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# Long-term stabilization and crystallization of ($Ca^{2+}$ + $Mg^{2+}$)-ATPase of detergent-solubilized erythrocyte plasma membrane

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**Conditions which were optimal for the stabilization of $Ca^{2+}$-transporting ATPase in solubilized sarcoplasmic reticulum membranes (Pikuła, S., Mullner, N., Dux, L. and Martonosi, A. (1988) J. Biol. Chem. 263, 5277–5286) were also found conducive for preservation of ($Ca^{2+}$ + $Mg^{2+}$)-ATPase activity in detergent-solubilized erythrocyte plasma membrane for up to 60 days. Of particular importance for the stabilization of calmodulin-stimulated $Ca^{2+}$-dependent activity of ($Ca^{2+}$ + $Mg^{2+}$)-ATPase of solubilized erythrocyte plasma membrane was the presence of $Ca^{2+}$ (10–20 mM), glycerol, anti-oxidants, proteinase inhibitors and appropriate detergents. Among eight detergents tested octaethylene glycol dodecyl ether, polyoxyethylene glycol(10) lauryl alcohol and polydocanol were found to be promotive in long-term preservation of the enzyme activity. Under these conditions ($Ca^{2+}$ + $Mg^{2+}$)-ATPase of erythrocyte ghosts became highly stable and developed microcrystalline arrays after storage for 35 days. Electron micrographs of the negatively stained and thin sectioned material indicated that crystals of purified, detergent-solubilized, lipid-stabilized erythrocyte ($Ca^{2+}$ + $Mg^{2+}$)-ATPase differ from those of $Ca^{2+}$-ATPase of detergent-solubilized sarcoplasmic reticulum microsomes.**

## Introduction

Erythrocyte plasma membrane contains $Ca^{2+}$-transporting ATPase (EC 3.1.6.38) which, however, represents only a minute amount of the total membrane protein [1]. Therefore, for structural studies the enzyme should be solubilized and purified to homogeneity; for this purpose the method of isolation by means of CaM-affinity chromatography was successfully used [2]. For purification of the active form of the enzyme a choice of appropriate detergent is essential. Several nonionic detergents were shown to solubilize erythrocyte ATPase. However, only with a few of them did it appear possible to preserve the enzyme activity for a period longer than several minutes [2–9].

It has been already demonstrated that the activity of another $Ca^{2+}$-transporting enzyme, the $Ca^{2+}$-dependent ATPase of SR membrane, is preserved for several months when the solubilization and storage medium is supplemented with glycerol or other polyhydroxy compounds and 10–20 mM $Ca^{2+}$ at the detergent/protein weight ratio not exceeding 2 [10–12]. In the presence of inhibitors of proteolysis, compounds preventing oxidation of SH groups and of double-bonds of phospholipid fatty acid moieties, as well as in the presence of bacteriostatic agents, the molecules of solubilized $Ca^{2+}$-ATPase tend to interact with each other to form three-dimensional oligomeric structures which are responsible for long-term stabilization of the SR enzyme activity [10–13]. Among 49 detergents tested, only $C_{12}E_8$, Brij 36T, Brij 56 and Brij 96 were found to promote self-association of the enzyme molecules and to preserve its activity [10,11]. Fluorescence spec-

Abbreviations: CaM, calmodulin; PS, phosphatidylserine; PC, phosphatidylcholine; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $C_{12}E_8$, octaethylene glycol dodecyl ether; Brij 36T, polyoxyethylene glycol(10) lauryl alcohol; Brij 56, polyoxyethylene glycol(10) cetyl alcohol; Brij 96, polyoxyethylene glycol(10) oleoyl alcohol; DDAO, *N*,*N*-dimethyloctadecylamine-*N*-oxide; SR, sarcoplasmic reticulum; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; K-Mops, potassium 3-(*N*-morpholino)propanesulfonate; $PhMeSO_2F$, phenylmethylsulfonyl fluoride.

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troscopy and high-performance liquid chromatography studies have provided evidence that stabilization of the solubilized enzyme activity is related to the appearance of dimers, tetramers and larger oligomers of $Ca^{2+}$-ATPase of SR membranes in the solubilizate [13].

Since $Ca^{2+}$-pumps in erythrocyte ghost and SR membranes belong to the same class of P-type ion-transporting enzymes [14,15], we investigated whether conditions which had been elaborated for $Ca^{2+}$-ATPase of SR preparations would also be suitable for stabilization of purified $(Ca^{2+} + Mg^{2+})$-ATPase of detergent-solubilized erythrocyte plasma membrane. Accordingly, we used several nonionic and zwitterionic detergents for solubilization, purification and storage of the erythrocyte enzyme. In addition, observations indicating formation of microcrystalline arrays of purified erythrocyte $(Ca^{2+} + Mg^{2+})$-ATPase, which differ from those obtained for $Ca^{2+}$-ATPase of solubilized SR membranes, are described.

