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Reconstitution of the sarcoplasmic reticulum Ca^{2+}-ATPase: mechanisms of membrane protein insertion into liposomes during reconstitution procedures involving the use of detergents

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The Ca^{2+}-ATPase from skeletal muscle sarcoplasmic reticulum was reconstituted into sealed phospholipid vesicles using the method recently developed for bacteriorhodopsin (Rigaud, J.L., Paternostre, M.T. and Bluzat, A. (1988) Biochemistry 27, 2677–2688). Liposomes prepared by reverse-phase evaporation were treated with various amounts of Triton X-100, octyl glucoside, sodium cholate or dodecyl octa(oxyethylene) glycol ether ($C_{12}E_8$) and protein incorporation was studied at each step of the liposome solubilization process by each of these detergents. After detergent removal by SM-2 Bio-Beads the resulting vesicles were analyzed with respect to protein incorporation by freeze-fracture electron microscopy, sucrose density gradients and Ca^{2+} pumping measurements. The nature of the detergent used for reconstitution proved to be important for determining the mechanism of protein insertion. With octyl glucoside, direct incorporation of Ca^{2+}-ATPase into preformed liposomes destabilized by saturating levels of this detergent was observed and gave proteoliposomes homogeneous in regard to protein distribution. With the other detergents, optimal Ca^{2+}-ATPase pumping activities were obtained when starting from Ca^{2+}-ATPase/detergent/phospholipid micellar solutions. However, the homogeneity of the resulting recombinants was shown to be dependent upon the detergent used and in the presence of Triton X-100 or $C_{12}E_8$ different populations were clearly evidenced. It was further demonstrated that the rate of detergent removal drastically influenced the composition of resulting proteoliposomes: upon slow detergent removal from samples solubilized with Triton X-100 or $C_{12}E_8$, Ca^{2+}-ATPase was found seggregated and/or aggregated in very few liposomes while upon rapid detergent removal compositionally homogeneous proteoliposomes were obtained with high Ca^{2+} pumping activities. The reconstitution process was further analyzed by centrifugation experiments and the results demonstrated that the different mechanisms of reconstitution were driven predominantly by the tendency for self-aggregation of the Ca^{2+}-ATPase. A model for Ca^{2+}-ATPase reconstitution was proposed which accounted for all our results. In summary, the advantage of the systematic studies reported in this paper was to allow a rapid and easy determination of the experimental conditions for optimal detergent-mediated reconstitution of Ca^{2+}-ATPase. Proteoliposomes prepared by the present simple method exhibited the highest Ca^{2+} pumping activities reported to date in Ca^{2+}-ATPase reconstitution experiments performed in the absence of Ca^{2+} precipitating agents.

Introduction

Reconstitution of membrane proteins into liposomes provides a powerful tool in structural as well as functional areas of membrane protein research. However, despite the thousands of publications concerning reinsertion of membrane proteins into liposomes (for reviews, see Refs. 1–7) the underlying processes which lead to the formation of specific reconstituted forms

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Abbreviations: OG, *n*-octyl β-D-glucopyranoside; TX-100, Triton X-100; $C_{12}E_8$, octaethylene glycol mono-*n*-dodecyl ether; EPC, egg phosphatidylcholine; EPA, egg phosphatidic acid; SR, sarcoplasmic reticulum; Pipes, 1,4-piperazinediethanesulfonic acid; R_{eff}, effective molar ratio of detergent to phospholipid; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

are not well understood and reconstitution still appears to be more art than science. A new experimental strategy has been recently developed in our laboratory [7,8] to provide more insight into the mechanisms that trigger protein insertion into liposomes during the most employed technique to prepare proteoliposomes, namely detergent-mediated reconstitutions.

The strategy employed was based on the idea that detergent-mediated reconstitution represented the reverse of membrane detergent solubilization [9–11]. Thus, stepwise solubilization of preformed liposomes was used to control the composition of the starting detergent/phospholipid mixtures in which the protein was incubated. After detergent removal the vesicles formed were characterized with respect to protein incorporation, orientation and biological activity. This method allowed a rapid and easy determination of experimental conditions, for optimal detergent-mediated reconstitution of two membrane proteins, bacteriorhodopsin [8] and H^+-ATPase [12]. Different mechanisms of association between lipids and proteins were evidenced and mainly related to the nature of the detergent used for reconstitution. We describe in this paper an extension of this procedure to study the reconstitution of the sarcoplasmic reticulum Ca^{2+}-ATPase. Various methods for incorporating this protein into liposomes have been published including detergent-mediated reconstitutions [13–19]. However, the mechanisms that trigger Ca^{2+}-ATPase incorporation into the lipid bilayers are still far from clear and the previous reconstitution studies indicate large variations in the resulting Ca^{2+} pumping activities of the different proteoliposome preparations.

The results presented here indicate that, as already reported for bacteriorhodopsin, Ca^{2+}-ATPase reconstitution into liposomes depends upon the nature of the detergent used. In particular, Ca^{2+}-ATPase can be directly incorporated into preformed liposomes saturated with octyl glucoside (OG). On an other hand, in the presence of cholate, TX-100 or octaethylene glycol mono-*n*-dodecyl ether ($C_{12}E_8$) optimal reconstitutions of Ca^{2+}-ATPase arise from initial micellar solutions of lipids, proteins and detergents. In these last reconstitution experiments, however, Ca^{2+}-ATPase molecules appear to be distributed heterogeneously among the liposomes leading to the formation of protein-rich vesicles and pure liposomes whose relative proportions vary with the nature of the detergent and its rate of removal. Freeze-fracture electron microscopy studies, transport data and sedimentation experiments demonstrate that a key factor in determining the final proteoliposome composition is the state of aggregation of Ca^{2+}-ATPase molecules whose propensity for self association in native membranes and in the presence of detergents is well documented (for a review, see Ref. 20). In summary, the results of the systematic studies presented here, allow us to propose a model describing the reconstitution process of the SR Ca^{2+}-ATPase. Generalization of this model to the reconstitution of other membrane proteins is further discussed in the light of other data dealing with the mechanims of lipid-protein associations during detergent-mediated reconstitutions [3,8,10,12,21].

Besides providing informations about the mechanisms of lipid-protein association during detergent-mediated reconstitutions, this work defines the important parameters involved in a functional reconstitution of the Ca^{2+}-ATPase. Therefore proteoliposomes, which satisfied most of the criteria for an efficient reconstitution, could be produced and sustained the highest Ca^{2+} transport activities, reported to date. Such proteoliposomes have already proved useful for the study of ions in the transport mechanism of sarcoplasmic reticulum Ca^{2+}-ATPase [22].

Materials and Methods

Materials

Phosphatidylcholine was extracted from egg-yolk according to Singleton et al. [23]. Phosphatidic acid was prepared from the former as described by Allgyer and Wells [24].

The detergents used in this study were as follows: Triton X-100 (Sigma), $C_{12}E_8$ (Nikko Chemical, Tokyo), octyl glucoside (Sigma and Calbiochem), sodium cholate (Calbiochem and Prolabo). SM-2 Bio-Beads were obtained from Bio-Rad and extensively washed before use as described by Holloway [25]. Polycarbonate filters were purchased from Nucleopore Corporation. All other reagents were of analytical grades.

Preparation of liposomes

Large unilamellar liposomes were prepared by reverse-phase evaporation as described previously [8,26]. A typical preparation contained 25 mg of phospholipids (EPC/EPA, 9:1) solubilized in 2 ml of diethyl ether and 0.5 ml of aqueous buffer (110 mM KCl, 10 mM Pipes-KOH, pH 7, 2). The resulting two phases system was sonicated for 2 min at 4°C. The organic solvent was then removed by rotary evaporation under reduced pressure (10–15 inch Hg) using a water aspirator. After about 15 min, a viscous gel formed which reversed to aqueous solution. At this point 1 ml of extra buffer was added and evaporation (30 inch Hg) allowed to proceed for a further 30 min to remove all trace of organic solvent. The liposomes (16 mg lipid/ml, i.e., 20 mM) were then sequentially extruded through 0.4 and 0.2 μm polycarbonate filters before use. The presence of 10% negative charges by avoiding fusion and/or aggregation of liposomes allowed to use the preparation for about 5 days.

Preparation of sarcoplasmic reticulum Ca^{2+}-ATPase

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle as described by Champeil et al. [27]. The vesicles were kept frozen in liquid N_2 and thawed before use. Solubilization of SR vesicles before reconstitution was performed by resuspending them at a concentration of 2 mg protein/ml in a buffer containing 110 mM KCl, 10 mM Pipes (pH 7.2) and 0.1 mM $CaCl_2$ supplemented with 6 mg/ml $C_{12}E_8$ (28) or TX-100 [29].

