A New "Gel-like" Phase in Dodecyl Maltoside–Lipid Mixtures: Implications in Solubilization and Reconstitution Studies

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ABSTRACT The interaction of dodecyl maltoside with lipids was investigated through the studies of solubilization and reconstitution processes. The solubilization of large unilamellar liposomes was analyzed through changes in turbidity and cryo-transmission electron microscopy. Solubilization was well described by the three-stage model previously reported for other detergents, and the critical detergent/phospholipid ratios at which lamellar-to-micellar transition occurred (R_{sat} = 1 mol/mol) and finished (R_{sol} = 1.6 mol/mol) were determined. The vesicle-micelle transition was further observed in the vitrified hydrated state by cryo-transmission electron microscopy. A striking feature of the solubilization process by dodecyl maltoside was the discovery of a new phase consisting of a very viscous "gel-like" sample. It is shown that this equilibrium cohesive phase is composed of long filamentous thread-like micelles, over microns in length. Similar structures were observed upon solubilization of sonicated liposomes, multilamellar liposomes, or biological Ca^{2+} ATPase membranes. This "gel-like" phase was also visualized during the process of liposome reconstitution after detergent removal from lipid-dodecyl maltoside micelles. The rate of detergent removal, controlled through the use of SM2 Bio-Beads, was demonstrated to drastically influence the morphology of reconstituted liposomes with a propensity for multilamellar liposome formation upon slow transition through the "gel-like" phase. Finally, on the basis of these observations, the mechanisms of dodecyl maltoside-mediated reconstitution of bacteriorhodopsin were analyzed, and optimal conditions for reconstitution were defined.

INTRODUCTION

Structural and functional studies of membrane proteins have made important advances during the past decade. However, in many instances, these studies are still limited because of the lack of reproducible methods for the solubilization, reconstitution and crystallization steps (for reviews, see Helenius and Simons, 1975; Silvius, 1992; Rigaud et al., 1995; Kühlbrandt, 1992; Dolder et al., 1996; Garavito et al., 1995). This bottleneck is mainly related to the amphiphilic character of membrane proteins, which require the use of detergents as a means of disintegrating the structure of native membranes in the initial step of their purification. Therefore, a comprehensive survey of the physicochemical properties and the use of detergents is still needed.

Dodecyl maltoside (DOM) is a nonionic detergent with a low critical micellar concentration (cmc) and is characterized by an intermediate length of the hydrophobic moiety and a bulky hydrophilic sugar headgroup. For the last ten years, DOM has gained widespread use in the solubilization of diverse functionally active membrane proteins (Suarez et al., 1984; Kragh-Hansen et al., 1993; Brandolin et al., 1993; Pourcher et al., 1995; Knol et al., 1996; Buchanan and Walker, 1996). In addition, this detergent has been used in reconstitution studies, and some reports have recently appeared in the literature dealing with the formation of active proteoliposomes (Groth and Walker, 1996; Knol et al., 1996) and 2D crystals (Rigaud et al., 1997). Surprisingly, despite all of these studies that employed DOM, very little information is available that quantitatively characterizes the solubilization and reconstitution processes using this detergent (Kragh-Hansen et al., 1993; De la Maza and Parra, 1997). Thus the main scope of the present investigation was to investigate the phase behavior of mixed DOM-phospholipid systems with the aim of providing a basis for developing rational, reproducible, and efficient reconstitution schemes that are useful for further functional and structural studies of membrane proteins.

In the present work, the transitional changes induced by the interaction of DOM on phosphatidyl choline/phosphatidic acid liposomes were studied by means of light-scattering and cryo-transmission electron microscopy (cryo-TEM). The turbidity data reported made it possible to accurately define the different steps of the solubilization process and to quantify the mixed bilayer–mixed micelle interconversion. The results were related to the three-stage model describing the interaction of detergents with membranes (Lichtenberg, 1985; Silvius, 1992; Rigaud et al., 1995). Then, using cryo-TEM, we have directly visualized the structures formed during the lamellar-to-micellar transition. It is worth noting that our data reveal an unexpected structural change during the solubilization process and report a new equilibrium "gel-like" phase specific to DOM-lipid interaction. It is shown that this cohesive viscous phase is composed of very long overlapping thread-like micelles, over microns in length, which has never been reported for other detergents.