## Materials and Methods

### *Chemicals*

Lactate dehydrogenase (EC 1.1.1.27) from rabbit muscle, pyruvate kinase (EC 2.7.1.40) from rabbit muscle, ATP, ADP, NADH and PEP were from Boehringer (Mannheim, F.R.G.). Dithiothreitol, albumin (bovine) and Mops were obtained from Sigma (St. Louis, MO, U.S.A.). The detergents were supplied as follows: $C_{12}E_8$ and Brij 36T ($C_{12}E_{10}$) by Sigma (St. Louis, MO, U.S.A.), Triton X-100 by Merck (Darmstadt, F.R.G.), DDAO by Millmaster Onyx International (Fairfield, NJ, U.S.A.), Lubrol PX (ethylene oxide condensates of fatty alcohols) and Triton WR 1339 (octylphenol-polyethyleneglycolether-formaldehyde polymer) by Serva (Heidelberg, F.R.G.), dodecyl-D-maltoside by Fluka (Ronkohoma, NY, U.S.A.), and polydocanol ($C_{12}E_9$) by Calbiochem (San Diego, CA, U.S.A.). Glutaraldehyde (8% aqueous) was from Polysciences (Warrington, PA, U.S.A.), and trasylol (aprotinin, 10000 IU/ml) was from Mobay Chemical (New York, NY, U.S.A.). Phosphatidylcholine was prepared from egg yolk as described by Sarzała and Michałak [16], and phosphatidylserine from bovine brain according to the method of Comfurius and Zwaal [17]. Calmodulin from bovine brain was purified as described by Brzeska et al. [18]. CNBr-activated Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden). All other chemicals were of the highest grade commercially available.

### *Preparation of erythrocyte ghosts and purification of $(Ca^{2+} + Mg^{2+})$-ATPase*

Porcine blood was freshly obtained from the slaughter-house and erythrocyte ghosts were prepared according to Steck and Kant [19] with modifications introduced by Wrzosek et al. [20]. The material (5–6 mg protein/ml) collected in 20 mM Hepes-Tris (pH 7.4)/100 mM KCl/0.1 mM $PhMeSO_2F$/0.4 mM dithiothreitol/0.1 mM EGTA was rapidly frozen and stored in polypropylene containers in liquid nitrogen for 2–3 weeks. Before use, the ghosts were thawed, diluted 20-fold with the appropriate medium without detergent and spun down at $105000 \times g$ for 60 min. In some experiments, as stated in the text, $CaCl_2$, glycerol, anti-oxidants and proteinase inhibitors were omitted from the medium, and 20 mM Hepes-Tris (pH 7.4) was replaced with 20 mM K-Mops (pH 6.0). The final protein concentration was always adjusted to 3 mg/ml. Conditions for solubilization of the erythrocyte ghost preparation are described in the text.

Erythrocyte ATPase was purified according to the method of Niggli et al. [2], as modified by Nelson and Hanahan [5]. The purified $(Ca^{2+} + Mg^{2+})$-ATPase preparation (0.3 mg protein/ml) was stored at 2°C under nitrogen in 20 mM K-Mops (pH 6.0)/100 mM KCl/3 mM $MgCl_2$/10–20 mM $CaCl_2$/20% glycerol/3 mM $NaN_3$/5 mM dithiothreitol/0.1 mM $PhMeSO_2F$ containing, per ml, 25 IU trasylol, 2 μg 1,6-di-*tert*-butyl-*p*-cresol and 0.25 mg PC, in the presence of 1 mg/ml of either $C_{12}E_8$, Brij 36T, Triton X-100 or polydocanol. The medium described above is designated throughout as the medium for crystallization.

### *Isolation of sarcoplasmic reticulum vesicles*

SR vesicles were isolated from predominantly white muscles of rabbits fasted for 24 h to deplete of glycogen according to Sarzała and Michałak [16]. 1 ml aliquots of 25–30 mg protein in 100 mM KCl/20 mM Hepes-Tris (pH 7.4)/300 mM sucrose were frozen in liquid nitrogen and stored in polypropylene containers for 3–4 weeks. Before use, samples were thawed, diluted 20-fold with the appropriate medium without detergent, centrifuged for 60 min at $30000 \times g$ and the pellet was resuspended in the same buffer solution with the detergent.

### *Enzyme activity measurements*

$(Ca^{2+} + Mg^{2+})$-ATPase activity in the erythrocyte ghost membrane or in solubilized and purified preparations was measured spectrophotometrically at 37°C using coupled enzyme assay system of Niggli et al. [2]. $Ca^{2+}$-ATPase activity in SR membranes or in the detergent-solubilized material was determined either using the coupled enzyme assay system or by measuring liberation of inorganic phosphate according to Pikuła et al. [11].

### *Electron microscopy*

Negative staining with 1% uranyl acetate (pH 4.3) was carried out at 2°C essentially as described by Dux et al. [10]. Thin-sectioned samples were prepared after overnight fixation with 1% glutaraldehyde, postfixation

with 2% osmium tetroxide and delipidation for 4 h at 2°C with 90% acetone or chloroform/methanol (2:1, v/v). Negatively stained and thin-sectioned material was viewed with JEOL 1200 EX (Japan) or JEM 100B (Japan) electron microscope at accelerating voltage of 60–80 kV.

*Other determinations*

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed as described by Laemmli [21] on 7.5% gels with 4% stacking gel. Coomassie blue staining and silver staining were done according to Laemmli [21] and Heukeshoven and Dernick [22], respectively. Protein concentration was assessed by the method of Lowry et al. [23]. Phospholipid content and composition were analyzed by solvent extraction according to Folch et al. [24], and thin-layer chromatography as described by Sarzała and Michalak [16]. Phospholipid phosphorus was determined according to Bartlett [25]. Concentrations of free $Ca^{2+}$ were calculated using a computer program based on the dissociation constants published by Fabiato and Fabiato [26].

