbols) of ATP. Ranges of signal intensity measurements were typically less than the size of symbols.

**DISCUSSION**

We have shown that the incorporation of a purified 110-kDa Mg$^{2+}$ATPase in egg PC vesicles can cause a rapid redistribution of PS* or PE*, but not of PC*, between the two leaflets of the artificial vesicles when Mg$^{2+}$ATP was present in the external medium. The redistribution was inhibited when the proteoliposomes had been previously incubated with N-ethylmaleimide. The fact that some activity remained in the presence of 1 mM N-ethylmaleimide is in accordance with the sensitivity of the transport in intact cells (21). *A priori* we have no indication of a selective orientation of the purified ATPase upon incorporation in the liposomes. However, the asymmetry of the system is provided by addition of ATP in the incubation medium after the formation of the sealed liposomes. This creates a functional asymmetry: only the ATPases with the ATP site exposed to the outer membrane leaflet are active. It is interesting to note that the transport of aminophospholipids could be obtained only if an anionic phospholipid, either PS or PI, was present on the outer monolayer (i.e., near the site of fixation of ATP). Indeed, if PS* was removed from the outer monolayer by bovine serum albumin before the incubation with ATP, no transport of PS* from the inner to the outer monolayer took place in PC liposomes. For the symmetrical distribution of PS*, the phospholipid substrate contributed also to the anionic environment and there was no requirement for another anionic phospholipid. But for PE*, the presence of PI was necessary. Nonlabeled PS could not be used as it is a better substrate than PE* and thus prevented the latter from being transported. The requirement for the presence of anionic phospholipids to obtain a transport activity is in accordance with the fact that anionic phospholipids are necessary for the maximum activity of this ATPase (5). Finally PC*, which is known not to be transported by the aminophospholipid translocase, could not be redistributed in the proteoliposomes.

In these transport experiments, we started with an almost symmetrical distribution of transport substrate (PS* or PE*). As a consequence only half of the labeled molecules were
available for transport from the inner to the outer monolayer. However, only 20% of the aminophospholipid initially present in the inner monolayer was effectively transferred to the outer monolayer. This can very well be explained by the low ratio of protein to lipid in our experiments. It is in fact probable that not all liposomes contained an active Mg\(^{2+}\) ATPase (Fig. 4). If the molecular mass of the ATPase is 110 kDa, the bulk suspension contained 5 \(\mu\)mol of lipids for 10 \(\mu\)g of protein (i.e., 0.09 nmol of Mg\(^{2+}\)ATPase), which corresponds to 55,000 lipid molecules per enzyme molecule. This is close to the value encountered in erythrocytes with 250 \(\times\) 10\(^6\) phospholipids and less than 10\(^7\) Mg\(^{2+}\)ATPases per cell (22). It is known that the size of the liposomes formed upon removal of Triton X-100 depends upon the rate of detergent elimination (20) and that the presence of proteins does not affect their size (23) and their volume (24). Under the reconstitution conditions, liposomes are heterogeneously distributed from 50 nm to 150 nm in diameter (22). Considering an area per phospholipid of 0.7 nm\(^2\), vesicles contained from 2 \(\times\) 10\(^4\) to 2 \(\times\) 10\(^5\) phospholipids and thus had at the maximum an average of 4 ATPases. Moreover, not all enzyme molecules could be properly oriented and a fraction of the purified ATPase molecules may be inactivated (Fig. 4).

In practice we may have a gaussian distribution of proteins among the vesicles and it is not improbable that half or less of the vesicles contained in fact a transporter. Another explanation for the incomplete reorientation of the analogues could be that another component is required to ensure a transport activity and that this component would exist as a contaminant of our preparation. However, no proteins other than the 110-kDa polypeptide were visualized by Coomassie or silver staining. Because of the small size of the vesicles containing the purified Mg\(^{2+}\) ATPase, the time for lateral diffusion of PS\(^*\) within the membrane leaflet must be very short, and in those vesicles containing an active Mg\(^{2+}\) ATPase, the rate-limiting step was certainly not the time to reach the active site of the protein. It is therefore not surprising that we were unable to study the kinetics of aminophospholipid translocation.

In conclusion, we have shown that egg PC vesicles containing a purified Mg\(^{2+}\) ATPase of molecular mass 110 kDa can cause the rapid reorientation of aminophospholipids in the presence of Mg\(^{2+}\) ATP. In addition to the requirement for Mg\(^{2+}\) ATP, we have shown that anionic phospholipids (either PS or PI) must be present on the side of the membrane where ATP binds. From these data, we conclude that the 110-kDa protein can be identified as at least a major part of the aminophospholipid translocase. This single polypeptide seems to be sufficient to translocate PS and PE. The possibility that in vivo other protein(s) play(s) a regulatory role is of course not excluded. A priori the net transfer of phospholipids in a liposome can cause important membrane shape change (25, 26), thus the shape change ATPase and the aminophospholipid translocase could in fact be the same protein. Direct proof should come from the investigation of the control of the shape of the reconstituted systems.

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