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RECONSTITUTION OF SARCOPLASMIC RETICULUM Ca^{2+}-ATPase WITH EXCESS LIPID

DISPERSION OF THE PUMP UNITS

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Sarcoplasmic reticulum Ca^{2+}-ATPase has been reconstituted with excess lipid (25–150 g egg phosphatidylcholine per g sarcoplasmic reticulum protein) by a procedure combining the use of a non-ionic detergent with cholate dialysis. The reconstituted vesicles were analyzed by sucrose density fractionation and freeze-fracture electron microscopy. At the lowest lipid to protein ratios some vesicles containing aggregated protein were observed. At a lipid to protein ratio of 150:1 (w/w) only 30–40% of the reconstituted protein sedimented through 7% (w/v) sucrose. The remainder of the latter prepration was characterized by a high Ca^{2+}-uptake capacity and a coupling ratio of 1.6 mol Ca^{2+} transported per mol ATP hydrolyzed. Intramembranous particles in this preparation occurred isolated in the membrane. In most cases only one particle could be seen on a fracture face. Cross-linking with cupric phenanthroline indicated that protein-protein contacts were drastically reduced by reconstitution. It is concluded that aggregation of intramembranous particles is not required for optimal Ca^{2+}-transport function. The dispersed preparation obtained by a combined reconstitution and sucrose density fractionation procedure is useful for further characterization of the Ca^{2+} pump.

Introduction

The active transport of Ca^{2+} through the membrane of sarcoplasmic reticulum is conducted by a (Ca^{2+} + Mg^{2+})-activated ATPase. This protein can be solubilized in monomeric form (M_r = 115000) with retention of full enzymatic activity [1,2]. However, the size of the Ca^{2+}-transporting unit is unknown. Several studies have provided evidence that the Ca^{2+}-ATPase polypeptides form oligomeric complexes in the membranous state (for a review of this topic see Ref. 3). The specificity and functional significance of such protein-protein interactions are difficult to evaluate due to the high density of peptide chains present in sarcoplasmic reticulum (lipid to protein ratio approx. 0.6 (w/w)). It is therefore important to be able to study the properties of Ca^{2+}-ATPase dispersed in an excess of lipid. Membrane reconstitution should offer an opportunity to accomplish this objective. However, recent attempts to dilute Ca^{2+}-ATPase by excess phospholipid in reconstituted vesicles have failed to produce functional vesicles with

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Abbreviations: $C_{12}E_8$, octaethyleneglycol monododecyl ether; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; SDS, sodium dodecyl sulphate; DTNB, 5,5′-dithiobis(2-nitrobenzoate); Tes, 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino)ethanesulphonic acid.

lipid to protein ratios higher than 2 (w/w) [4,5].

In the present communication we describe the preparation of reconstituted Ca^{2+}-ATPase dispersed in a large excess of lipid. Evidence is presented that the active Ca^{2+}-transport is performed by the individual intramembranous particles observed after freeze-fracture. The preparation should prove useful for a delineation of the size and other properties of the Ca^{2+} pump.

Materials and Methods

Lipids and detergents. Egg phosphatidylcholine was the product of Lipoid KG, Papenburg (nominal purity 99%). Thin-layer chromatography on Silica gels did not reveal any impurities. Sarcoplasmic reticulum lipids were prepared according to Wang et al. [4]. $C_{12}E_8$ was obtained from Nikko Chemicals, Tokyo, and the (1-^{14}C)-labelled $C_{12}E_8$ from C.E.A. Saclay. Sodium cholate (Merck AG, Darmstadt) was used without further purification. [^{14}C]cholic acid was from The Radiochemical Centre, Amersham.

Ca^{2+}-ATPase preparations. Sarcoplasmic reticulum vesicles were isolated according to De Meis and Hasselbach [6], and stored in 1 mM Hepes (pH 7.5) and 0.3 M sucrose at −80°C. Soluble Ca^{2+}-ATPase was prepared by mixing 3 mg sarcoplasmic reticulum protein with 6 mg $C_{12}E_8$ in 750 μl of buffer A (30 mM Tris, 0.4 M KCl, 0.4 M sucrose, 4 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol, 1 mM NaN_3 (pH 7.1)) followed by centrifugation in a Beckman Airfuge for 20 min at 130000 × *g*. The clear supernatant comprising about 80% of the total protein was used for reconstitution.

Reconstitution. To obtain a preparation with a lipid to protein ratio of approx. 150 : 1 (w/w) 100 μl of the $C_{12}E_8$ solubilized Ca^{2+}-ATPase was mixed with 60 mg egg phosphatidylcholine or sarcoplasmic reticulum lipid, dissolved in 3 ml of buffer A containing 1% (w/v) sodium cholate. The clear mixture was dialyzed for 60 h at 5°C against 2 litre of buffer B (0.1 M sodium phosphate, 4 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol, 1 mM NaN_3 (pH 7.1)) which was changed six times during the dialysis period. Addition of radioactive tracers of the detergents showed that the preparation contained less than 0.1 mg/ml cholate and approx. 0.1 mg/ml $C_{12}E_8$ after 48 h of dialysis. This corresponds to removal of 99% and 60% of the two detergents, respectively. Frequent buffer changes were more important as a means of removing detergent than addition of Amberlite (XAD-2) or Bio-Beads (SM-2, BioRad). In a few experiments the lipid to protein ratio was lower than described above. This was accomplished by increasing the added volume of solubilized protein. When samples were prepared for cross-linking experiments dithiothreitol was omitted from buffer B durin the last 24 h of dialysis. This ensured a complete removal of dithiothreitol as indicated by the absence of DTNB-reactive sulfhydryl groups in control liposomes prepared without protein and solubilized in SDS [7].