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We have also investigated the mechanisms of DOM-mediated liposome reconstitution upon detergent removal from lipid-DOM micellar solutions. The optimal conditions for removing this low-cmc detergent by hydrophobic adsorption onto SM2 Bio-Beads were determined. The rate of detergent removal was shown to critically affect the morphology of resulting liposomes, demonstrating the role of kinetic factors in liposome reconstitution. In particular, our data indicated that a slow transition through the "gel-like phase" led to the formation of multilamellar liposomes instead of homogeneous unilamellar liposomes upon rapid transition. Finally, on the basis of these observations, we have studied the process of DOM-mediated membrane protein reconstitution according to a general method developed in our laboratory (Rigaud et al., 1995), and using bacteriorhodopsin (BR) as a prototypic membrane protein. The optimal conditions for the use of this glycosylated detergent in reconstitution experiments are defined and integrated into the general model proposed for other detergents.

MATERIALS AND METHODS

Materials

Egg phosphatidyl choline (EPC) and egg phosphatidic acid (EPA) of the highest purity were purchased from Avanti. The nonionic surfactant *n*-dodecyl-β-D-maltoside (DOM) was obtained from Sigma, and its radioactive derivative, 1-[^{14}C]dodecylmaltoside, was from CEA Saclay (France). SM2 Bio-Beads (20–50 mesh; BioRad) were extensively washed with methanol and water before use (Holloway, 1973). Polycarbonate filters were purchased from Nucleopore Corporation. All other reagents were of analytical grade.

Methods

Liposome preparation

Unilamellar liposomes of defined size were prepared by reverse-phase evaporation, followed by sequential extrusion through 0.4-μm and 0.2-μm Nucleopore filters (Paternostre et al., 1988). Small unilamellar vesicles were prepared by sonicating the large vesicles for 30 min under argon at 4°C. Multilamellar vesicles were prepared by rehydration of a dry lipid film. The liposomes were prepared with 9:1 molar mixtures of EPC and EPA as lipids, in a buffer composed of 100 mM K_2SO_4 and 10 mM KH_2PO_4 (pH 7.2).

Liposome solubilization

Liposomes were adjusted to the desired lipid concentration (from 1 to 10 mM). Solubilization of liposomes was carried out by adding increasing amounts of DOM to aliquoted vesicle suspensions, under constant stirring. After equilibration at 20°C, the turbidities of the different phospholipid-detergent suspensions were measured at 400–500 nm with a Unicam UV2 spectrophotometer.

Cryo-transmission electron microscopy

A few microliters of the lipid-DOM mixtures were applied to a holey carbon film on a copper grid that was held by tweezers mounted on a shaft above a liquid ethane bath cooled by liquid nitrogen (Leica EM CPC). The sample was blotted with filter paper and immediatly plunged into the liquid ethane. The vitreous specimens were transferred under liquid nitrogen to the cryo-TEM cold stage (model 626 Gatan), which was inserted into the Philipps CM120 electron microscope and maintained at −170°C throughout specimen observation. Specimens were imaged at 120 kV by low dose techniques, and micrographs were recorded on Kodak SO163 films with a magnification of 45,000× and a 1-μm defocus.

Membrane protein reconstitution

Purple membrane was isolated from *Halobacterium halobium* strain S9, according to the method of Oesterhelt and Stoeckenius (1974). Monomers of BR in detergents were prepared as described previously (Seigneuret et al., 1991). BR-containing proteoliposomes were reconstituted by a step-by-step procedure, according to the method previously reported (Rigaud et al., 1988, 1995). Liposomes were first treated with different amounts of DOM to reach the desired stage in the solubilization process. In a second step, solubilized proteins were added to the equilibrated detergent-phospholipid mixtures. Finally, the detergent was removed by direct addition of SM2 Bio-Beads (Levy et al., 1990a,b; Rigaud et al., 1997).