## Results

*Solubilization of $(Ca^{2+}+Mg^{2+})$-ATPase and stabilization of its activity in the presence of detergents*

The influence of eight detergents on the activity of membrane-bound $(Ca^{2+}+Mg^{2+})$-ATPase was studied (Fig. 1) in order to evaluate optimal conditions for further purification and crystallization of the enzyme.

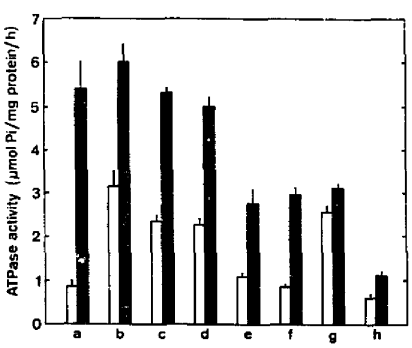


Fig. 1. Activity of $(Ca^{2+}+Mg^{2+})$-ATPase of erythrocyte ghosts solubilized with various detergents. Erythrocyte ghosts (3 mg of protein/ml) were incubated for 10 min at 2°C in 100 mM KCl/20 mM Hepes-Tris (pH 7.4)/0.4 mM dithiothreitol/0.1 mM $PhMeSO_2F$/1 mM $MgCl_2$/0.1 mM $CaCl_2$ and 3 mg/ml of following detergents: (a) none; (b) $C_{12}E_8$; (c) Brij 36T; (d) polydocanol; (e) Triton X-100; (f) Triton WR 1339; (g) Lubrol PX; (h) dodecyl D-maltoside. Then, the enzyme activity was measured at 37°C using coupled enzyme assay system containing 10 μM free $Ca^{2+}$, in the absence (empty bars) or presence (filled bars) of 0.2 μM CaM. Values are means for two different ghost preparations. Three to five determinations per each preparation were performed, which varied within ±S.E. of 5–7%.

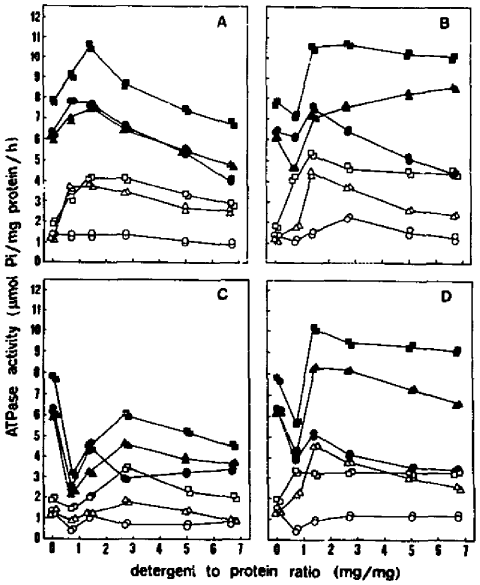


Fig. 2. $(Ca^{2+}+Mg^{2+})$-ATPase activity of erythrocyte ghost membrane solubilized with $C_{12}E_8$ (A), Brij 36T (B), Triton X-100 (C) and polydocanol (D) at various detergent/protein weight ratios and in the presence of 0.1 mM or 20 mM $CaCl_2$. Erythrocyte ghosts (3 mg protein/ml) resuspended in 100 mM KCl/20 mM Hepes-Tris (pH 7.4)/3 mM $MgCl_2$/0.1 mM $CaCl_2$/0.1 mM $PhMeSO_2F$/3 mM $NaN_3$/5 mM dithiothreitol containing, per ml, 25 IU trasylol and 2 μg 1,6-di-*tert*-butyl-*p*-cresol (buffer 1, ○, ●), or in the same buffer but with 20 mM $CaCl_2$ and 20% glycerol (buffer 2, △, ▲), or in the same medium as buffer 2 but with 20 mM K-Mops (pH 6.0) replacing 20 mM Hepes-Tris (pH 7.4) (buffer 3, □, ■) were solubilized for 10 min at 2°C with various detergents at the detergent/protein weight ratio indicated at the abscissa. ATPase activity was measured immediately after solubilization in the absence (open symbols) or presence (closed symbols) of 0.2 μM CaM. Concentration of free $Ca^{2+}$ in the medium for the enzyme activity measurements was 10 μM and protein concentration was 90 μg/ml (what indicates 33-fold dilution of the sample in the assay medium). Results for two separate preparations are shown.

The following concomitant effects of the detergents tested were observed: (a) alterations of the enzyme activity assayed in the presence of $Ca^{2+}$; (b) changes in the sensitivity to CaM; (c) a decrease in the degree of stimulation of the enzyme by CaM.

Under conditions described in the legend to Fig. 1, three chemically related nonionic detergents, $C_{12}E_8$, Brij 36T and polydocanol, activated the ATPase assayed in the absence of CaM with a parallel lowering of the degree of stimulation by CaM to about 2. Two zwitterionic detergents, DDAO and dodecyl D-maltoside, strongly inhibited the enzyme, while Triton X-100 and Triton WR 1339 decreased the ATPase activity stimulated by CaM with no significant influence on the activity assayed without CaM. The effect of Lubrol PX was somehow between that of polyoxyethylene glycol

ethers and Tritons. This detergent increased ($Ca^{2+}$ + $Mg^{2+}$)-ATPase activity measured in the presence of $Ca^{2+}$ alone, but almost completely inhibited that stimulated by CaM. The described effects could not be reversed by the addition of phospholipids to the assay mixture (results not shown).

Short-term effect of detergents on the enzyme activity strongly depended on the type of detergent used, the detergent/protein weight ratio, the presence of $Ca^{2+}$ and glycerol, and pH of the solubilization medium.