Measurement of Ca^{2+}-transport and ATP-hydrolysis. Reconstituted vesicles (10 μg protein/ml) were incubated at 25°C in buffer C (30 mM imidazol, 0.3 M NaCl, 4 mM $MgCl_2$, 1 mM EDTA, 1 mM NaN_3 (pH 7.1)) to which had been added 0.1 mM $^{45}CaCl_2$, 5 mM MgATP, 5 mM phospho*enol*pyruvate, NADH, pyruvate kinase and lactate dehydrogenase as previously described [8]. The ATP hydrolysis rate was followed by the decrease in absorbance at 340 nm, and aliquots were taken at different time intervals for simultaneous measurement of Ca^{2+} uptake. The samples were layered on Sephadex G-50 columns (1 × 25 cm) (Pharmacia) at 0°C and eluted with buffer C at 0°C. The radioactivity eluting together with the vesicles in the void volume was collected within 2–3 min and measured by liquid scintillation counting. Active Ca^{2+} uptake was calculated after subtraction of the radioactivity associated with vesicles incubated in the absence of ATP. With this procedure Ca^{2+} uptake is measured in the presence of intravesicular phosphate as a Ca^{2+}-precipitating agent, while phosphate is absent from the outer medium. This could not be successfully performed with sarcoplasmic reticulum vesicles, since these are leaky to phosphate. Therefore, Ca^{2+} uptake by sarcoplasmic reticulum vesicles was measured in buffer B (without dithiothreitol) instead of buffer C. When this procedure was applied to the reconstituted systems the measured uptake rates decreased to approx. 70%.

Gel chromatography. The reconstituted vesicles were characterized by chromatography on a Seph-

arose 2B column (1.5 × 90 cm) (Pharmacia) equilibrated with buffer B without dithiothreitol. The dithiothreitol-free preparation described above was used in this case.

Electron microscopy. After concentration on an Amicon M3 device the reconstituted vesicles were equilibrated for 3–4 h at 4°C with glycerol added to a final concentration of about 25% (v/v). Aliquots were quickly frozen in Freon 22 cooled by liquid nitrogen. Freeze-fracture was carried out in a Balzers BAF 300 freeze-fracture apparatus (Balzer AG, Lichtenstein). The specimens were fractured at −120°C and immediately shadowed with platinum at an angle of 45° and replicated with carbon. After cleaning in sodium hypochlorite, rinsing in water and further cleaning in a mixture of 50% ether in methanol the replicas were placed on 200-mesh grids and examined in a Jeol 100 CX electron microscope at 80 kV.

The diameters of the fracture faces from equatorially fractured vesicles were measured as described before [9]. The diameters of the intramembranous particles were measured perpendicular to the shadow direction.

Sucrose density fractionation. In some experiments the preparation was examined after removal of the most protein rich vesicles. This was accomplished by centrifugation on 7% (w/v) sucrose (in buffer B) for 17 h in an S.W. 50.1 rotor at 48 000 r.p.m. in a Beckman preparative ultracentrifuge. After centrifugation the vesicles formed a sharp band on top of the 7% sucrose which was carefully collected.

Cross-linking and SDS-gelelectrophoresis. To 10 ml of reconstituted vesicles (approx. 0.1 mg protein per ml) was added 0.1 mM $CuSO_4$ and 0.3 mM 1,10-phenanthroline (EGA-Chemie). The reaction was stopped after 5 min by addition of 5 mM EDTA. Then the sample was solubilized in 3% (w/v) SDS and heated in boiling water until clarification was complete (approx. 5 min). After concentration on the Amicon M3 device to approx. 2 ml the sample was applied to a Sepharose 6B column (1.5 × 90 cm) equilibrated with 50 mM NaCl, 10 mM Tes, 0.2% SDS, 1 mM NaN_3 (pH 7.5). The protein which eluted before the lipid peak was collected (including the void volume) and concentrated to approx. 700 µl. Then SDS-gel electrophoresis (in absence of reducing agent) was performed in 3.0% polyacrylamide slab gels containing 0.5% agarose [10]. A control sample not treated with cupric phenanthroline was also run. Sarcoplasmic reticulum vesicles (1 mg/ml in buffer B without dithiothreitol) were treated for 5 min with the same concentrations of $CuSO_4$ and phenanthroline as described above, and applied directly to the gel after solubilization and heating in SDS in the absence of reducing agent. The degree of cross-linking obtained in this case was largely independent of the protein concentration. A similar procedure was used for sarcoplasmic reticulum which was solubilized in $C_{12}E_8$ (2 mg/mg protein in buffer B without dithiothreitol) and centrifuged on the Beckman Airfuge as described above.