ATPase activies were determined using the linked enzyme assay of Froud et al. [30] in which ADP production was detected by measurement of NADH oxidation at 340 nm.

Reconstitution procedure

The reconstitution procedure derived from the method previously described for bacteriorhodopsin [8] is carried out in three different steps.

(a) Stepwise solubilization of preformed liposomes. Liposomes prepared by reverse-phase evaporation were resuspended at 4 mg lipid/ml (i.e., 5 mM) and treated with the desired amount of detergent through all the range of detergent addition that causes the transformation of lamellar structures into mixed micelles. Previous studies of the interactions of detergents with model membranes indicated that the solubilization process could be described by a 'three-stage' model and accurately visualized through changes in turbidity of the lipid-detergent suspensions [11,31–33]. In stage I, detergents incorporate into the liposomes until they saturate the phospholipid bilayers and induce slight changes in turbidity. Stage II corresponds to a gradual solubilization of lipids resulting in a large decrease in turbidity. Stage III is characterized by a complete solubilization of lipids into mixed micelles and the solution becomes optically transparent. Turbidity versus detergent concentration curves can be related to the effective detergent to phospholipid molar ratios at the onset of solubilization ($R_{eff} = R_{sat}$ when the turbidity starts to decrease noticely) and at total solubilization ($R_{eff} = R_{sol}$ for optically transparent solution). The saturation levels of detergents bound to the membrane (R_{sat}) were found to be 0.64, 0.66, 1.3 and 0.3 mol of detergent/mol of phospholipid for Triton X-100, $C_{12}E_8$, OG and cholate, respectively. The molar detergent to phospholipid ratios in micelles (R_{sol}) were 2.5, 2.2, 3.6 and 0.9 for Triton-X100, $C_{12}E_8$, OG and cholate, respectively [11,33].

It should also be noted that although most of the reconstitution experiments reported in this paper have been performed at 5 mM phospholipid, similar results were obtained between 1.25 and 20 mM phospholipid.

(b) Ca^{2+}-ATPase addition. In the second step of the reconstitution procedure Ca^{2+}-ATPase is added to the equilibrated detergent-phospholipid mixture. It was found essential that the protein was added as a solution of detergent-solubilized monomeric Ca^{2+}-ATPase. Therefore SR vesicles were first solubilized with $C_{12}E_8$ (detergent/protein, 3(w/w)) in the presence of 0.1 mM Ca^{2+} to protect the solubilized enzyme against denaturation [28,29,34]. Then aliquots of $C_{12}E_8$-solubilized active Ca^{2+}-ATPase were added under vortex to the detergent-lipid mixtures to give the desired lipid to protein ratio. Alternatively, addition of all the detergent first to SR vesicles and then addition of this solubilized SR to the untreated liposome suspensions led to comparable results with all the detergents analyzed except with octyl glucoside. Indeed, solubilization of SR vesicles with octyl glucoside caused immediate and irreversible inactivation of Ca^{2+}-ATPase activity (data not shown; Refs. 19 and 35). However inactivation of the Ca^{2+}-ATPase can be delayed by addition of phospholipids. In preliminary experiments, we found that in the presence of 5 mM phospholipids inactivation of Ca^{2+}-ATPase activity by solubilizing OG concentrations (40 mG OG, i.e., $R_{eff} \approx 4$) was limited to 25% after 1 h incubation. This inhibition was found to be further delayed with decreasing OG concentrations: at a R_{eff} of 1.3, only a 10% inactivation was observed after 1 h incubation. On another hand addition of non-solubilized SR vesicles to lipid/detergent mixtures led always to unsatisfactory and irreproducible results. Thus, the order of addition adopted in our reconstitution experimental procedure, i.e., first presolubilization of Ca^{2+}-ATPase in $C_{12}E_8$, then addition to detergent-treated liposomes and incubation for 5–10 min before detergent removal proved to be superior and more reproducible than other alternatives. It was also checked in control experiments that the small amount of $C_{12}E_8$ added together with the protein did not affect results of TX-100, cholate or octyl glucoside-mediated reconstitutions. First, the turbidity changes of the liposome suspensions with increasing detergent concentrations were not affected by the presence of this small amount of detergent. Second, identical Ca^{2+} transport activities were measured whatever reconstitutions were performed with presolubilization of SR vesicles in $C_{12}E_8$ or Triton X-100.

(c) Detergent removal. The third step in our reconstitution procedure is related to detergent removal from the lipid-protein detergent mixtures. We have adapted the batch procedure using SM_2 Bio-Beads to remove detergent as originally described by Holloway [25]. This was generally performed by successive additions: 80 mg beads/ml for 3 h incubation followed by two successive additions of 80 mg beads/ml for 1 h. In experiments dealing with the influence of detergent removal rate (see part IV) the amount of beads present during the first incubation was varied according to Lévy et al. [11,36]. Finally, titration of SM-2 Bio-Beads with ^{3}H-lipid and iodo[^{14}C]acetamide-labelled Ca^{2+}-

ATPase indicated no protein adsoption onto the beads and a negligible loss of lipids of about 1 mg lipid/g wet beads [8,11,36].

Proteoliposome characterizations

After reconstitution, proteoliposomes were submitted to discontinuous floatation gradients as previously described [8] with successive layers containing 30, 20, 15, 10, 5 and 2.5% sucrose (w/w). It is important to supplement the 30% sucrose layer with 0.05% TX-100, due to the impermeability of the proteoliposomes to sucrose. After centrifugation at 30000 rpm for 3 h in a Beckman SW 41 rotor, the fractions were collected and analyzed for lipid and protein contents. Lipid content was determined using [^{3}H]phosphatidylcholine. Protein content was determined using iodo[^{14}C]acetamide-labelled Ca^{2+}-ATPase [37].

Freeze-fracture electron microscopy was performed as described previously [38] using platinum-carbon shadowing.

Protein orientation in the membrane of reconstituted proteoliposomes was determined from gel electrophoresis patterns before and after trypsin treatment of the reconstituted samples. To this end proteoliposomes (100 μg protein/ml) were incubated at room temperature for 5 min in the presence of trypsin (trypsin/protein, 1:10 (w/w)). The reaction was stopped by a 2-fold weight excess of soybean trypsin inhibitor and 0.1 mM phenylmethylsulfonyl fluoride. In some experiments the samples were delipidated before electrophoresis according, to Wessel and Flüge [39]. SDS-PAGE was performed on 7.5-15% gradient acrylamide gels. Scans of Commassie blue-stained gels were performed with a LKB laser densitometer (2202 Ultrascan) supplemented with an integral computer (spectra physic 4100). Percent of protein orientation was determined from the ratio of the intensities of the Ca^{2+}-ATPase band (M_r 120000) before and after trypsin digestion. Intensities were normalized using the intensity of the trypsin inhibitor band as a reference of the sample concentration deposited on the gel.

Ca^{2+} uptake measurements

Ca^{2+} uptake by the reconstituted vesicles was followed by dual wavelength spectrophotometry using murexide to monitor changes in external Ca^{2+} concentration. Reconstituted liposomes were diluted in the same buffer used for their preparation and supplemented with 5 mM $MgCl_2$ and 80 μM murexide (final lipid concentration: 1–1.6 mg/ml). Calibration was established by addition of known Ca^{2+} concentrations to the sample prior to initiation of the uptake (final Ca^{2+} concentration: 40–100 μM). Ca^{2+} uptake was initiated by addition of 0.2–0.4 mM ATP in a buffer pH 7.2. Uptake of Ca^{2+} was followed by measuring the changes in absorbance at 487–550 nm using a DW_2 Aminco spectrophotometer.

Results

I. Octyl glucoside-mediated reconstitutions

Fig. 1A shows the effect of initial octyl glucoside concentration on the Ca^{2+} transport activity of reconstituted Ca^{2+}-ATPase proteoliposomes. Liposomes (5 mM lipid) were treated with different amounts of octyl glucoside and incubated 5 min with $C_{12}E_8$-solubilized Ca^{2+}-ATPase. After detergent removal by successive Bio-Beads additions, the ATP-dependent Ca^{2+} accumulation in the resulting proteoliposomes were measured by following the changes in external murexide adsorbance. At this point, it should be stressed that all samples were always analyzed, in the presence of FCCP and valinomycin. Due to the low ionic permeability of the proteoliposones and the operation of a Ca^{2+}-H^+ countertransport during ATPase functioning, the presence of these ionophores was shown to be necessary for maximal Ca^{2+} pumping efficiency [22].