At pH 7.4 in the presence of 0.1 mM $CaCl_2$ and absence of glycerol, the four detergents tested had practically no effect on the ATPase activity measured in the absence of CaM, while the degree of CaM stimulation was affected to varying extent depending on the detergent/protein ratio (Fig. 2A–D, buffer 1). Most striking differences were observed when Triton X-100 was used or when the concentration of other detergents exceeded 20 mg/ml. Only under harsh conditions including either elevated temperature of incubation with the detergent (22°C), pH of the solubilization buffer above pH 8.0, presence of 1 mM EGTA, absence of glycerol or other polyhydroxy compounds, or high detergent concentration (above 20 mg/ml) there was almost a complete inhibition of the ATPase already after 10 min of incubation (results not shown).

Conditions which were found optimal for solubilization of the active form of ($Ca^{2+}$ + $Mg^{2+}$)-ATPase were as follows: the detergent/protein weight ratio not exceeding 5, the presence of 20% glycerol and 10–20 mM $CaCl_2$, low temperature for solubilization (0–2°C) and pH of the buffer solution between 6.0 and 7.5. Under these conditions activation of the ATPase assayed in the absence of CaM was observed. However, the CaM-stimulated enzyme activity as well as the degree of stimulation by CaM decreased after solubilization (Fig. 2A–D, buffers 2 and 3). Omission of anti-oxidizing agents from the solubilization medium had no effect on short-term preservation of the enzyme activity (results not shown).

Influence of $Ca^{2+}$ and glycerol on stabilization of detergent-solubilized ($Ca^{2+}$ + $Mg^{2+}$)-ATPase was tested for erythrocyte ghosts solubilized with either $C_{12}E_8$, Brij 36T, Triton X-100 or polydocanol at the detergent/protein weight ratio of 0.7 or 2.7. The solubilized material was centrifuged for 20 min at 105000 × *g* and the resulting supernatants were kept at 2°C under nitrogen without phospholipids added. Under these conditions 11–20% of total protein and 24–32% of total phospholipids of erythrocyte plasma membrane were solubilized at the detergent/protein weight ratio of 0.7, and 23–32% of protein and 37–46% of phospholipids at the detergent/protein weight ratio of 2.7, depending on the medium constituents.

When assayed in the absence of CaM, the enzyme activity was up to 3-fold stimulated by detergents. It

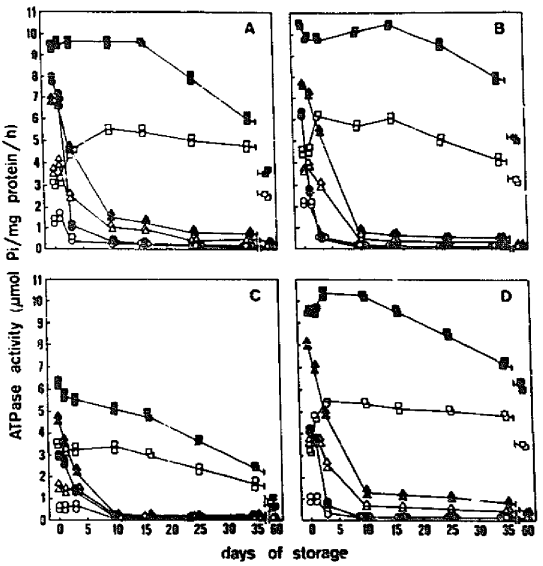


Fig. 3. Effect of $Ca^{2+}$ and 20% glycerol on the stability of ($Ca^{2+}$ + $Mg^{2+}$)-ATPase of erythrocyte ghost membranes solubilized with $C_{12}E_8$ (A), Brij 36T (B), Triton X-100 (C) and polydocanol (D) at the detergent/protein weight ratio of 2.7. Erythrocyte ghosts (3 mg protein/ml) were solubilized for 10 min at 2°C with four detergents listed, in the media described in the legend to Fig. 2 as buffers 1 (○, ●), 2 (△, ▲) and 3 (□, ■) and the nonsolubilized material was removed by centrifugation (20 min at 2°C, 105000 × *g*). The supernatants were kept under nitrogen at 2°C and the enzyme activity in the supernatants was measured immediately or after storage up to 60 days using coupled enzyme assay system with 10 μM free $Ca^{2+}$ in the absence (open symbols) or presence (closed symbols) of 0.2 μM CaM. ($Ca^{2+}$ + $Mg^{2+}$)-ATPase activity was recalculated on the basis of total protein concentration in the sample before centrifugation (3 mg/ml). It is worth mentioning that fully active form of the enzyme was always found in the supernatants and never in the pellets.

remained relatively high for at least 60 days only if the solubilized ATPase was stored in buffer 3, containing 20 mM $CaCl_2$ and 20% glycerol at pH 6.0. This was true either for detergent/protein ratio of 0.7 (not shown) or 2.7 (Fig. 3), and for all detergents tested, except for Triton X-100 which did not prevent the loss of enzymatic activity (Fig. 3C). Lowering $Ca^{2+}$ concentration to 0.1 mM, excluding glycerol from the solubilization medium or raising pH of the storage buffer resulted in rapid inactivation of the ATPase. The enzyme activity measured in the presence of CaM was preserved only in buffer 3; however, increasing the detergent/protein ratio in the solubilization medium above 5 caused a loss of the activity even in the presence of 20 mM $CaCl_2$ and 20% glycerol at pH 6.0.