Other methods. Protein was determined by a modified Lowry procedure to avoid the interfering influence of lipids [11], and phospholipids were measured according to Bartlett [12]. Tryptophan fluorescence of protein was measured on a Perkin-Elmer spectrofluorometer (excitation and emission wavelengths 295 and 325 nm, respectively). Liquid scintillation counting was done with a Packard scintillation counter, using Lumagel (BN Plastics) as scintillation fluid.

Results

Ca^{2+} uptake

Fig. 1 shows the time course of active Ca^{2+} uptake in sarcoplasmic reticulum and in Ca^{2+}-ATPase vesicles reconstituted with either egg phosphatidylcholine or extracted sarcoplasmic reticulum lipid. The Ca^{2+} uptake in Ca^{2+}-ATPase/egg phosphatidylcholine vesicles centrifuged on 7% sucrose (see Materials and Methods) is also shown. The reconstituted vesicles are characterized by Ca^{2+}-uptake capacities per mg protein which are higher than for sarcoplasmic reticulum. Especially the reconstituted vesicles which have been centrifuged on 7% sucrose have a high uptake capacity. Equilibration is not attained after 1 h in the reconstituted systems whereas it is reached within 10–15 min in sarcoplasmic reticulum. Protein determination indicated that 30–40% of the total protein sedimented into the 7% sucrose. The sedimented material may represent vesicles of high protein content and therefore relatively low uptake

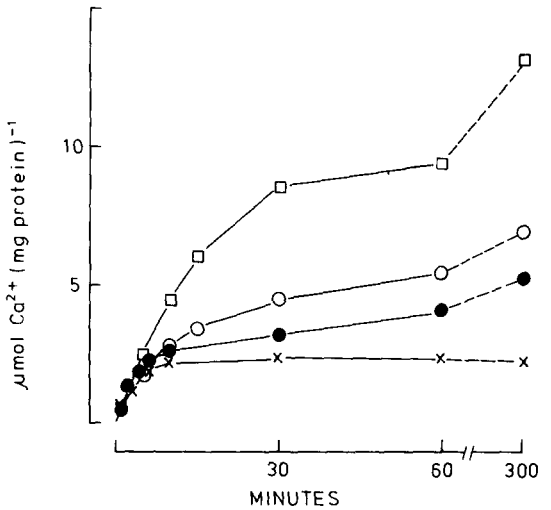

Fig. 1. Active Ca^{2+}-uptake in sarcoplasmic reticulum vesicles (×), Ca^{2+}-ATPase vesicles reconstituted with extracted sarcoplasmic reticulum lipid (100 mg phospholipid/mg protein) (●), Ca^{2+}-ATPase vesicles reconstituted with egg phosphatidylcholine (150 mg/mg protein) (○) and in the same preparation as (○) after centrifugation on 7% sucrose (□).

capacity per mg protein. Alternatively, it represents unincorporated protein. However, the latter possibility alone cannot account for the large increase in uptake capacity induced by centrifugation (see Fig. 2 and Table I).

Table I shows Ca^{2+}-ATPase activity and Ca^{2+}-transport rates measured within the first minute after addition of ATP to the preparations described above. The initial Ca^{2+}-uptake rates per mg protein do not differ significantly among the various reconstituted preparations shown and are somewhat lower for these than for the native sarcoplasmic reticulum. ATP hydrolysis rates measured during Ca^2 transport were about half that of the native preparation. However, after solubilization with $C_{12}E_8$ no significant difference in enzyme activity was observed, indicating that the protein was not partially inactivated during reconstitution. The lower ATP hydrolysis rate in reconstituted vesicles may reflect random insertion of ATPase in the membrane resulting in inaccessibility of ATP to half of the phosphorylation sites. The calculated coupling ratios (ratio between Ca^{2+} uptake and ATP hydrolysis in the membranous state) range between 1 and 2. These coupling ratios are higher than those obtained in most other reports [4,13–17]. Lower lipid to protein ratios (less than 50 : 1 (w/w)) as well as omission of dithiothreitol during dialysis led to a significant

TABLE I

FUNCTIONAL CHARACTERIZATION OF RECONSTITUTED Ca^{2+}-ATPase AND SARCOPLASMIC RETICULUM VESICLES

All data are average values of determinations made on at least two similar preparations. Measurements were performed as described in Materials and Methods.

Preparation	Initial Ca^{2+}-transport rate ($nmol \cdot mg^{-1} \cdot min^{-1}$)	Ca^{2+}-ATPase ($\mu mol \cdot mg^{-1} \cdot min^{-1}$)		Ca^{2+}/ATP (mol/mol)
		Membranes	Solubilized [a]	
Sarcoplasmic reticulum vesicles	711	0.60	2.88	1.19
Vesicles reconstituted with sarcoplasmic reticulum lipid (100 mg/mg protein)	462	0.37	2.59	1.25
Vesicles reconstituted with egg phosphatidylcholine (150 mg/mg protein)	437	0.33	3.03	1.32
Vesicles reconstituted with egg phosphatidylcholine (150 mg/mg protein) and centrifuged on 7% sucrose	566	0.35	3.21	1.62

[a] 2 mg $C_{12}E_8$/ml was present during the assay.