From Fig. 1A, it is obvious that the Ca^{2+} pumping efficiency after reconstitution is drastically dependent upon the initial octyl glucoside/phospholipid ratio. The turbidities of phospholipid/detergent/Ca^{2+}-ATPase mixtures before detergent removal are plotted in the inset of Fig. 1A. In accordance with our previous results [31], octyl glucoside concentrations of 24 mM and 40 mM corresponded, respectively, to the onset of (R_{sat} = 1.3) and to total (R_{sol} = 4) solubilization of the initial liposome suspensions. Point is that incorporation of Ca^{2+}-ATPase takes place in proteoliposomes reconstituted from initial detergent concentrations below those necessary for saturating the initial preformed liposomes. Low Ca^{2+} accumulation was measured at about 20 mM octyl glucoside. Then Ca^{2+} pump efficiencies rose drastically and were maximal in proteoliposomes reconstituted from initial liposome suspensions containing 26 mM octyl glucoside. For reconstitution experiments performed above this critical concentration, no significant change in Ca^{2+} pumping efficiency was observed up to 40 mM OG corresponding to reconstitution from isotropic micellar solution. Thus, the most striking feature of the data presented in Fig. 1A is that maximal incorporation of Ca^{2+}-ATPase occurred approximately at the onset of the solubilization of the preformed pure liposomes present in the incubation medium.

Some representative sucrose density gradients patterns of vesicle preparations reconstituted from different detergent/lipid ratios are shown in Fig. 1B. When reconstitutions were performed in the absence of octyl glucoside, i.e., by simply adding the solubilized protein to pure liposomes, all the protein was found at the bottom of the gradient and most of the lipid floating

(Fig. 1Ba). For reconstitutions at OG concentrations of 20 mM, all the protein initially present was recovered associated with about only 10% of the total phospholipids, the remaining lipids floating as protein free liposomes (Fig. 1Bb). When the OG concentration in the initial suspension was further increased, more lipids became associated with the protein at the expense of the protein-free liposome population. In samples reconstituted from an initial detergent concentration of 26 mM, the reconstituted material was essentially collected at the interface between 5 and 2.5% (w/w) sucrose in one band containing 90–95% of the protein (Fig. 1Bf). The protein band found at the 20–15% (w/w) sucrose boundary clearly indicated that only 5–10% of the protein could not be incorporated into detergent-saturated liposomes. Only vesicles reconstituted from isotropic micellar solution were collected on floatation sucrose gradient as a single band containing all lipid and protein (Fig. 1Bi). Thus, density centrifugation experiments not only confirmed the previous observations that maximal Ca^{2+} pumping activities occurred in samples reconstituted from liposome-OG suspensions at the onset of solubilization but also revealed that protein incorporation was nearly complete and relatively homogeneous among the liposomes. This has been corroborated by freeze-fracture electron microscopy studies (data not shown).

II. Cholate-mediated reconstitution

Fig. 2 shows the influence of the cholate concentration on the transport activities of the resulting Ca^{2+}-ATPase proteoliposomes. No Ca^{2+} pumping activities were detected in samples reconstituted from initial cholate concentrations below those corresponding to

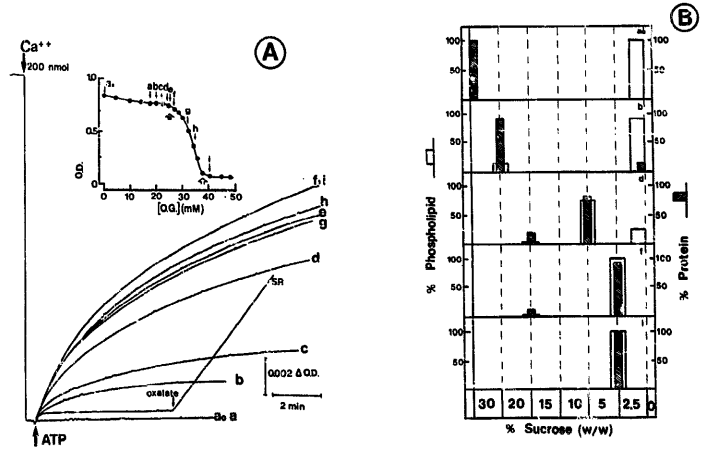

Fig. 1. Octyl glucoside-mediated reconstitution of Ca^{2+}-ATPase. Liposomes prepared by reverse-phase evaporation were first treated with variable amounts of OG (as lettered in the figure); Then Ca^{2+}-ATPase previously solubilized as described in Materials and Methods was added under vortex mixing (Final lipid/protein = 80 w/w; incubation medium: 130 mM KCl, 10 mM Pipes-KOH, pH 7.2). After a 5 min incubation, OG was removed by SM-2 Bio-Bead treatment: 80 mg beads/ml for 3 h followed by two successive additions of 80 mg beads/ml for 1 h. Panel A. ATP-dependent Ca^{2+} accumulation by reconstituted proteoliposomes. Ca^{2+}-ATPase proteoliposomes were resuspended at 1.6 mg lipid/ml in the same buffer used for their preparations. The reaction mixtures were supplemented with 5 mM $MgCl_2$, 80 μM murexide, 0.5 μM valinomycin and 0.25 μM FCCP. Final volume in the cuvette: 2.5 ml. After 5 min equilibration in a stirred and thermostated cuvette (20°C), 80 μM $CaCl_2$ was added. Then Ca^{2+} uptake was initiated by the addition of 150 μM ATP and monitored by changes in murexide absorbance. SR: Ca^{2+} accumulation by native sarcoplasmic reticulum vesicles (20 μg protein /ml). Inset: Turbidities (λ = 600 nm) of octyl glucoside/phospholipid/Ca^{2+}-ATPase mixtures before detergent removal. Black and white arrows indicate the threshold values for onset and total liposome solubilization, respectively. Panel B. Floatation of OG-reconstituted proteoliposomes in discontinuous sucrose gradients. Proteoliposomes prepared in the presence of [^{3}H]phosphatidylcholine and Iodo[^{14}C]acetamide-labelled Ca^{2+}-ATPase were submitted to floatation in sucrose gradients as described under Materials and Methods. Proteoliposomes reconstituted from initial OG concentrations of 0 (a), 20 (b), 22.5 (d), 26 (f) and 40 (i) mM OG. (100%, total Ca^{2+}-ATPase (hatched bars) or total phospholipid (open bars) concentrations in each sucrose gradient).

the onset of phospholipid solubilization (5.5 mM cholate, corresponding to a $R_{eff} = R_{sat} \approx 0.3$). Only for reconstitutions performed above this critical ratio were Ca^{2+} pumping activities measured and total Ca^{2+} accumulation increased progressively with increasing initial cholate concentrations up to 11 mM ($R_{eff} = 0.9$) corresponding to the total solubilization by this detergent. Consequently for cholate-mediated reconstitutions the efficiency of Ca^{2+}-ATPase reconstitution was related to the initial percentage of phospholipid solubilization. Optimal Ca^{2+} uptake was obtained in samples reconstituted from an isotropic Ca^{2+}-ATPase/cholate/phospholipid micellar solution. It can also be noted that although the total extent of Ca^{2+} uptake increases with initial cholate concentration, the initial rates of Ca^{2+} pumping are already maximal at R_{eff} 0.3 and then independent of the cholate concentration. Therefore the most likely mechanism for cholate-mediated reconstitution is similar to that found for bacteriorhodopsin [8]: Ca^{2+}-ATPase proteoliposomes are only formed from ternary micelles; raising the initial cholate concentration increases the number of ternary mixed micelles and consequently the number of Ca^{2+}-ATPase-containing liposomes. This interpretation was

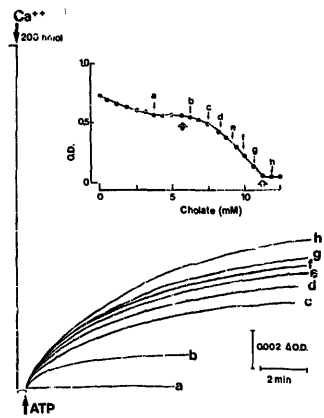

Fig. 2. Sodium cholate-mediated reconstitution of Ca^{2+}-ATPase. Liposomes prepared by reverse-phase evaporation were treated by the indicated amounts of sodium cholate. Reconstitutions and ATP-dependent Ca^{2+} accumulations by reconstituted proteoliposomes were performed as described in Fig. 1. Inset: Turbidities of phospholipid/cholate/Ca^{2+}-ATPase mixtures before detergent removal. Black and white arrows indicate the threshold values for onset and total liposome solubilization, respectively.