Glycerol was necessary for long-term stabilization of ($Ca^{2+}$ + $Mg^{2+}$)-ATPase activity in detergent-solubilized systems. Omission of glycerol from the incubation medium induced irreversible loss of the activity, and

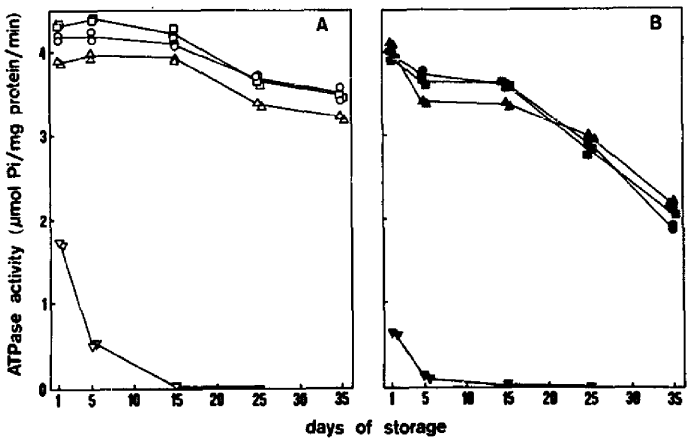


Fig. 4. Time-dependent rate of inactivation of detergent-solubilized lipid-stabilized ($Ca^{2+} + Mg^{2+}$)-ATPase purified from erythrocyte ghosts. The enzyme purified as described under Materials and Methods was kept at 2°C under nitrogen in the medium for crystallization containing 1 mg/ml of the following detergents: Triton X-100 (▽, ▼), $C_{12}E_8$ (○, ●), Brij 36T (□, ■) and polydocanol (△, ▲). Protein concentration was 0.3 mg/ml. (A) $Ca^{2+}$-dependent ATPase activity. (B) CaM-stimulated ATPase activity (calculated as a difference between CaM + $Ca^{2+}$-dependent and $Ca^{2+}$-dependent ATPase activities). Values are means for two separate enzyme preparations, which varied within ± S.E. of 10%. Three determinations per each preparation were performed (± S.E. of 2–4%).

raising $Ca^{2+}$ concentration to 20 mM could not substitute for glycerol in preservation of the ATPase activity. Therefore, as in the case of $Ca^{2+}$-ATPase of detergent-solubilized SR vesicles [10,11], stabilization of the erythrocyte enzyme activity is the result of combined effect of high $Ca^{2+}$ and relatively high glycerol concentration. Glycerol could be substituted in the storage medium by 20% ethylene glycol or 20% sucrose (results not shown).

The standard medium used for stabilization of solubilized ($Ca^{2+} + Mg^{2+}$)-ATPase included dithiothreitol (5 mM) and 1,6-di-*tert*-butyl-*p*-cresol (2 μg/ml) to prevent oxidation of SH groups and lipids, respectively, trasylol (25 IU/ml) and $PhMeSO_2F$ (0.1 mM) as inhibitors of proteinases, and $NaN_3$ (3 mM) as a bacteriostatic agent. The samples were always incubated under nitrogen with a limited access of daylight. Omission of all these protective compounds from the medium diminished the activity of the solubilized enzyme within few days of storage. This may indicate oxidative damage of the enzyme and lipid molecules and significant proteolysis of ($Ca^{2+} + Mg^{2+}$)-ATPase during long-term incubation without protectives.

*Evidence for the formation of three-dimensional crystalline arrays of purified, detergent-solubilized and phospholipid-stabilized ($Ca^{2+} + Mg^{2+}$)-ATPase*

($Ca^{2+} + Mg^{2+}$)-ATPase of erythrocyte ghost membrane was solubilized and purified by means of CaM-affinity column chromatography as described under Materials and Methods. Triton X-100 used for enzyme solubilization was replaced on the column by $C_{12}E_8$, Brij 36T or polydocanol and the purified enzyme was stored in the presence of PC, 20 mM $CaCl_2$ and 20% glycerol. Under these conditions the activity of purified ($Ca^{2+} + Mg^{2+}$)-ATPase was stable for at least 35 days (Fig. 4). It has to be pointed out that the electrophoretical pattern of the stored ATPase revealed no proteolysis of the 138 kDa polypeptide chain.

After 5 days of incubation in the medium described in the legend to Fig. 4 at 2°C under nitrogen, electron microscopy of specimens negatively stained with uranyl acetate showed random dispersion of the solubilized protein. During incubation for the next 3–4 weeks some well-organized structures developed which varied in size between 20 and 80 nm (Fig. 5). Fixation with 1% glutaraldehyde for 24 h at 2°C preserved the spatial features of these structures and glutaraldehyde-fixed preparations extracted for 4 h at 2°C with 90% acetone or with chloroform/methanol (2:1, v/v) were indistinguishable from nonextracted fixed ones. On the other hand, removal of phospholipids, elevated temperatures and high pH of the storage medium disrupted unfixed preparations.