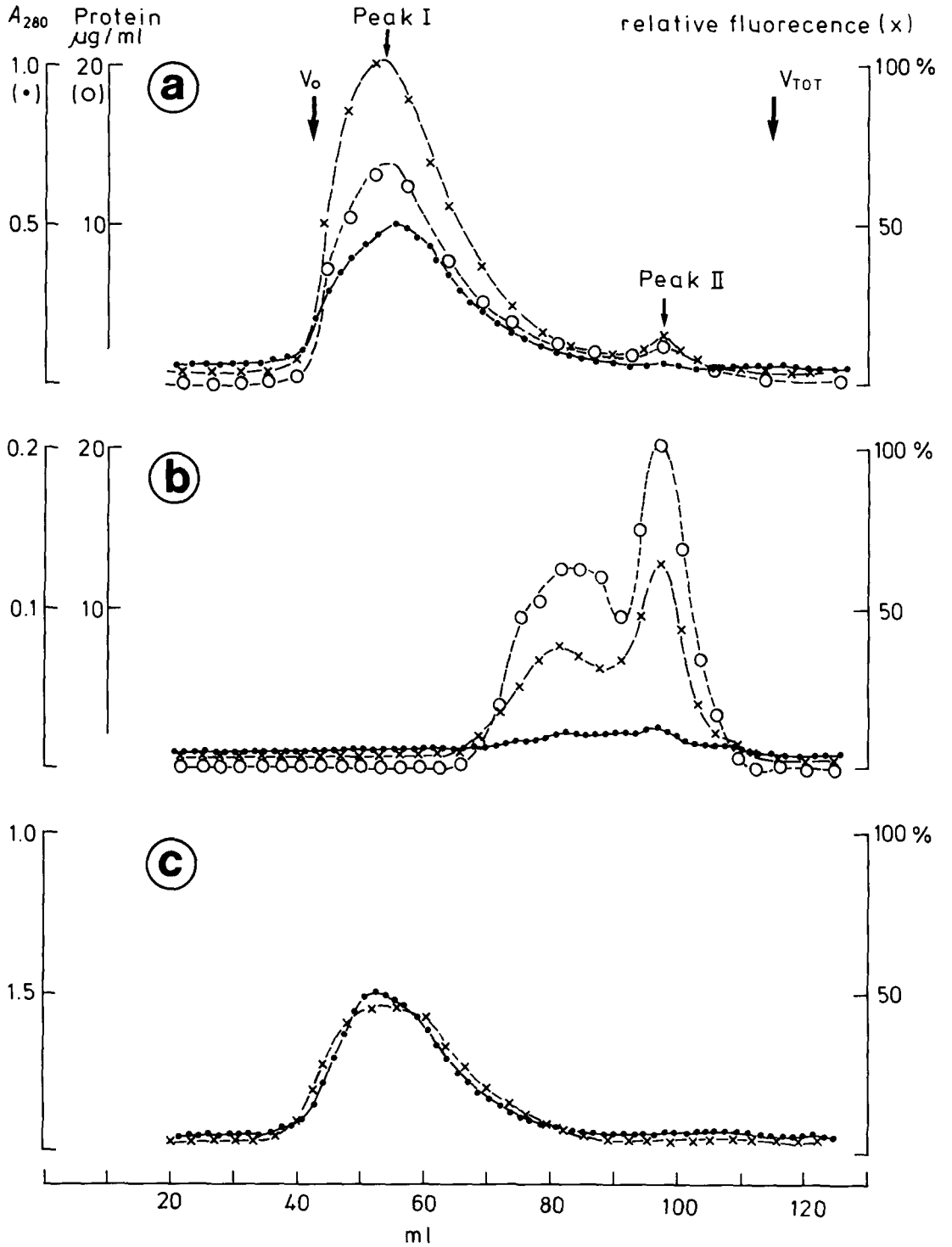

Fig. 2. Sepharose 2B chromatography of (a) 0.37 mg Ca^{2+}-ATPase reconstituted with egg phosphatidylcholine (100 mg phospholipid/mg protein), (b) 0.5 mg Ca^{2+}-ATPase solubilized by 1 mg $C_{12}E_8$ and (c) 40 mg egg phosphatidylcholine vesicles reconstituted without protein. Fluorescence intensity (λ_{ex} 295 nm, λ_{em} 325 nm) is shown relative to the peak value in (a).

decrease in the coupling ratio (not shown). Also the ATPase activity measured after solubilization was reduced in these conditions.