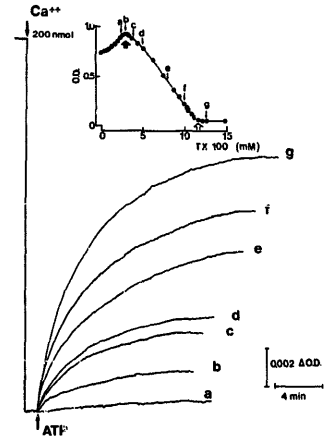

Fig. 3. Triton X-100-mediated reconstitution of Ca^{2+}-ATPase. Liposomes prepared by reverse-phase evaporation were treated by the indicated amounts of Triton X-100. Reconstitutions and ATP-dependent Ca^{2+} accumulations by reconstituted proteoliposomes were performed as described in Fig. 1. Inset: Turbidities of phospholipid/Ca^{2+}-ATPase/Triton X-100 mixtures before detergent removal. Black and white arrows indicate the threshold values for onset and total liposome solubilization.

corroborated by density gradient centrifugation analysis of the different vesicle preparations. No protein-lipid association could be detected for samples reconstituted below R_{sat}. Above this critical ratio, all the protein was found associated with phospholipids, the amount of associated phospholipids increasing proportionally with the initial cholate concentration. Only vesicles reconstituted from pure micellar solutions were collected in floatation gradient as single band containing all the protein and phospholipid (data not shown and Fig. 4).

III. Triton X-100-mediated reconstitutions

Fig. 3 depicts the ATP-dependent Ca^{2+} accumulation of recombinants from different initial TX-100 concentrations. Ca^{2+} pumping activities were only observed in samples reconstituted at a TX-100 to phospholipid ratio higher than that corresponding to the onset of phospholipid solubilization ($R_{sat} = 0.64$, i.e., 3 mM TX-100). Above this critical ratio the efficiency of Ca^{2+} uptake increased progressively with increasing initial detergent concentration up to a R_{eff} of 2.5 i.e., $\approx$ 12.5 mM TX-100 where it became independent on

the detergent concentration. Thus, in the presence of TX-100, maximal reconstitution of Ca^{2+}-ATPase occurred, as previously described with cholate, when starting from totally solubilized samples. This finding is clearly in contrast with those previously reported for TX-100-mediated reconstitution of bacteriorhodopsin [8] and H^+-ATPase [12] using the same methodology and experimental conditions as described in this paper. Indeed, with these two membrane proteins, optimal reconstitutions were obtained when starting from initial TX-100 to phospholipid ratios of about 1 (which corresponded to 20–30% of initial liposome solubilization) and a time-dependent incorporation of the proteins was observed suggesting a transfer of the proteins initially present in the micelles to detergent-saturated liposomes still present in the incubation medium. Experiments have been performed with the Ca^{2+}-ATPase where the time of incubation of the protein in the presence of different phospholipid/TX-100 mixtures was varied between 5 to 60 min before detergent removal. Whatever the initial detergent concentrations, the final Ca^{2+} pumping activities were independent on this time of incubation (data not shown) indicating that the mechanism reported for bacteriorhodopsin and H^+-ATPase was inefficient in the case of SR Ca^{2+}-ATPase.

When the Ca^{2+}-ATPase proteoliposomes formed from TX-100-solubilized samples were analyzed by sucrose density centrifugation, two populations were evident (Fig. 4a). If all the protein initially present was found associated with phospholipids it nevertheless appeared distributed inhomogeneously in two major populations. One population which comprised about 75% of the phospholipids associated with 50% of the protein was found at the interface between 2.5 and 5% (w/w) sucrose while the other comprising the remaining lipids and proteins banded at the 5–10% (w/w) sucrose interface. In some experiments more inhomogeneous populations were observed: above and below the two major bands previously reported, some protein-free and protein-rich liposomes, respectively, were detected. Thus, the sucrose density patterns indicated that Ca^{2+}-ATPases and phospholipids did not mix homogeneously after TX-100 removal from solubilized samples in contrast to what observed above with OG (Fig. 1Bi) and cholate (Fig. 4c).

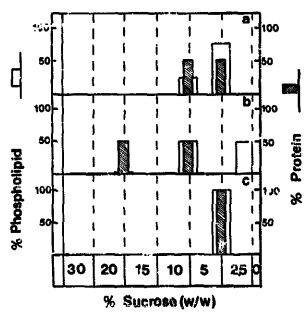

Fig. 4. Floatation of proteoliposomes in discontinuous sucrose gradients. Proteoliposomes were reconstituted from phospholipid/detergent/Ca^{2+}-ATPase solubilized samples containing 15 mM Triton X-100 (a), 12 mM $C_{12}E_8$ (b) or 10 mM cholate (c) and submitted to sucrose gradients as described in Materials and Methods. (100%, total protein (hatched bars) or total lipid (open bars) concentrations in each sucrose gradient).

A series of conditions were varied to determine what factors affect the reconstitution of Ca^{2+}-ATPase. First another detergent was tested: $C_{12}E_8$, which displays physico-chemical properties (cmc, micellar size) roughly analog to those of TX-100 and has the advantage of being an homogeneous species. Optimal reconstitutions of Ca^{2+}-ATPase in the presence of $C_{12}E_8$ occurred again from totally solubilized samples (data not shown). When these recombinants were submitted to sucrose gradient analysis, the inhomogeneity of the protein distribution appeared even more obvious than in the presence of TX-100 (Fig. 4b). A large fraction of vesicles (50 ± 10% of total phospholipids) appeared as protein-free liposomes floating on the top of the gradient. The remaining phospholipids were detected associated with about 50 ± 10% of the protein at the 5–10% (w/w) sucrose interface. Finally, about 50% of the protein was found associated with very few lipids (less than 5%) banding at the 15–20% (w/w) interface.

Another variable that was examined was the order of mixing of the components. One reconstitution was performed as described under Materials and Methods, i.e., detergent was added to a liposomal suspension at the concentration needed for total solubilization, followed by addition of presolubilized Ca^{2+}-ATPase. A second reconstitution was performed by first solubilizing the Ca^{2+}-ATPase in the total amount of detergent and subsequent addition to the liposomal suspension. In the first preparation both detergent/protein and detergent/phospholipid micelles may initially exist while in the second preparation the order of mixing ensures that phospholipid will be directly incorporated into micelles containing the Ca^{2+}-ATPase. After detergent removal both reconstituted systems exhibited similar Ca^{2+} pumping efficiencies. (data not shown for $C_{12}E_8$ and TX-100-mediated reconstitution). As additional checks, although freeze-fracture electron micrographs gave good indications of complete dispersal of Ca^{2+}-ATPase and phospholipids at R_{sat} values of 2.5 and 2.0 for TX-100 and $C_{12}E_8$ respectively, reconstitution experiments were performed starting from R_{eff}

values two times higher to ensure complete distribution of the protein among the micelles in monomeric form. These procedures had no significant effects on the Ca^{2+} pumping efficiency of the reconstituted samples. Thus from all these experiments, it appears that the composition of the final products of TX-100 and $C_{12}E_8$ reconstitutions does not merely depend upon the initial mixed micelle composition.

IV. Effects of the rate of detergent removal

Kinetic factors have been reported to be important in determining the mode of protein-lipid association during detergent-mediated reconstitutions [3,21]. We thus analyzed the effects of the rate of detergent removal upon the final composition of proteoliposomes.

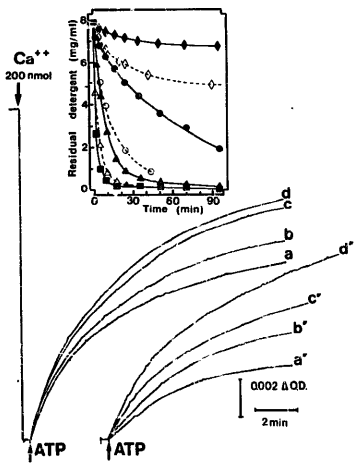

Fig. 5. Effects of the rate of detergent removal on the Ca^{2+} pumping efficiencies of Ca^{2+}-ATPase proteoliposomes. After 10 min incubation, protein/lipid/Triton X-100 (R_{sol} = 3) or protein/lipid/$C_{12}E_8$ (R_{sol} = 2.6) micellar solutions were treated with different amounts of SM-2 Bio-Beads and analyzed for their Ca^{2+} pumping activities (same experimental conditions as in Fig. 1). Traces a, b, c, d and traces a', b', c', d' refer to Triton X-100 and $C_{12}E_8$-mediated reconstitutions, respectively. Traces a, a': three successive additions of 20 mg beads/ml for 1 h, followed by 440 mg beads/ml for 2 h. Traces b, b': 80 mg beads/ml for 3 h followed by 420 mg beads/ml for 2 h. Traces c, c': 200 mg beads/ml for 3 h followed by 300 mg beads/ml for 2 h. Traces d, d': 500 mg beads/ml for 5 h. Inset: Time course of detergent removal during one period incubation (90 min) at different Bio-Beads concentrations. Micellar phospholipid/protein/detergent solutions containing ^{3}H-Triton X-100 (closed symbols) or ^{14}C-$C_{12}E_8$ (open symbols) were treated by 20 (◆, ◇), 80 (●, ○) 200 (▲, △) or 500 (■) mg beads/ml. Aliquots from the supernatant were collected as a function of time and analyzed for their radioactivities.