Negatively stained (Fig. 5B, D, J) and thin sectioned (Fig. 5A, C, E–H, K) specimens revealed multilamellar character of crystalline arrays in various orientations. Two distinct patterns were observed in sectioned and negatively stained specimens. In one view (Fig. 5A–C, K), images of densities are seen that repeat every 38–40 Å. These probably represent side views of the stacked lamellae of the ATPase molecules. There are no images representing the interacting head portions of ($Ca^{2+} + Mg^{2+}$)-ATPase molecules on the surfaces of lamellae

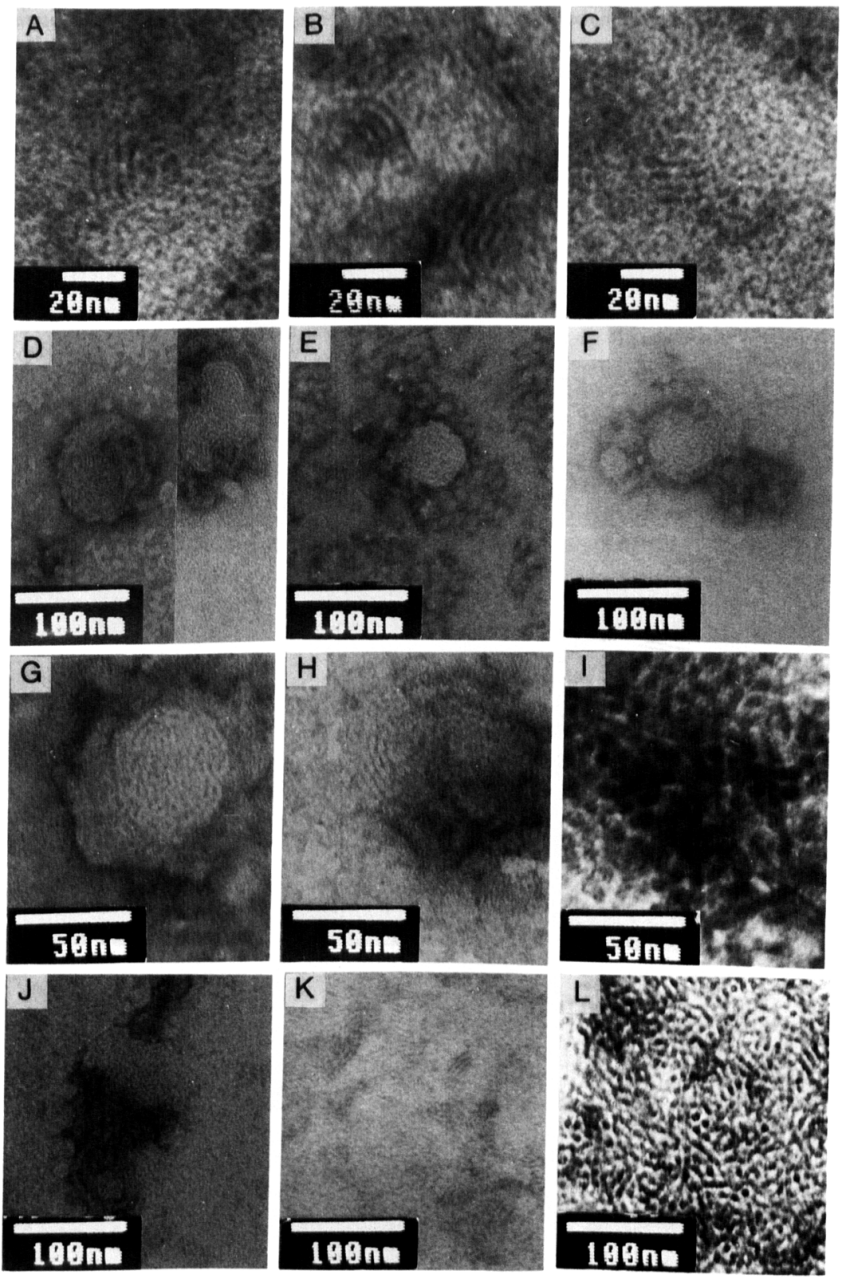


Fig. 5. Electron microscopy of detergent-solubilized and lipid-stabilized purified ($Ca^{2+}$-$Mg^{2+}$)-ATPase of erythrocyte ghosts. The solubilized enzyme (0.3 mg protein/ml) was stored in the medium for crystallization (see Materials and Methods) containing either $C_{12}E_8$ (3.3 mg/mg protein) (A–F) or Brij 36T (3.3 mg/mg protein) (G, H, J, K), in the presence of 0.2 μM CaM (E, H) or 0.005% PS (A–C, K), or with no further additions (D, F, G, J). The samples were incubated under nitrogen at 2°C for 35 days. For comparison, protein-free medium was stored under the same conditions as for the solubilized enzyme. It contained 0.25 mg PC/ml, 1 mg $C_{12}E_8$/ml and 0.005% PS (I, L). Then, the material was negatively stained (B, D, J) or thin sectioned (A, C, E–H, K). In some experiments the specimens were fixed with 1% glutaraldehyde overnight (A, F–H) or fixation was followed by extraction with either chloroform/methanol (2:1, v/v) (C, E) or 90% acetone (K) for 4 h at 2°C.

exposed to negative stain. In the second view (Fig. 5D–F, G, H, J), the projected image normal to the plane of the lamella (down the *z*-axis) shows stain-excluding particles, presumed to represent the view of interacting, cytoplasmic domain portions of the enzyme molecules (densities repeat every 36–41 Å). It is obvious