Chromatographic characterization of the reconstituted vesicles

The degree of incorporation of Ca^{2+}-ATPase protein into the reconstituted vesicles was examined by gel filtration on Sepharose 2B. Measurement of tryptophan fluorescence (excitation at 295 nm and emission at 325 nm) was used as a sensitive method to be able to observe traces of protein. Fig. 2a shows an experiment with Ca^{2+}-ATPase/egg phosphatidylcholine vesicles having an average lipid to protein ratio of 100 : 1 (w/w). The reconstituted Ca^{2+}-ATPase elutes as a broad peak (Peak I), which starts at the void volume and has the same elution characteristics as a preparation of pure egg phosphatidylcholine liposomes (Fig. 2c). The measured fluorescence is much higher in the reconstituted Ca^{2+}-ATPase vesicles than in the liposomes. Since we did not find evidence for protein impurities in the egg phosphatidylcholine, the apparent fluorescence detected in the latter case presumably is caused by scattered light [18]. A miniscule peak (Peak II) of material that is Lowry positive and fluorescent is observed close to the total volume of the column. This peak contributes less than 10% of the protein content of Peak I and probably represents some Ca^{2+}-ATPase or non-ATPase protein from the sarcoplasmic reticulum which remained solubilized after reconstitution. When solubilized Ca^{2+}-ATPase is applied directly to the column (Fig. 2b) without prior reconstitution the protein emerges as two peaks at positions corresponding to oligomeric and monomeric/dimeric Ca^{2+}-ATPase (cf. Ref. 19). The monomer/dimer elutes at the same position as the 'Peak II protein' of Fig. 2a. When reconstitution was performed with 4 times as much protein as in Fig. 2a (i.e. a lipid to protein ratio of 25 : 1 (w/w)) significant amounts of 'Peak II protein' were observed (approx. 35%, not shown).

Electron microscopy

Freeze-fracture of Ca^{2+}-ATPase vesicles reconstituted with egg phosphatidylcholine in a ratio of 150 mg phospholipid per mg protein revealed a uniform population of unilaminar vesicles (Fig. 3). The average diameter of the vesicles fractured near their equatorial plane was about 700 Å (Figs. 3 and 5). Ca^{2+}-ATPase reconstituted with extracted sarcoplasmic reticulum lipid showed a higher degree of heterogeneity with respect to vesicle diameter, which varied from 300 Å to 5000 Å (Fig. 4). Occasionally multilamellar structures were seen. In both kinds of preparations intramembranous particles were only observed on a small fraction of the vesicle fracture faces (2–3%). In vesicles reconstituted with egg phosphatidylcholine the particles were equally distributed between convex and concave fracture faces. The ratio between the number of particles on convex and concave fracture faces was 1.03 when counted on 5800 fracture faces showing 124 particles. The diameter of the intramembranous particles measured on a separate series of high resolution electron micrographs was 90 ± 10 Å (mean $\pm$ S.D., $n = 44$). This value is similar to the diameter of the intramembranous particles in native sarcoplasmic reticulum [20].

Freeze-fracture studies were performed on the egg phosphatidylcholine vesicles both before and after the sucrose density frctionation (Figs. 5 and 6). Most of the particles in the uncentrifuged specimen (lipid to protein ratio 150 : 1 (w/w)) and all particles observed after centrifugation on 7% sucrose occurred isolated in the membrane (minimum separation 250 Å). In the uncentrifuged preparation two fracture faces containing aggregates of intramembranous particles were observed per 2600 fracture faces. The aggregates were found more constantly in preparations with a lower lipid to protein ratio (Fig. 6). After centrifugation of the preparation with a lipid to protein ratio of 150 : 1 (w/w) on 7% sucrose we found a total of 74 particles per 3200 fracture faces. Of these 62 were observed as one particle on one fracture face while 12 occurred as two well separated particles on one fracture face. None of the observed fracture faces contained more than two particles.

Intramembranous particles were never observed when egg lecithin vesicles were reconstituted in absence of Ca^{2+}-ATPase protein.

Cross-linking

The dispersion of Ca^{2+}-ATPase in the unfractionated egg phosphatidylcholine vesicles was also tested by the use of cupric phenanthroline which