For BR proteoliposomes reconstituted in a medium containing 100 mM K_2SO_4, 10 mM PIPES (pH 7.2), light-induced transmembrane pH gradients were measured as changes in the fluorescence intensity of 9-aminoacridine (Cladera et al., 1996). Fluorescence was monitored with a Perkin-Elmer LS50B fluorimeter, using 400 and 460 nm for excitation and emission, respectively. Illumination was performed with a 250-W xenon lamp through a flexible glass fiber guide equipped with a low wavelength cutoff at 500 nm and a heat filter.

RESULTS

Solubilization of liposomes by dodecyl maltoside

Turbidity measurements

Previous studies demonstrated that changes in optical density of liposome suspensions upon the addition of detergent constituted a convenient technique for a survey of the bilayer solubilization by detergents (Lichtenberg, 1985; Paternostre et al., 1988; Silvius, 1992; Rigaud et al., 1995). Accordingly, the solubilizing perturbation produced by incremental addition of DOM to EPC/EPA liposomes was monitored by this technique.

However, because detergent was externally added, we have first determined the time needed for full equilibration. Control kinetic studies of the interaction of DOM with liposomes indicated that up to ~2–3 h was needed to achieve a constant light-scattering level when DOM was added to preformed liposomes (data not shown). In the following, to ensure complete detergent equilibration, the interaction of DOM with liposomes was studied through the changes in the OD of detergent-lipid suspensions equilibrated 24 h before measurement (see also De La Maza and Parra, 1997).

Fig. 1 *A* shows the solubilization curves of liposomal suspensions with increasing DOM concentrations (lipid concentration in the range 2.5–10 mM). The shape of each curve is similar for all lipid concentrations, which makes it possible to identify several points that represent a unique