Previous systematic studies indicated that the batch procedure using SM-2 Bio-Beads as detergent removing agent was well suited for controlling the rate of detergent removal [11,36]. Thus to determine if time was important either for rearrangement or for tranfer of lipid and protein between micelles during detergent removal, aliquots of solubilized Ca^{2+}-ATPase/detergent/phospholipid mixtures were treated with different amounts of SM-2 Bio-Beads and assessed in regard to their Ca^{2+} pumping efficiencies. Fig. 5 shows the time courses of Ca^{2+} uptakes by proteoliposomes reconstituted from TX-100 or $C_{12}E_8$ at different detergent removal rates. For both detergents it appeared that the rate of detergent removal (illustrated in the inset of Fig. 5) had a drastic influence on the rate and/or total extent of Ca^{2+} accumulation. For reconstitutions performed from $C_{12}E_8$-solubilized samples, the initial Ca^{2+} pumping rate of proteoliposomes reconstituted at the slowest detergent removal rate amounted to about 0.3 μmol Ca^{2+}/min per mg protein and only 40 nmoles of Ca^{2+} could be accumulated. Increasing the rate of detergent removal, increased both the rate and total extent of Ca^{2+} uptake in the reconstituted proteoliposomes. When $C_{12}E_8$ was removed in less than 5 min (in the presence of 500 mg beads/ml) up to 125 nmoles of Ca^{2+} could be now accumulated with an initial rate of 0.8 μmol Ca^{2+}/min per mg protein. In the case of TX-100-mediated reconstitutions, rates of Ca^{2+} pumping were slightly affected by the initial conditions varying from 0.85 to 1 μmol Ca^{2+}/min per mg protein, only the total amount of accumulated Ca^{2+} increased significantly with the rates of detergent removal. These important findings can be interpreted assuming that increasing the rate of detergent removal during TX-100 and $C_{12}E_8$-mediated reconstitutions of Ca^{2+}-ATPase improves the efficiency of the Ca^{2+} transport activity (as evidenced by an increase in the initial Ca^{2+} pumping rates * and/or the dispersal of protein among the liposomes, i.e., the internal volume in which Ca^{2+} is accumulated (as evidenced by an increase in the total extent of Ca^{2+} uptake). Such experiments were also carried out in the case of OG and cholate-mediated reconstitutions but the rate of detergent removal was found to have little influence on the Ca^{2+} pumping activities of the resulting proteoliposomes. In particular there were no dis-

* Reconstitution by slow removal of TX 100 or $C_{12}E_8$ gave vesicles with high rates of ATP hydrolysis but low Ca^{2+} uptakes. Ca^{2+}/ATP coupling ratios of 1 and 0.5 were, respectively, measured as compared to coupling ratios of about 1.8 using rapid detergent removal (data not shown).

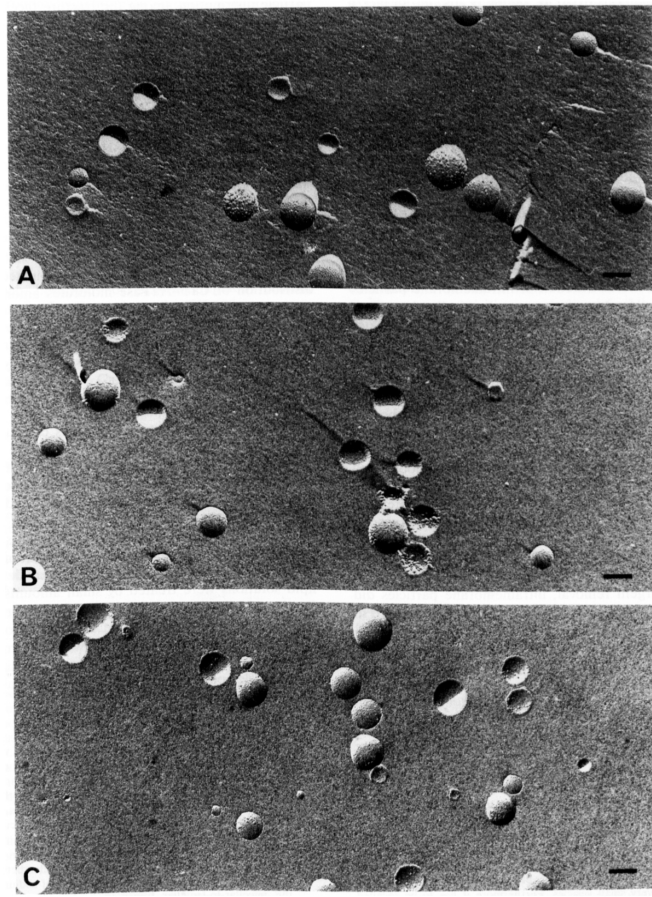

Fig. 6. Freeze-fracture views of Ca^{2+}-ATPase proteoliposomes reconstituted at different rates of detergent removal. Solutions of Ca^{2+}-ATPase/phospholipid/$C_{12}E_8$ (lipid: 8 mg/ml; protein: 200 μg/ml; $C_{12}E_8$: 16 mg/ml) were treated with different amounts of SM-2 Bio-Beads. Panels A and B. Three successive additions of 40 mg beads/ml for 1 h, followed by 480 mg beads/ml for 2 h. Panel C. 600 mg beads/ml for 5 h. Magnification is the same for all micrographs. The bar in (A) represents 100 nm.

cernable differences in the Ca^{2+} pumping activities of proteoliposomes reconstituted by OG elimination from micellar solutions by 20 to 500 mg beads/ml. In fact, a small decrease ($\approx$ 10–20%) in the initial rate of Ca^{2+} uptake was observed upon very slow detergent removal (dialysis for 5 h at room temperature, followed by incubation with 500 mg beads/ml) but could be simply related to the inhibition of the Ca^{2+}-ATPase activity after long incubation time in the presence of this detergent (see Materials and Methods).

Freeze-fracture electron microscopy was used for further analyzing the nature and the composition of reconstituted samples depending upon the rate of detergent removal. Fig. 6 shows typical electron micrographs of Ca^{2+}-ATPase liposomes obtained after slow and rapid detergent removal from $C_{12}E_8$-solubilized samples. Whatever the rate of detergent removal, most of the material appeared as spherical unilamellar vesicles * but the distribution of intramembrane particles among the fracture faces was drastically dependent upon the rate at which $C_{12}E_8$ had been removed. After slow detergent removal (panel A) intramembrane particles were observed on a small fraction ($\approx$ 15%) of the vesicle fracture faces which appeared densely particulated. The remaining fracture planes appeared smooth as in the case of liposomes reconstituted in the absence of Ca^{2+}-ATPase. Interestingly, in some fracture faces (Panel B in Fig. 6) intramembrane particles were aggregated, leaving the remaining surface smooth and devoid of particles. On the contrary, electron micrographs of samples reconstituted by rapid detergent removal (panel C in Fig. 6) revealed a large increase in the proportion of fracture faces containing intramembrane particles. Indeed, particles were found in up to 70% of the fracture faces **. Furthermore, upon rapid detergent removal, intramembrane particles did not occur clustered or aggregated but well separated in the fracture faces. In the case of TX-100-mediated reconstitutions, similar increase in the homogeneity of particle distribution among the fracture faces and disappearance of particle clustering were also linked to an increase in detergent removal rate. However, at comparable rates of detergent removal, Triton X-100-mediated reconstitutions led to more homogeneous preparations than did $C_{12}E_8$-mediated reconstitutions: the fraction of fracture faces containing intramembrane particles increasing from about 40% up to 90% for samples reconstituted at the slowest and highest TX-100 removal rates, respectively. Finally, there were no significant differences in particle distribution of the reconstituted vesicles prepared at different OG removal rates from micellar OG/Ca^{2+}-ATPase/lipid solutions (data not shown).

Thus, the freeze-fracture data indicate that the rate of detergent removal has a strong influence on the distribution of Ca^{2+}-ATPase molecules among the liposomes during $C_{12}E_8$ and TX-100-mediated reconstitutions in complete agreement with the Ca^{2+} pumping data reported in Fig. 5. They also illustrate the tendency for aggregation of Ca^{2+}-ATPase particles upon slow detergent removal.