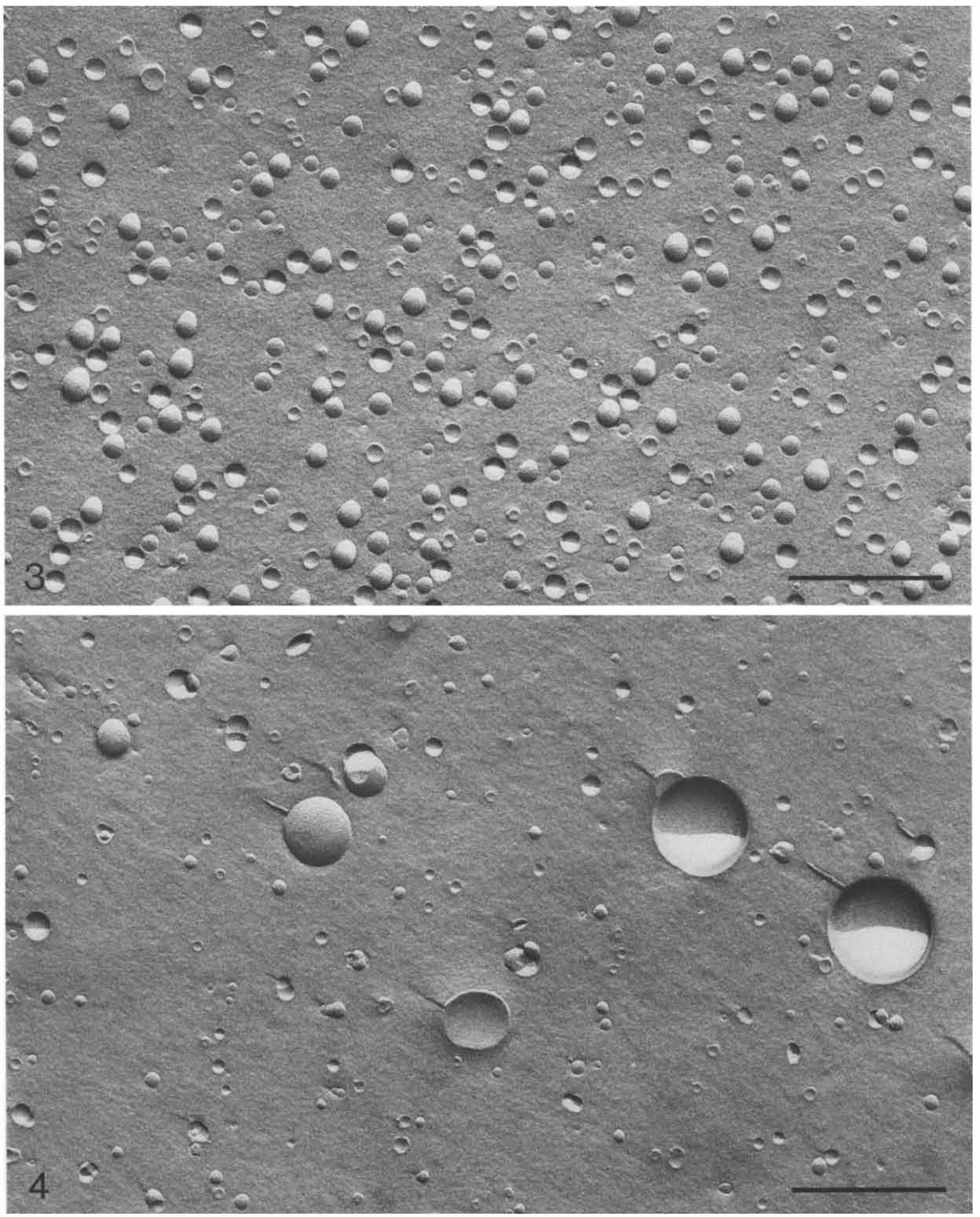

Fig. 3. Electron micrograph of a freeze-fracture replica of egg phosphatidylcholine vesicles reconstituted with Ca^{2+}-ATPase (150 mg phospholipid/mg protein) showing a uniform population of vesicles. Shadow direction from below. Bar 0.5 μm. Magnification 50000×.

Fig. 4. Electron micrograph of freeze-fracture replica of sarcoplasmic reticulum lipid vesicles reconstituted with Ca^{2+}-ATPase (100 mg phospholipid/mg protein) showing a high degree of heterogeneity in size of the vesicles. Direction of shadowing from below. Bar 0.5 μm. Magnification 50000×.