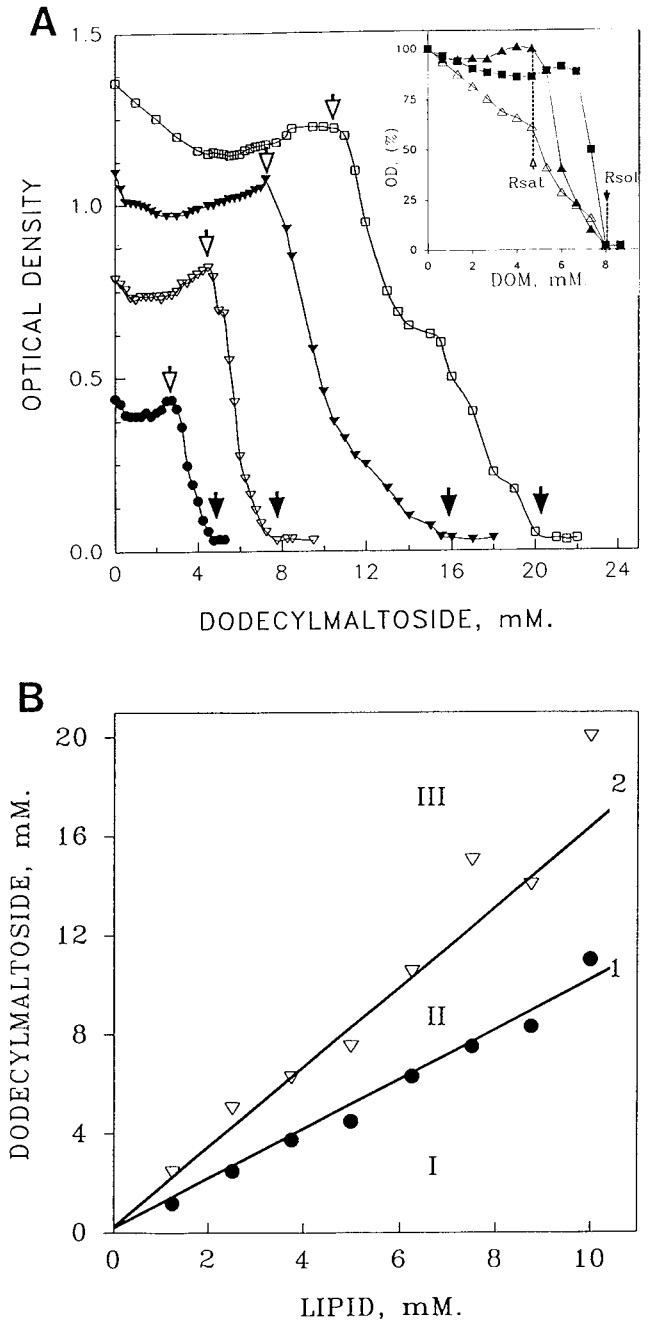

FIGURE 1 Solubilization of liposomes by dodecyl maltoside. (*A*) Changes in turbidity of liposome suspensions upon addition of increasing amounts of DOM. Liposomes, prepared by reverse-phase evaporation and subsequently filtered through 0.2-μm Nucleopore filters, were resuspended at concentrations of 2.5 (●), 5 (▽), 7.5 (▼), and 10 mM (□) phospholipid and treated with different amounts of DOM. Optical densities were measured at 450 nm after 24 h of equilibration at 20°C. White and black arrows indicate onset and total solubilization, respectively. (*Inset*) Percentage changes in turbidity of multilamellar (△), sonicated (■), and reverse-phase (▲) liposomes upon DOM addition. Total lipid concentration in all samples: 5 mM (i.e., 4 mg/ml). (*B*) Lipid-DOM phase diagram. Total DOM concentrations corresponding to the onset (●; *curve 1*) and total (▽; *curve 2*) solubilization were plotted as a function of the total phospholipid concentration. The plots are derived from data in *A* and other experiments. I, II, and III refer to the three stages of the solubilization process: liposomes with different amounts of incorporated detergent (I); mixture of DOM-saturated liposomes and mixed micelles (II); mixed micelles (III).

assembly of macromolecular structures. Previous studies indicated that each curve can be interpreted according to a three-stage model (Lichtenberg, 1985; Rigaud et al., 1995). Stage I involves partitioning of detergent monomers between the aqueous medium and the lipid bilayer, a process recently analyzed in detail by De La Maza and Parra (1997). During this stage, optical density shows a slight decrease followed by a slight increase. These changes in OD are difficult to interpret because they can be related to many parameters, such as changes in the average diameter, in the number of particles, in viscosity, and/or in refractive index. At the end of this stage (denoted by *white arrows*), liposomes are saturated with detergent. During stage II, detergent addition promotes gradual liposome solubilization and lipid-detergent micelles begin to form, inducing a large and significant decrease in OD. Stage III is characterized by complete solubilization of lipid into small mixed micelles, and the suspension becomes optically transparent (denoted by *black arrows*).

The process of solubilization was further analyzed quantitatively by plotting the DOM concentrations at which onset and total solubilizations occurred, versus the lipid concentrations. From the linear relationships obtained in Fig. 1 *B*, the process of solubilization can be described by the general equation

$$D_{\text{total}} = D_{\text{water}} + R_{\text{eff}} \cdot [\text{Lipid}]$$

where D_{total} and [Lipid] are the total detergent and lipid concentrations, respectively; D_{water} is the aqueous monomeric detergent concentration; and R_{eff} is the effective DOM/lipid ratio in the mixed aggregates (liposomes or micelles). From the slope of curve 1 in Fig. 1 *B*, the DOM/lipid molar ratio in the detergent saturated liposomes ($R_{\text{eff}} = R_{\text{sat}}$) is found to be ~1 mol DOM/mol lipid (0.625 w/w). Similarly, the slope of curve 2 in Fig. 1 *B* corresponds to the DOM/lipid molar ratio in the mixed micelles, i.e., the ratio at which the lamellar to micellar transition finishes ($R_{\text{eff}} = R_{\text{sol}} = 1.6$ mol/mol, i.e., 1 w/w)). Extrapolations of curves 1 and 2 to zero lipid concentration give D_{water} values of ~0.3 mM, which represent the aqueous concentration of DOM monomers in equilibrium with the saturated liposomes and the mixed saturated micelles, i.e., the cmc of DOM in the presence of lipids.