V. Centrifugation experiments

From the freeze-fracture data, it is concluded that kinetic factors are of key importance for the achievement of homogeneous association of Ca^{2+}-ATPase with excess lipid. This suggests a different behavior, i.e., a different stability of lipid/detergent and lipid/detergent/protein micelles, upon detergent removal [41,42]. The reconstitution process was thus characterized in further details by ultracentrifugation experiments. To this end micellar Ca^{2+}-ATPase/detergent/lipid solutions were treated by small amounts of Bio-Beads and aliquots were pipetted off at different time intervals. After centrifugation at 400 000 $\times g$ for 45 min the amounts of lipid and protein were determined in the pellets. Under these centrifugation conditions bilayer vesicles pellet while mixed micelles remain in the supernatant [11] ***.

* As already reported for reconstitution of pure liposomes, it can be noted that the sizes of the proteoliposomes depends upon the rate of detergent removal [11,36]. Size distribution diagrams indicated that at the slowest rate of detergent removal analyzed, the vesicles consisted of a fairly homogeneous vesicle population with mean diameters of about 150 and 80 nm for TX-100 and $C_{12}E_8$ reconstitutions, respectively. At high detergent removal rates, a second population of small vesicles were formed with mean diameters around 25–40 nm (however calculation of phospholipid distribution indicated that the small liposomes comprised $\approx$ 5% of total phospholipid molecules).

** It is obvious that the proportion of the fractures containing intramembranous particles can be even higher since it is difficult by freeze-fracture electron microscopy to distinguish between fully smooth and trully particulate fracture planes in the case of very small vesicles. Furthermore it has to be recalled that there is a ratio of 8 to 12 between the number of protein molecules per liposome and the number of particules occurring in fracture faces since only a portion of the liposome surface is actually observable after freeze-fracture [38,40].

*** Sedimentation experiments have been shown to be well suited for investigating the composition and the relative proportions of micelles and bilayers present in lipid/TX 100 or lipid/$C_{12}E_8$ samples (Ref. 11; see also Fig. 7). In contrast, in the case of octylglucoside no phase separation between micelles and bilayers occurred during liposome solubilization or vesiculation: all material was found in the pellet or in the supernatant below or above an R_{eff} of 4, respectively. This observation can be related to the high density of mixed lipid/OG micelles between R_{eff} values of 1.5 and 4 [43]. Finally in the case of sodium cholate, the micellar-to-lamellar transition is difficult to analyze by centrifugation since large and variable amounts of bilayers (up to 30–40%) never sediment in our experimental conditions, probably due to the small size of liposomes formed upon cholate removal.

Fig. 7 is a plot of the results obtained during TX-100-mediated reconstitution of the Ca^{2+}-ATPase. Starting from micellar solution no pellet is obtained; as detergent is removed phospholipids appear in the pellets whose amounts increase concomitantly with detergent removal. Onset of vesiculation occurs at detergent to phospholipid ratio of about 2.5 (mol/mol) while complete vesiculation occurs at a ratio of about 0.6 (mol/mol). The main finding in Fig. 7 is that phospholipids and protein show different pelleting behaviors during detergent removal. Clearly a greater percentage of protein relative to phospholipid pellet at the early beginning of the vesiculation process. For example, after 40 min incubation with a low amount of beads almost all the Ca^{2+}-ATPase initially solubilized is found in the pellet associated with only 20% of the total phospholipids. Thus, it is obvious from our results that at the beginning of the micellar-to-lamellar transition protein-rich liposomes or lipid-'aggregated' protein complexes are first formed. As the TX-100 concentration is further lowered, the remaining lipids pellet upon centrifugation. Curves comparable to those depicted in Fig. 7 were obtained for $C_{12}E_8$-mediated reconstitutions of the Ca^{2+}-ATPase (data not shown). These important results explain the observation of reconstituted proteoliposomes with phospholipid-rich and protein-rich vesicles on the freeze-fracture electron micrographs reported in Fig. 6. They also illustrate the tendency for aggregation of Ca^{2+}-ATPase molecules upon removal of a small amount of detergent from the initial detergent/phospholipid/protein micelles and point out the importance of the aggregation state of the protein in determining the final proteoliposome composition.

VI. Ca^{2+} pumping efficiencies

The reproducible preparation of impermeant proteoliposomes of defined size with an homogeneous and asymmetric protein orientation was a necessary objective for their use in Ca^{2+}-ATPase reconstitution. Table I summarizes some characteristics of proteoliposomes reconstituted from different detergents using the procedure described in this paper. The main conclusion to be drawn from Table I is that fairly high Ca^{2+} transport activities are observed for all the detergents used, although at a lower degree for cholate-mediated reconstitutions, thus proving the efficiency and general validity of the procedure. Noteworthy high Ca^{2+} uptakes, up to 20 times than that of SR vesicles, can be measured in the absence of precipitating anions

TABLE I

Characteristics of Ca^{2+}-ATPase reconstituted proteoliposomes and sarcoplasmic reticulum vesicles

Liposomes prepared by reverse-phase evaporation (5 mM lipid) were first treated by solubilizing concentrations of TX-100 (12 mM), $C_{12}E_8$ (7 mM), cholate (9 mM), or saturating concentration of OG (26 mM). Then $C_{12}E_8$-presolubilized Ca^{2+}-ATPase was added to each sample to give a final lipid to protein ratio of 40 w/w. After detergent removal by SM-2 Bio-Beads, the reconstituted systems were characterized for their Ca^{2+} pumping activities (changes in murexide absorbance), size (freeze-fracture electron microscopy), and protein sideness (trypsin digestion followed by gel electrophoresis). Intravesicular aqueous volumes were calculated from the lipid and protein contents, the mean size of the vesicles, a phospholipid surface area of 0.7 nm^2/molecule, a phospholipid molecular weight of 800 and a membrane thickness of 40 Å. The calculated values are of course indicative and do not take into account the fraction of protein-free liposomes in the case of TX-100 and $C_{12}E_8$ reconstitutions

Samples	Ca^{2+} transport activities		Size (nm)	Internal volume (μl/mg protein)	Protein orientation (% right-side-out)
	initial rates (μmol Ca^{2+}/min per mg)	total extent (μmol Ca^{2+}/mg protein)			
	no oxalate	no oxalate			
Octyl glucoside-reconstituted proteoliposomes	1.25 ± 0.25	1.75 ± 0.25	100	135	80 ± 5
Triton X-100-reconstituted proteoliposome	1.0 ± 0.1	2.00 ± 0.2	150	225	75 ± 5
$C_{12}E_8$-reconstituted proteoliposome	0.9 ± 0.1	1.25 ± 0.2	80	102	75 ± 5
Cholate reconstituted proteoliposome	0.6 ± 0.1	0.7 ± 0.1	60	63	70 ± 5
	no oxalate	no oxalate			
	undetermined	≅ 0.10			
Sarcoplasmic reticulum vesicles	+oxalate	+oxalate	120	5–7 (Ref. 48)	95
	0.6 ± 0.1	∞			

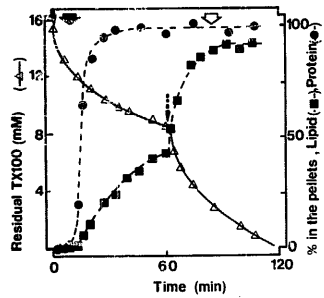

Fig. 7. Time-course of the Ca^{2+}-ATPase-phospholipid association during detergent removal. A lipid/Ca^{2+}-ATPase/Triton X-100 micellar solution containing [^{3}H]phosphatidylcholine and iodo[^{14}C] acetamide-labelled protein was treated by 40 mg beads/ml for 1 h followed by a second addition of 80 mg beads/ml. Aliquots (200 μl) were pipetted off as a function of time and centrifuged at 400000 × g for 45 min. The pellets were resuspended in 200 μl buffer and analyzed for their radioactivities. Protein (●) and phospholipid (■) concentrations are reported as a function of the incubation time with biobeads. Time course of detergent removal (△) was performed under the same experimental conditions in a separate experiment with unlabelled protein and phospholipid but in the presence of ^{3}H-Triton X-100. Dotted, arrows indicate successive additions of Bio-Beads. Black and white arrows indicate the onset and the total lipid vesiculation respectively.

such as oxalate or phosphate. Based on the internal volumes reported in Table I, these loads correspond to intravesicular Ca^{2+} concentrations of 10–15 mM, comparable to those measured in native SR vesicles in absence of precipitating anions (see also Ref. 48). Furthermore the initial rates of Ca^{2+} pumping in proteoliposomes reconstituted from OG, TX 100 or $C_{12}E_8$ are about 2-fold higher than those measured in native SR vesicles in the presence of oxalate (see also Fig. 1). This observation reflects the good incorporation and orientation of the Ca^{2+} pump in the reconstituted systems. The delayed Ca^{2+} uptake in SR vesicles could be related to the limitation of Ca^{2+} precipitation by oxalate [44]. Presumably there are several reasons for this higher Ca^{2+} pumping efficiency of our proteoliposomes as compared to other publications [13–19].