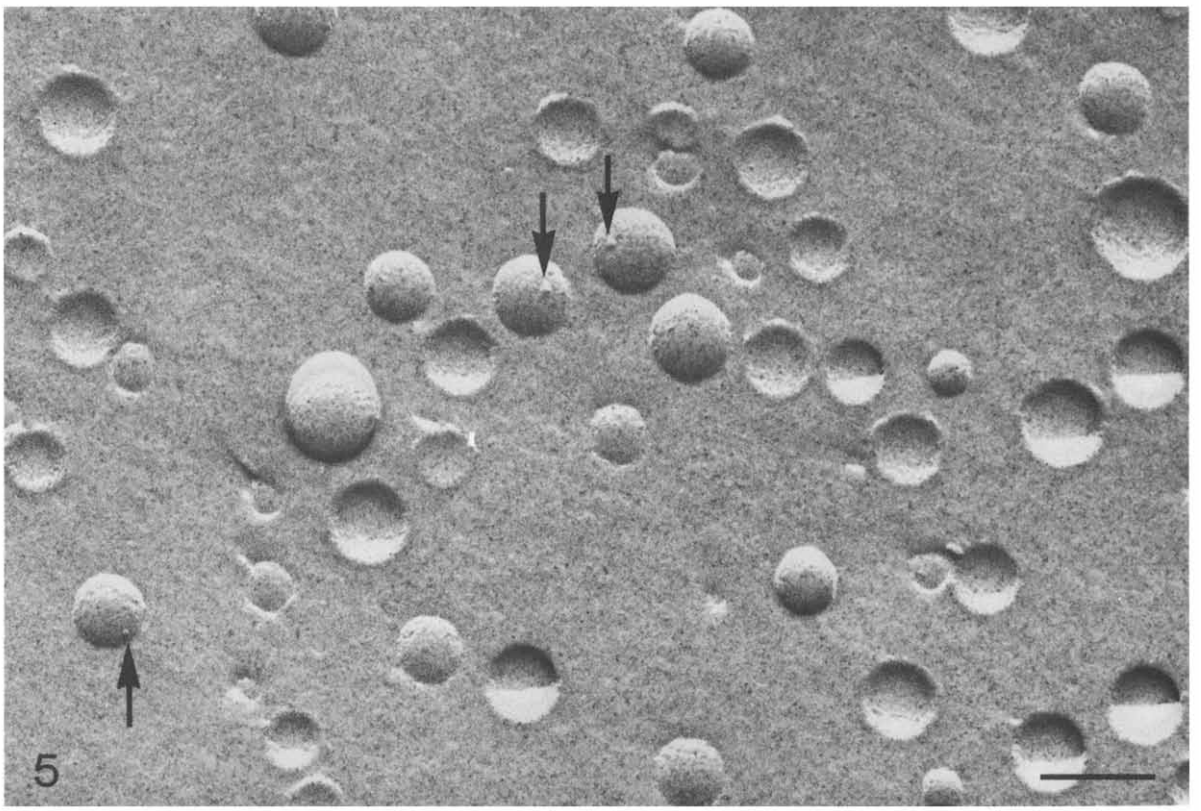

Fig. 5. Higher magnification of the same preparation as shown in Fig. 3 after sucrose density density fractionation. Arrows indicate single intramembranous particles. Bar 0.1 μm. Magnification 150000×.

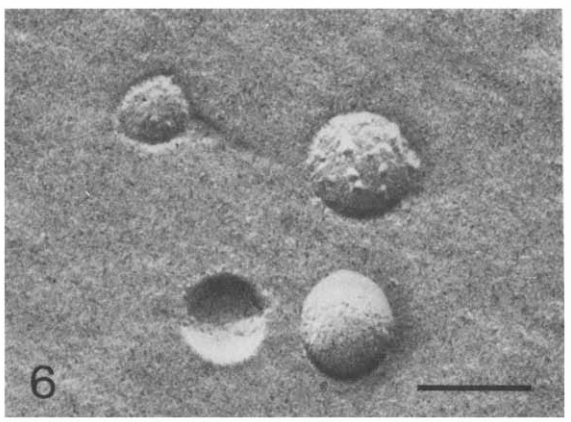

Fig. 6. Fracture-face with aggregate of intramembranous particles found in a preparation of egg phosphatidylcholine vesicles reconstituted with Ca^{2+}-ATPase (100 mg phospholipid/mg protein) before sucrose density fractionation. Bar 0.1 μm. Magnification 150000×.

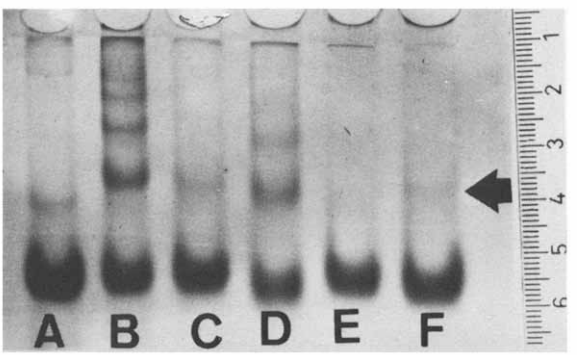

Fig. 7. SDS-gel electrophoresis of cupric phenantholine-treated (B, D, F) and non-treated (A, C, E) prepartions. (A, B) native sarcoplasmic reticulum vesicles; (C, D) Ca^{2+}-ATPase solubilized in $C_{12}E_8$ at a ratio of 2:1 (w/w); (E, F) Ca^{2+}-ATPase reconstituted with egg phosphatidylcholine (150 mg phospholipid/mg protein). Arrow indicates the dimeric band.

has been shown to be an efficient cross-linker of Ca^{2+}-ATPase in sarcoplasmic reticulum [21–24]. Fig. 7 shows that sarcoplasmic reticulum is extensively cross-linked with distinct formation of dimers, trimers and higher aggregates of the Ca^{2+}-ATPase. By contrast the protein in reconstituted vesicles (egg phosphatidylcholine to protein ratio 150 : 1 (w/w)) is much less susceptible to cross-linking under the same conditions. A very faint band corresponding to a dimer of Ca^{2+}-ATPase is observed (Fig. 7F), which is totally absent in the sample not treated with Cu^{2+}-phenanthroline (Fig. 7E). Under similar conditions $C_{12}E_8$ solubilized Ca^{2+}-ATPase is less extensively cross-linked than the sarcoplasmic reticulum but more so than the reconstituted sample: the proportion of dimer is higher and a distinct trimeric band is also observed (Fig. 7D).