For comparison, the changes in turbidity of multilamellar and sonicated liposomes caused by the addition of DOM were measured under similar conditions (Fig. 1 *A*, *inset*). Although the detergent concentrations at which stage II started (R_{sat}) and finished (R_{sol}) were identical for each liposome preparation, some changes in the behavior of turbidity were observed, depending on the mode of preparation of the liposomes. In particular, for sonicated liposomes, instead of the progressive decrease in OD observed during stage II with liposomes prepared by reverse-phase evaporation, one can observe a slight increase followed by an abrupt decrease just before the end of the transition. In this context, much more drastic increases in turbidity have

been reported for DOM during stage II (Groth and Walker, 1996; Knol et al., 1996), which could be related to different lipid compositions and/or different modes of liposome preparations.

It is noteworthy that one of the most striking features not visible in the turbidity curves was a very drastic change in the consistency of the suspensions for R_{eff}'s around 1.4–1.5 mol/mol (i.e., near the end of the lamellar-to-micellar transformation). This change, consisting of an increase in the viscosity of the suspension, was clearly observed for phospholipid concentrations above 2 mM, and thickening progressed with phospholipid concentration to give a so-called gel-like sample: at a phospholipid concentration of 8 mM, when the sample was turned upside down, it remained stacked at the bottom of the tube. The new optically transparent phase was stable at room temperature for weeks, appearing to be isotropic between cross-polarizers. It should also be stressed that stirring enhanced the gel consistency and that during stirring, the interface moved inward and climbed at the center: this flow behavior is reminiscent of the so-called Weissenberg effect (Weissenberg, 1947) that is observed in semidilute polymer solutions and which implies the presence of highly entangled objects.

Cryo-transmission electron microscopy

We have correlated the different stages observed by optical density measurements with long-lived structures observed in the vitrified hydrated state using cryo-TEM. This technique, which avoids the artifacts of staining and drying procedures, permits the observation of undistorted samples.

As depicted in Fig. 2 *a*, the EPC/EPA liposomes formed by reverse-phase evaporation, followed by filtration through 0.2-μm Nucleopore filters, appeared by cryo-TEM to be spherical shells with delineated 4–5-nm lipid bilayer walls. No multilamellar vesicles were observed, and liposomes had a monomodal distribution centered around 120 nm (Fig. 3 *A*), in agreement with previous studies by freeze-fracture electron microscopy (Gulick-Krzywicki et al., 1987).

Vesicular structure was maintained after the addition of subsolubilizing DOM concentrations (Fig. 2 *b*), with slight but significant changes in the size distribution. As shown in Fig. 3 *A*, adding detergent up to the onset of solubilization led to the progressive appearance of larger unilamellar liposomes up to 0.3–0.4-μm diameter. Nevertheless, it should be noted that these large liposomes constitute only 3–5% of the total liposome population and could be attributed to vesicle rebuilding processes after local addition of highly concentrated detergent solutions to the liposome suspensions.

Adding DOM above the onset of solubilization (R_{sat}) induced a decrease in the number of liposomes which, up to a detergent/lipid ratio of $\sim$1.2 mol/mol, still appeared as closed unilamellar vesicles. Above this ratio, the number of liposomes still decreased, but now appeared more and more as highly open structures (Fig. 2 *c*). Upon a further increase in detergent concentration, long stringlike structures were seen to coexist with the intact and open vesicles (Fig. 2 *d*). Because these strings appear to have the same thickness as the vesicle wall, it could be suggested that they are strips of broken lamellar phases. However, these strings could also be attributed to mixed micelles of lipid and DOM, as proposed in the case of octylglucoside (*n*-octyl-β-D-glucopyranoside, OG), where long flexible cylindrical structures, 200–500 nm in length and similar in thickness to that of a phospholipid bilayer, were observed (Vinson et al., 1989). Whatever the final interpretation, it should be noted that the stringlike structures in Fig. 2 *d* appear, in our case, as contrasted as liposome bilayers.