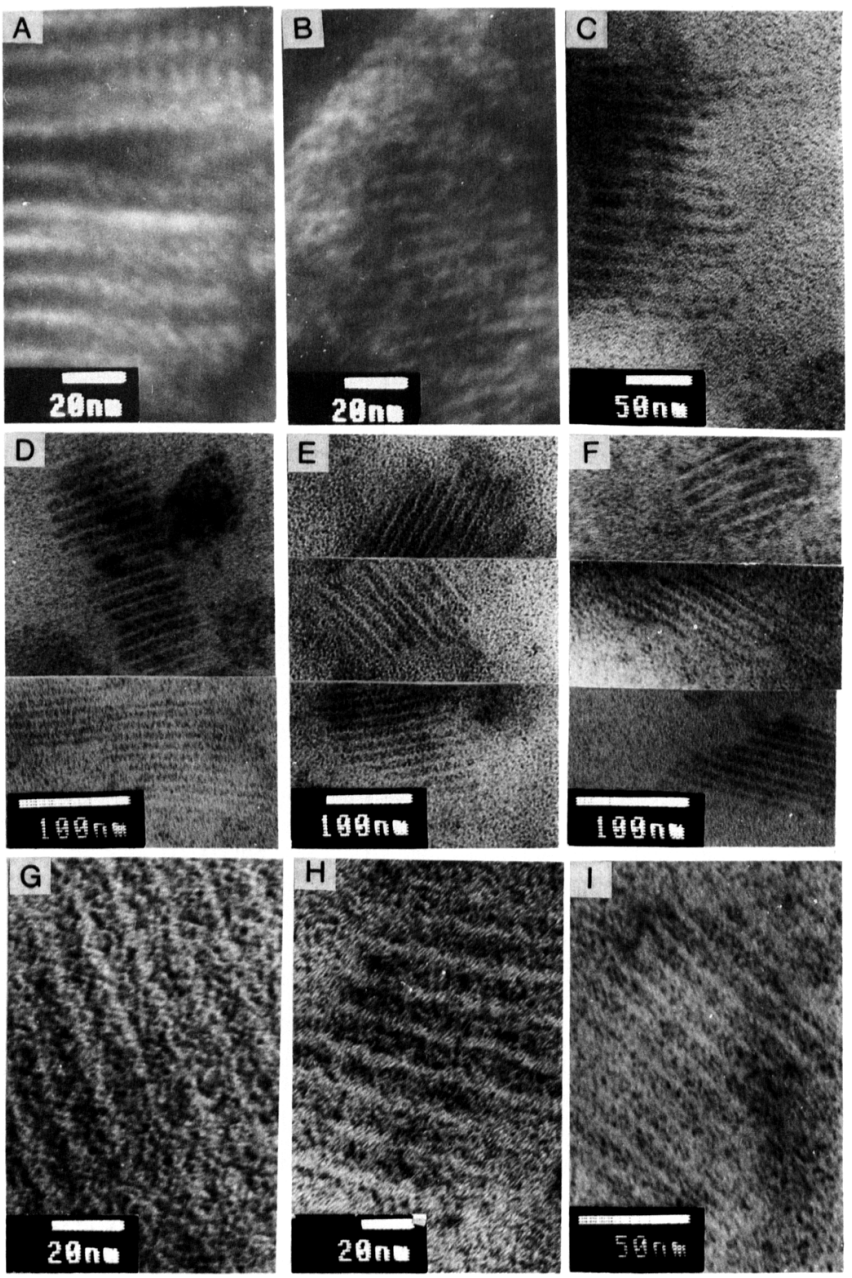


Fig. 6. Electron microscopy of SR $Ca^{2+}$-ATPase crystals of negatively stained (A, B) and thin sectioned material (C–I). SR membranes (2 mg protein/ml) were solubilized with either $C_{12}E_8$ (2 mg/mg protein) (A, B, D, E, G, H) or Brij 36T (2 mg/mg protein) (C, F, I), in the medium for crystallization with no exogenous phospholipids. Then, the samples were incubated under nitrogen at 2°C for 29 days and processed for electron microscopy as described under Materials and Methods.

that the lamellae contain detergent-lipid phase into which the hydrophobic fragments of the enzyme molecules are inserted. The lamellae then aggregate to form stacked, three-dimensional structures. Similar structures were formed in the presence of $C_{12}E_8$, Brij 36T and polydocanol, but not Triton X 100, at the detergent/protein weight ratio of 3.3. Addition of CaM or PS had no effect on the formation of microcrystalline arrays of the ATPase (Fig. 5A–C, E, H, K). Their three-dimensional character is evident from electron microscopy of thin sectioned material (Fig. 5A, C, E–H, K) and from serial sections that resolve the structure of the crystals in the third dimension (not shown).

For comparison, lipid/detergent mixtures stored in the same medium were viewed and they exhibited no similarities to the structures formed by the enzyme molecules (Fig. 5I and L; images of densities repeat every 86–88 Å). Therefore, lipid or lipid-detergent systems of the kind that may have been encountered in the solubilized erythrocyte enzyme preparation did not produce the type of ordered arrays that may be misinterpreted as protein crystals. These observations, of course, do not rule out a possible requirement for phospholipids in the formation and stabilization of the structures observed.

In Fig. 6 electron microscopy images of three-dimensional crystals of $Ca^{2+}$-ATPase of detergent-solubilized SR membranes are presented, providing comparison with the structures obtained with purified ($Ca^{2+}$ + $Mg^{2+}$)-ATPase of erythrocyte ghost membrane. Similarities in the behavior of both enzymes after solubilization include the stabilization of their activity in the presence of the same class of nonionic detergents under conditions described for $Ca^{2+}$-ATPase of SR [10–12].