Discussion

It has previously been observed that Ca^{2+}-ATPase protein tends to be distributed heterogenously in reconstitution experiments, forming a mixture of protein-rich vesicles and pure liposomes [4,5]. This has raised the question whether the tendency for aggregation of Ca^{2+}-ATPase particles is an inherent feature of the structure and Ca^{2+}-transport function of the protein in the membrane.

Our results demonstrate that Ca^{2+}-ATPase can be reconstituted with more than 60% of the protein occurring in a dispersed state. Our preparation exhibited both a high Ca^{2+}-uptake capacity and coupling ratio (mol Ca^{2+} transported per mol ATP hydrolyzed). The Ca^{2+}-uptake capacity was further increased after centrifugation on 7% sucrose ($\rho = 1.03$ g/cm^3). In the centrifuged specimen all intramembranous particles observed occurred isolated in the bilayer, most of them as one particle on a fracture face. Thus the presence of intramembranous particles in aggregated form as in native sarcoplasmic reticulum does not seem to be required for high Ca^{2+}-transport activity.

The observed frequency of intramembranous particles in the centrifuged preparation was 74 per 3200 fracture faces. Since the observed fracture faces represent a random sample and can be estimated to constitute about 10% of the total fracture face area of the vesicle membrane it is evident that the proportion of the vesicles, which contain intramembranous particles, is at most 23%. Of these as many as 90% should contain only one particle, assuming a random distribution. However, in our experiments we observed 6 fracture faces with two particles out of 68 fracture faces with particles, which is more than expected. Thus it cannot be excluded that a relatively large fraction of the vesicles contains more than one particle.

We have evidence that the use of a high lipid to protein ratio is essential for dispersion of Ca^{2+}-ATPase since aggregation was more frequently observed at lower lipid to protein ratios. In the studies by Wang et al. [4] and Konigsberg [5] the maximal lipid to protein ratios used were much lower than in our experiments. Results similar to the present data have previously been obtained for $(Na^+ + K^+)$-ATPase, also by the use of a large excess of phospholipid [9]. Other factors of importance for the present results may have been the use of a high concentration of dithiothreitol and of a nonionic detergent for solubilization of the Ca^{2+}-ATPase, which ensures a predominantly monomeric state of the soluble protein before reconstitution [19].

Our transport data contrast with those of Wang et al. [4], who reported that the coupling ratio measured in presence of oxalate decreased when the lipid to protein ratio of their reconstituted vesicles was increased. However, oxalate was added after reconstitution and the oxalate permeability of the membrane may vary with the lipid to protein ratio. It was subsequently found by these authors that in the absence of oxalate the coupling ratio was independent of the lipid to protein ratio [25]. In our study, using phosphate as a Ca^{2+} precipitating anion, a sufficient concentration in the intravesicular space was ensured by the presence of phosphate in the dialysis buffer.

Since the Ca^{2+}-uptake capacity was considerably increased by sucrose density fractionation the predominant part of Ca^{2+}-uptake capacity presumably is localized in the vesicles with a low protein content. However, the results of Table I indicate that the initial rate of uptake (in terms of μmol Ca^{2+}/mg protein per min) is almost the same before and after centrifugation. This suggests that the protein-rich vesicles do participate in Ca^{2+}

transport, but are saturated at a lower level of intravesicular Ca^{2+} per mg protein. This is consistent with the idea that the intravesicular volume per Ca^{2+}-transporting unit is an important determinant of the Ca^{2+}-uptake capacity. The intravesicular volume of sarcoplasmic reticulum vesicles is about 10-times larger than that of the reconstituted egg phosphatidylcholine vesicles (the average diameter of sarcoplasmic reticulum vesicles is indicated to be about 1500 Å [20]). In spite of this difference the Ca^{2+}-uptake capacity of the fractionated reconstituted egg phosphatidylcholine vesicles was approx. 5-times higher than that of sarcoplasmic reticulum vesicles. This is in accordance with an extensive dilution of the Ca^{2+}-transporting units in the bilayer lipid.

Cross-linking has been used by several authors to obtain information about the aggregational state of the Ca^{2+}-ATPase polypeptides in the native membrane [21–24]. The cross-linking data shown in Fig. 7 further attest to the dispersed state of the Ca^{2+}-ATPase protein in the reconstituted vesicles. The reconstituted Ca^{2+}-ATPase (Fig. 7F) was less susceptible to cross-linking by cupric phenanthroline than both the native membrane (Fig. 7B) and detergent solubilized Ca^{2+}-ATPase (Fig. 7D) which under the conditions of the cross-linking assay consists of approx. 70% monomer and 30% of higher aggregates (unpublished ultracentrifugation data). The small amount of covalently coupled dimer observed in the reconstituted preparation may represent associations between polypeptide chains contained within the individual intramembranous particles. However, it should be noted that cross-linking was performed on unfractionated preparations of reconstituted Ca^{2+}-ATPase. Hence it cannot be excluded that cross-linking resulted from the presence of vesicles containing aggregated particles.

In conclusion, by various criteria we have obtained evidence for dispersion of the Ca^{2+} pumps by our reconstitution procedure. Further studies on this preparation should be useful for the assessment of the subunit structure of the Ca^{2+} pump in the membrane.

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