Near the end of stage II ($R_{eff} \approx 1.5$), the new transitional "gel-like" state was detected, and vitrified specimens of these samples were observed by cryo-TEM (Fig. 2 *e*). The most striking features are very long filaments that almost completely fill the field of view. In some images, these filaments appear to emerge from the few liposomes still present (see, for example, Fig. 4, *a* and *b*). They reach over a few microns in length and overlap. Such extended structures, never reported for other detergent-lipid mixed systems (Vinson et al., 1989; Walter et al., 1991; Edwards et al., 1993), appear similar, however, to the very long threadlike micelles described by Danino et al. (1997) in highly diluted micellar solutions of phosphoglucolipids.

At R_{sol}, when the solution became clear, indicating that the structures were small, no large structures could be visualized, and no attempt to resolve these tiny structures was made (data not shown). Above this ratio, only micelles are present in the aqueous solution, which have been observed for other detergents as small threadlike or spheroidal structures (Vinson et al., 1989; Walter et al., 1991).

It is worth noting that the "gel-like" phase has also been encountered during the lamellar-to-micellar transition of different preparations of liposomes. Fig. 4, *a* and *b*, reports some representative micrographs, observed in the "gel-like" phase ($R_{eff} \approx 1.5$) for multilamellar and sonicated liposomes, respectively. In both cases, many long filamentous structures, characteristic of DOM-lipid mixtures, are visualized.

For comparison, we have also analyzed the process of solubilization by DOM of biological membranes, namely sarcoplasmic reticulum (SR) vesicles from skeletal muscle. One important finding was the absence of the "gel-like" phase reported during the solubilization of liposomes by DOM. Nevertheless, analysis by cryo-TEM of the structures formed near the end of the lamellar-to-micellar transition of SR vesicles indicated the presence of long filamentous structures (Fig. 4 *c*). However, it has to be stressed that these structures, although rather similar to those observed in the "gel-like" phase of DOM-liposome mixtures, were much less numerous and progressively broke down, decreasing in length, before full solubilization was obtained.