TABLE I

*Comparison of crystalline arrays growing in solubilizate of SR $Ca^{2+}$-ATPase and of erythrocyte ($Ca^{2+}$ + $Mg^{2+}$)-ATPase*

Purified ($Ca^{2+}$ + $Mg^{2+}$)-ATPase of erythrocyte ghosts and $Ca^{2+}$-ATPase of SR vesicles were solubilized with $C_{12}E_8$ and stored under conditions described in the legend to Figs. 5 and 6, respectively. Then, electron micrographs of thin sectioned or negatively stained material were taken and distribution of densities were measured independently by three persons on the basis of at least ten different micrographs. Two distinct patterns were observed, one representing side views of stacked lamellae of enzyme molecules and the second showing a single lamella viewed down the *z*-axis. The average diameter of the clusters of the crystalline arrays is also shown.

| Source of crystals | Average diameter (nm) | Repetition of densities | |
|---|---|---|---|
| | | in side view (Å) | down the *z*-axis (Å) |
| SR $Ca^{2+}$-ATPase | >150 | 142–148 | 62–65 |
| Erythrocyte ($Ca^{2+}$ + $Mg^{2+}$)-ATPase | 20–80 | 38–40 | 36–41 |

However, crystalline arrays appearing during the storage of solubilized proteins were found to be different (Table I). Differences between both ion-pumping enzymes in spatial organization of observed crystals are probably due to differences in secondary and tertiary structure of reticular and plasma membrane ATPases.

## Discussion

($Ca^{2+}$ + $Mg^{2+}$)-ATPase of erythrocyte ghosts is remarkably stable in the membrane-bound form, apparently because the lipid surrounding of the protein molecules ensures perfect conditions for preservation of the enzyme functional conformation. Solubilization of this enzyme is frequently accompanied by the loss of its hydrolytic activity [2–9] and changes in the protein structure [20]. On the other hand, solubilization constitutes an obligatory step in formation of three-dimensional crystals of the protein. In order to obtain the enzyme in the active form, we looked for solubilization conditions which would stabilize ($Ca^{2+}$ + $Mg^{2+}$)-ATPase and promote the formation of crystals. It has already been demonstrated that the effect of detergents on the stability of $Ca^{2+}$-ATPase of SR membranes in solution can vary enormously, from preserving the structure and function for months [10,11] to complete inactivation and irreversible denaturation within minutes or hours [10–12,27–30]. It has been postulated that solubilization by detergents alters the structure of SR $Ca^{2+}$-ATPase, resulting in a decrease in enzyme stability [10–12,28]. This was also reflected by changes in circular dichroism spectra [31,32] and reactivity of SH groups [27].

We have confirmed observations made on SR $Ca^{2+}$-ATPase and found that the concentration of $Ca^{2+}$ and glycerol was of particular importance for the stability of detergent-solubilized erythrocyte ATPase. Calcium ions presumably induce formation of ATPase oligomers as described for $Ca^{2+}$-ATPase of solubilized SR membranes [10,11,13]. Glycerol may increase hydrophobic interactions in the nonpolar regions of the protein as postulated for oligomerization and further crystallization of detergent-solubilized SR $Ca^{2+}$-ATPase [10–12]. The presence of anti-oxidizing agents, inhibitors of proteolysis and exogeneous phospholipids, which presumably preserve the enzyme native structure, was also indispensable for long-term stabilization of solubilized ($Ca^{2+}$ + $Mg^{2+}$)-ATPase purified from erythrocyte plasma membrane. In the presence of PC the rate of inactivation was several-times slower than in its absence.

Under conditions described for stabilization of purified erythrocyte ($Ca^{2+}$ + $Mg^{2+}$)-ATPase formation of crystalline structures of the enzyme was observed. The three-dimensional character of these structures was suggested by electron microscopy of thin sectioned material

and from serial sections. Similar structure was observed neither in extracted erythrocyte membrane lipids nor in lipid/detergent mixtures. Moreover, these structures, fixed with glutaraldehyde, were resistant to extraction with organic solvents. They presumably represent the so-called pseudo three-dimensional crystals of membraneous proteins (for review see Ref. 33). The three-dimensional structures of purified detergent-solubilized ($Ca^{2+}$ + $Mg^{2+}$)-ATPase of erythrocyte ghosts obtained in the present study appeared essentially different from structures formed under similar conditions from $Ca^{2+}$-ATPase of solubilized SR membranes [10–12]. It seems that molecules of the erythrocyte enzyme are more densely packed within the crystalline structure than those of $Ca^{2+}$-ATPase of SR. The reason for this difference in packing density is obscure so far. One can speculate that molecules of the erythrocyte ATPase intercalate asymmetrically into the lipid-detergent layer. For these reasons the observed crystals may appear as structures consisting of densely stacked particles confined into a smaller unit cell than that of SR enzyme crystalline arrays.

It is already well established that membrane proteins can form three-dimensional [34–36] as well as two-dimensional crystals described for example for SR $Ca^{2+}$-ATPase [33,37–40]. Pseudo-three-dimensional crystals can be thought of as stacks of two-dimensional crystals. As pointed out by Michel [35], it is difficult to grow large crystals of this type, because conditions that facilitate polar interactions between layers tend to destabilize hydrophobic interactions within them. Therefore, these crystals are usually too small for X-ray crystallography, but suitable for structural studies by electron microscopy. For this reason, further effort is required to grow real three-dimensional crystals of erythrocyte ($Ca^{2+}$ + $Mg^{2+}$)-ATPase which would resemble those of soluble proteins [33,35,36].

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