In the first place, the procedure used allows a thorough mixing of phospholipids and ATPase molecules, thus providing an extensive dilution of pumping units in the lipid bilayers. The intravesicular volume per Ca^{2+} pumping unit is much larger than that of SR vesicles and consequently much more Ca^{2+} can be translocated into proteoliposomes before inhibition of ATP hydrolysis by internal Ca^{2+} concentration. In this framework it is obvious that an other advantage of our procedure relies on the efficient reconstitution of proteoliposomes with a high lipid to protein ratio. Table II reports Ca^{2+} transport activities of such proteoliposomes reconstituted at different lipid to protein ratios. For lipid to protein ratios ranging from 160 to 10 (w/w) the initial Ca^{2+} pumping rates of OG-reconstituted proteoliposomes increased proportionally with the amount of protein initially present (note that this would have led to constant initial rates if expressed per mg protein). On the other hand, the amount of Ca^{2+} taken up by the different proteoliposomes was found to be independent on the lipid to protein ratio. These results reflect an increasingly efficient, total and homogeneous protein insertion into liposomes, the internal volume in which Ca^{2+} ions are pumped being the same in this range of lipid to protein ratios. For lipid to protein ratios below 10 (w/w) (i.e., 1000:1, mol/mol) the rates of Ca^{2+} accumulation do not increase proportionally with the initial protein content reflecting limitation in incorporation of high amount of protein into preformed liposomes. On the other hand, the decrease in total Ca^{2+} uptake may be related to an increase in the passive permeability or to a decrease in the size of the resulting proteoliposomes.

A third reason that makes our procedure more suitable than previous ones may be related to the use of Bio-Beads as detergent removing agent. Besides

TABLE II

Influence of the lipid to protein ratio on the pumping efficiency of Ca^{2+}-ATPase proteoliposomes reconstituted by direct incorporation of the protein into octyl glucoside-saturated liposomes

Liposomes prepared by reverse-phase evaporation (4 mg lipid/ml, i.e. 5 mM) were first treated by saturating levels of octyl glucoside (26 mM); then presolubilized Ca^{2+}-ATPase was added to give the desired lipid to protein ratio. After detergent removal by SM-2 Bio-Beads, Ca^{2+} accumulations by reconstituted proteoliposomes were performed as described in Fig. 1 (for all reconstitution samples: 4 mg lipid in 2.5 ml of the reaction medium for Ca^{2+} accumulation measurements). The values presented are mean values ± S.D. from 10 different experiments.

Note that Ca^{2+} accumulations are not expressed per mg protein to avoid misleading interpretations when different lipid to protein ratios are used since as shown here the steady-state Ca^{2+} accumulations are in a large range (between 160 and 10 w/w) independent of protein content (in this range the initial rates of Ca^{2+} pumping increase linearly with the protein content).

Samples	Lipid/protein (w/w)	Ca^{2+} accumulation	
		initial rate (μmol Ca^{2+}/min)	total extent (μmol Ca^{2+})
Octyl glucoside reconstituted Ca^{2+}-ATPase proteoliposomes	160	0.025	0.150
	80	0.06 ± 0.01	0.175 ± 0.25
	40	0.125 ± 0.025	0.175 ± 0.25
	20	0.220 ± 0.04	0.175
	10	0.400	0.140
	5	0.500	0.100
	2.5	0.640	0.080

providing a reproducible and easy way to achieve unilamellar and well sized proteoliposomes it is well suited for varying and controling the rate of detergent removal. Furthermore, it allows complete detergent removal and using radioactive labels less than 7 TX-100 molecules or 5 $C_{12}E_8$ molecules per 100 lipid molecules were detected after reconstitutions. Implication of the almost complete detergent removal is the low ionic permeability of the reconstituted Ca^{2+}-ATPase proteoliposomes [22] allowing generation of large Ca^{2+} gradient concentration in the absence of oxalate. It was also checked in control experiments that, after accumulation into proteoliposomes, Ca^{2+} does not readily leak out of vesicules (a 5% decrease in murexide steady-state absorbance was observed after one hour in conditions where ATP initially added was exhausted).

Other factors of importance for the present results may have been: (i) presolubilization of the protein before reconstitution which ensures a predominantly monomeric state of soluble protein before reconstitution; (ii) the sequential addition of protein, detergent and lipid adopted which for example avoids complete inactivation of the Ca^{2+}-ATPase in OG-mediated reconstitutions and makes this detergent one of the most efficient for reconstitution; (iii) the nature of the phospholipids used. In this context considerable greater Ca^{2+} transport activities were observed for vesicles reconstituted with mixtures of EPC and EPA compared to those reconstituted with EPC alone (data not shown). The possibility that an appreciable amount of Ca^{2+} uptake was the result of adsorption to the negatively charged surface of the vesicules could be ruled out by the finding that the Ca^{2+} ionophore A23187 released all the Ca^{2+} previously accumulated indicating the occurrence of a transmembrane process (data not shown). Thus, the enhancement of Ca^{2+} transport activity by EPA could be related to the amount of protein incorporation, to the percentage of protein orientation (see below) and/or to a specific role of negatively charged lipids on the function of the Ca^{2+}-ATPase.

Another important aspect when dealing with membrane protein reconstitution mechanisms concerns the final orientation of the protein in the bilayer. In many cases where protein reconstitution occurred through a mechanism of direct incorporation into preformed liposomes, proteins were found almost unidirectionnally oriented whereas proteoliposomes with more random orientation were obtained when incorporation and vesicle formation occured simultaneously [3,8,10]. Unfortunately, our studies on Ca^{2+}-ATPase orientation do not allow us to analyze the possible relationships between the orientation of the protein and its reconstitution mechanism. Whatever the detergent used and its rate of removal rather similar orientations of the Ca^{2+}-ATPase were detected: they corresponded to about $80 \pm 10\%$ of the protein with right-side out orientations. Steric restrictions and/or electrostatic interactions between positively charged groups of the Ca^{2+}-ATPase with negatively charged groups of phospholipids could account for the fact that in any cases somewhat more than 70% of the proteins are finally found in right-side-out orientation. Additionally, it could be that the methodology to measure Ca^{2+}-ATPase orientation is not enough accurate to analyze slight variations ($\approx 15\%$) in protein orientation. Regardless of the ultimate explanation of this anomalous observation, it should, however, be stressed that the most efficient proteoliposomes were obtained using OG-mediated reconstitutions where direct incorporation of Ca^{2+}-ATPase into preformed liposomes was evidenced.

Discussion

The results obtained from Ca^{2+} pumping activities, sucrose density gradients, freeze-fracture electron microscopy and centrifugation experiments suggest a particular sequence of events that lead to incorporation of Ca^{2+}-ATPase into liposomes. These are schematically depicted in Fig. 8. Initially, mixed micelles composed of either lipid-detergent or lipid-protein-detergent are present. Depending upon the rate of detergent removal two main processes may occur. Upon slow detergent removal (process II in Fig. 8) it is proposed that as the detergent is initially removed, protein-rich structures are first formed. This is consistent with the observation that a large fraction of protein sedimented at detergent concentrations well below those at which the micellar-to-lamellar transition for phospholipids was complete. As the detergent concentration is further lowered, micelles containing lipids are disrupted and pellets consequently contain an increasing amount of phospholipid. It is likely that these phospholipids exist as liposomes saturated with detergent. At this stage, it is proposed that the dispersal of the protein among the liposomes, will depend upon the nature of the detergent and its ability to incorporate the 'aggregated' protein into the preformed liposomes. In the case of $C_{12}E_8$-mediated reconstitution, a large fraction of protein-free liposomes coexist with a small fraction of protein-dense liposomes (see Figs. 4 and 6). These observations indicate that in the presence of $C_{12}E_8$ the protein is, not transferred from the initially formed protein-rich structures to the phospholipid-rich liposomes (process IIB in Fig. 8). On the contrary, the results of OG-mediated reconstitutions (process IIA in Fig. 8) would indicate that the preformed lipid-protein complex can be incorporated into the phospholipid-rich recombinant since compositionally homogeneous proteoliposomes are formed upon slow removal of this detergent. This interpretation is further corroborated

by the results from the step by step reconstitution of the Ca^{2+}-ATPase demonstrating that Ca^{2+}-ATPase can be 'directly' incorporated into preformed liposomes destabilized by saturating levels of OG. Freeze-tracture electron micrographs, sucrose density gradients and also high ATP-dependent Ca^{2+} accumulation all indicate a total and homogeneous incorporation of the protein into OG-saturated liposomes. Finally, in the case of TX-100-mediated reconstitutions, it could be assumed that part of the protein is transferred to the liposomes. Thus although two vesicle populations were present the amount of protein-free liposomes was much less than in the case of $C_{12}E_6$-mediated reconstitutions and Ca^{2+} pumping efficiencies were in between those measured in OG- and $C_{12}E_8$-mediated reconstitutions.