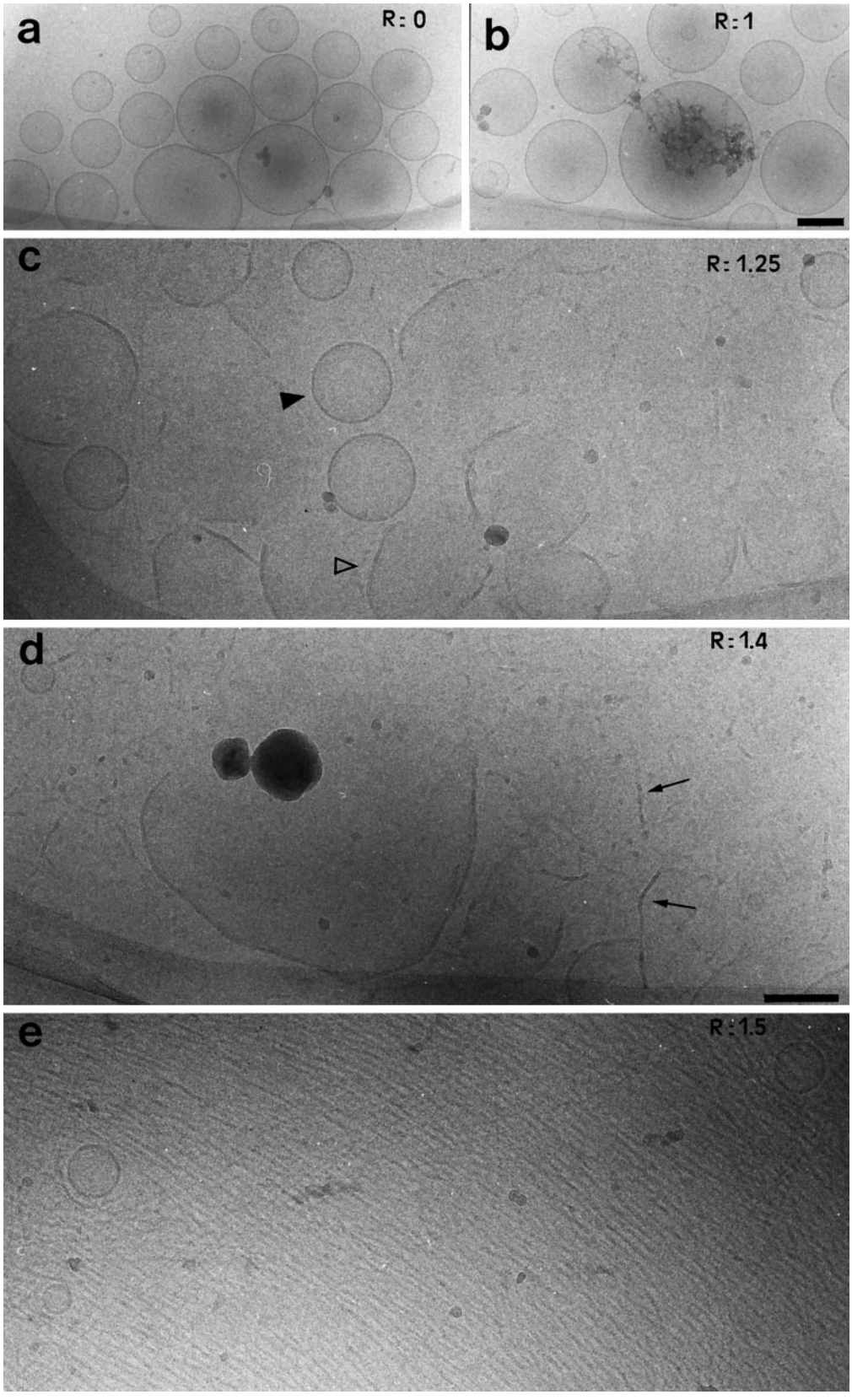

FIGURE 2 Cryo-TEM micrographs of liposome-dodecyl maltoside mixtures. Liposomes prepared by reverse-phase evaporation and subsequently filtered through 0.2-μm nucleopore filters were resuspended at a concentration of 5 mM lipid and treated with different amounts of DOM. (*a*) Sample corresponding to liposomes in the absence of detergent, $R = 0$. (*b*) Liposomes with average DOM/lipid molar ratio of (R) = 1. (*c*) $R = 1.25$: black and white arrowheads indicate close and highly open vesicles, respectively. (*d*) $R = 1.4$: arrows indicate long strings of broken lamellae or of lipid-DOM micelles. (*e*) $R = 1.5$: gel-like phase. Bar = 100 nm.