Upon rapid detergent removal (process I in Fig. 8) it was shown by transport data and freeze-fracture electron microscopy that homogeneous protein distributions among liposomes were reached. The simplest explanation is that upon rapid detergent removal, the disruption of both types of micelles initially present is simultaneous, resulting in a mixing of their components. Under these conditions, whatever the nature of the detergent used, the resulting reconstituted liposomes are more nearly a reflection of the overall composition of the starting solutions, and thus more homogeneous. Finally, in the case of cholate-mediated reconstitutions, although it has been difficult to accurately follow lipid-protein association during detergent removal, it is proposed that Ca^{2+}-ATPase proteoliposomes only arise from micelle coalescence whatever the rate of detergent removal. This proposal is based on the following observations: (i) the rate of detergent removal has no significant effect on the homogeneity and Ca^{2+} pumping efficiencies of the reconstituted proteoliposomes; (ii) no protein heterogeneity can be detected by electron microscopy even at slow detergent removal rate; (iii) protein cannot be incorporated into preformed liposomes, eliminating the possibility of a protein transfer from putative protein-rich structures to preformed liposomes during slow detergent removal.

It is interesting to compare the mechanisms we propose for Ca^{2+}-ATPase reconstitution with those reported for other membrane proteins. Eytan [3] proposed that two mechanisms might occur during detergent removal: (1) detergent could be removed homogeneously from all micelles which disrupted and coalesced resulting in a mixing of their components; (2) 'loose' structures resembling liposomes could be first formed upon detergent removal, the remaining detergent catalyzing a direct incorporation of lipid-protein complexes into these 'preformed' liposomes. Significantly, it was proposed that the rate of detergent removal might be involved in determining one of the mechanisms: thus rapid detergent removal could lead to rapid and simultaneous micellar coalescence while upon slow detergent removal, liposome formation could preceed protein incorporation (see also Ref. 42). Thus, the mechanisms proposed by these authors are in line with those illustrated in Fig. 8. However, our experimental results clearly demonstrate that slow detergent removal lead to the formation of Ca^{2+}-ATPase-rich

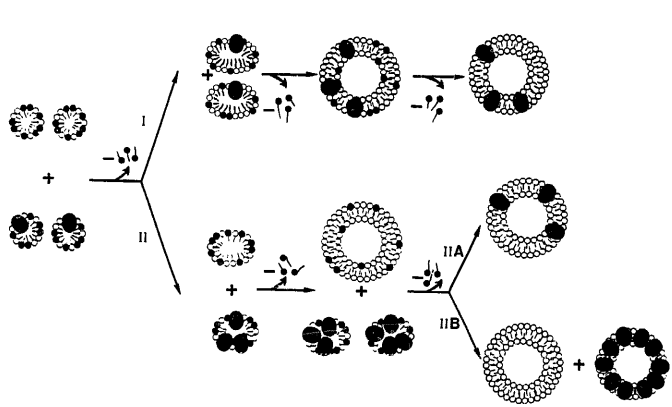

Fig. 8. Schematic representation of the suggested mechanisms by which Ca^{2+}-ATPase can associate with phospholipids upon detergent elimination from initial micellar detergent/lipid/protein solutions.

structures prior to liposomes formation, the efficiency of the reconstitution depending then on the ability of the detergent to incorporate the protein into the preformed liposomes.

What happens exactly at the beginning of detergent removal is not clear. Indeed this could be due to one or more of the following factors: (i) detergent/Ca^{2+}-ATPase/lipid micelles are less stable than the lipid/detergent micelles so that as the detergent concentration is initially lowered it is preferentially removed from the ternary micelles resulting in the initial formation of protein-rich liposomes; (ii) detergent is removed from both types of micelles but protein molecules tend to aggregate upon destabilization of the ternary micelles. Although we have no definite proofs, it is tempting to associate the appearance of protein-rich structures to the propensity of the Ca^{2+}-ATPase to self-aggregate when ternary micelles are detergent-depleted or become saturated with phospholipid. Indeed, although Ca^{2+}-ATPase can be solubilized in different detergents as defined monomers, it has been demonstrated that there is an increased tendency for self-association of the protein depending upon small variations in detergent, protein and/or phospholipid concentration (for a recent review, see Ref. 20). Thus, in detergent-mediated reconstitutions it becomes obvious that this propensity towards oligomeric association must be taken into account since proteoliposomes are formed by disruption of these 'unstable' protein/detergent/phospholipid micelles.

Extension of the model proposed in this report for SR-ATPase to other membrane proteins is also supported by our previous findings obtained under similar experimental conditions with bacteriorhodopsin and H^+-ATPase from chloroplast. In particular, it was demonstrated that these two membrane proteins could be incorporated into OG-saturated preformed liposomes [7,8] and that spontaneous insertion of crystalline arrays of bacteriorhodopsin (purple membrane sheets) into OG-saturated liposomes occurred. Since in many instances OG proved to be useful in faciliting the direct incorporation of membrane proteins into bilayer membranes even in the aggregated state [10,45,46], it is tempting to extend this mechanism to the reconstitution of all membrane proteins that tend to form aggregates or oligomers in the presence of low amounts of detergents. The results from TX-100 mediated reconstitutions of bacteriorhodopsin and H^+-ATPase [12] indicated that these two membrane proteins could also be incorporated into preformed liposomes but by a mechanism which required the presence of micellar structures. A time-dependent transfer of protein initially present in mixed micelles to TX-100-saturated liposomes was observed and shown to be dependent upon the number and/or composition of the mixed micelles present in the incubation medium. In view of the results reported here for Ca^{2+}-ATPase reconstitution, it could be proposed that the amount of detergent above that needed for liposome saturation was necessary to allow the dissociation of protein oligomers and/or their further incorporation into liposomes: in the case of Ca^{2+}-ATPase, due to its high propensity for self association, the size and/or the stability of these protein complexes is so important that they could not be dissociated by TX-100 and very few proteins could be directly incorporated. Finally, the results of reconstitution studies with sodium cholate demonstrated that H^+-ATPase and bacteriorhodopsin proteoliposome formation only arose from micelle coalescence according to the mechanism proposed here for Ca^{2+}-ATPase.

Conclusion

In order to obtain more information about the parameters involved in the reconstitution process we have recently developed a new experimental strategy [8] allowing a 'snapshot' on all the situations that may occur during detergent-mediated reconstitutions. It was obvious from our previous studies [8,11,12,31,36] that the nature of the detergent used for reconstitution was a key factor in determining the mode of optimal lipid-protein association. The purpose of the present report was to evaluate how the nature and the structure of the protein to be reconstituted influenced the reconstitution process and consequently proteoliposome characteristics. Therefore, studies similar to those reported for bacteriorhodopsin were conducted with another prototypic membrane protein, the Ca^{2+}-ATPase from sarcoplasmic reticulum. Although the products of reconstitution are clearly shown to be dependent upon the particular detergent used, the most important finding reported here is that the tendency for self-association of Ca^{2+}-ATPase molecules was a key factor in determining the composition of the final proteoliposomes during detergent-mediated reconstitution of this protein. The broad and systematic assessment reported here, offers a model for SR-ATPase reconstitution which can be extended to other membrane proteins.

Besides providing information by which proteins may associate to phospholipids during detergent-mediated reconstitutions, we believe that an important benefit of our study is the finding that the reconstitution described in this paper is a method of choice for the reconstitution of the Ca^{2+}-ATPase. The Ca^{2+} pumping capabilities (total Ca^{2+} uptake, initial rates of Ca^{2+} pumping) are the highest among those obtained without using Ca^{2+} precipitating anions in the reconstitution studies reported to date and comparable to those measured in the native SR vesicles. The good asymmetric orientation of the protein as well as the low ionic permeability of the reconstituted liposomes are

also definite advantages for further functional and structural studies of the Ca^{2+}-ATPase.

All the results emphazize the need for a systematic study of the kind reported here for the purpose of understanding reconstitution mechanisms and producing biologically efficient proteoliposomes. For example from the data of the literature, it appears that most reconstitution studies on SR ATPase have been essentially limited to the bile salt detergents while TX-100 [14], $C_{12}E_8$ [15] and OG [47] have not been used frequently. Furthermore, in all cases low transport activities were reported in the absence of precipitating anions. In addition, since most of these reconstituted systems have not been characterized in details with respect to their size distribution, protein distribution, protein sideness and passive permeability, it is difficult to offer explanations of the widespread, often unsatisfactory, results reported in the literature. Furtner detailed studies of the reconstitution processes whith other membrane proteins and other detergent are currently in progress in our laboratory, leading to an optimistic perspective in the formulation of general sets of principles for reconsititutions experiments.

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