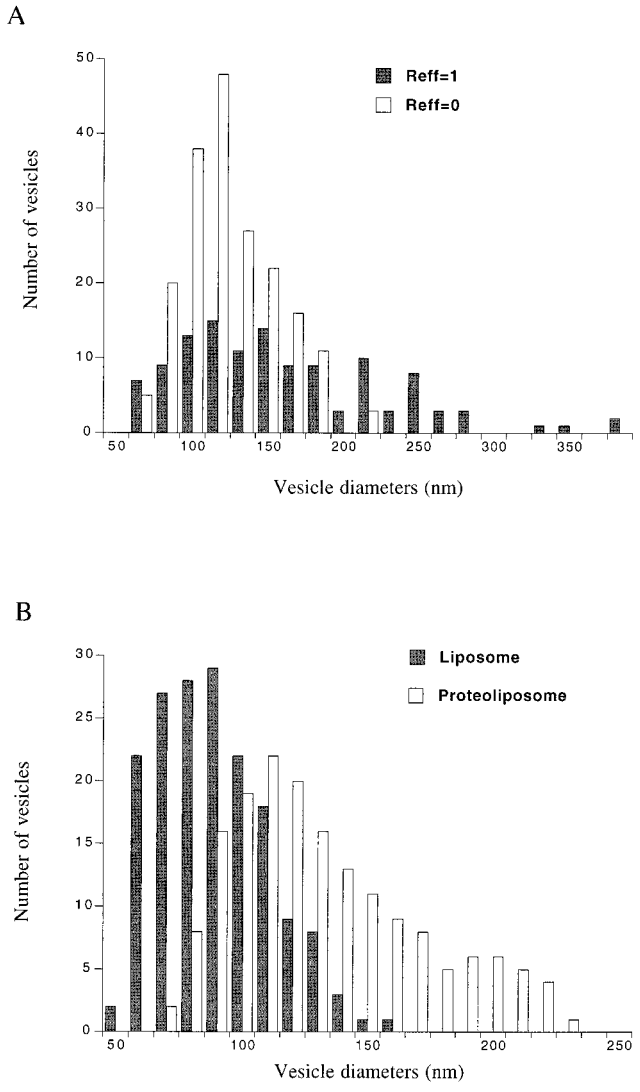

FIGURE 3 Size distribution of EPC/EPA liposomes. (*A*) Liposomes prepared by reverse-phase evaporation (*white columns*) and liposomes with an average DOM/lipid molar ratio of 1 (*grey columns*). (*B*) Liposomes (*grey columns*) and bacteriorhodopsin proteoliposomes (*white columns*) reconstituted by fast detergent removal from DOM-lipid micellar solutions as described in the legends of Figs. 5 and 6 (samples correspond to electron micrographs in Fig. 6, *b* and *d*, respectively).

Liposome and proteoliposome reconstitution

Liposome reconstitution

Bilayer formation upon detergent removal from mixed detergent-phospholipid micelles has been demonstrated to be the symmetrical opposite of bilayer solubilization (Levy et al., 1990b). However, although three steps in the reconstitution process have been observed at detergent/lipid ratios similar to those observed during solubilization, there is no information about the intermediate structures formed during reconstitution, which may be very different in the two symmetrical processes. In addition, kinetic factors have been shown to be important in determining the size distribution of reconstituted liposomes (Levy et al., 1990a,b, 1992). Because these aspects are important for further reconstitution experiments, we have studied in more detail the morphology of liposomes formed after detergent removal from DOM-lipid micellar solutions.

Because of the very low cmc of DOM, dialysis or dilution as means of detergent removal is relatively inefficient. We have thus adapted to this detergent the method previously developed for the study of liposome formation from mixed micellar solutions containing Triton X-100 or $C_{12}E_8$. This method relies on hydrophobic adsorption of detergents onto polystyrene beads. Previous studies indicated that this batch procedure was well suited for almost complete removal of different detergents and for controlling the rate of detergent removal by simply controlling the amount of beads (Levy et al., 1990a,b, 1992; Rigaud et al., 1997).

The standard procedure was the following. Solutions of mixed lipid-detergent micelles were obtained by adding 8 mM ^{14}C DOM to a suspension containing 5 mM lipid. After 24 h of incubation to ensure complete solubilization, SM2 Bio-Beads were added directly at different bead/detergent ratios, and detergent removal was analyzed as a function of time. Fig. 5 shows time courses of DOM removal after additions of different amounts of beads. DOM removal was essentially complete in ~30 min in the presence of an excess of beads (*open triangles* in Fig. 5) or extended to many hours by successive additions of small portions of beads at different time intervals (*filled triangles* in Fig. 5).

The size and unilamellarity of reconstituted liposomes have been investigated by cryo-TEM. Fig. 6 demonstrates that the rate of detergent removal drastically affects the lamellarity of reconstituted liposomes. Upon slow detergent removal, a large proportion of multilamellar liposomes are formed (Figs. 6 *e*), whereas rapid detergent removal leads to a relatively homogeneous population of unilamellar liposomes 100 nm diameter (Figs. 6 *d* and 3 *B*).

Importantly, although the different structures formed during DOM removal have not been analyzed in detail, it should be noted that the gel-like phase composed of very long filamentous threadlike micelles observed during solubilization were also present during the reverse process of reconstitution (see, for example, Fig. 6 *a*). Because this peculiar phase at the early micelle-bilayer transition is specific for dodecyl maltoside, the propensity for multilamellarity of liposomes reconstituted upon slow DOM removal could be related to a slow transition through the "gel-like" phase.

Proteoliposome reconstitution

We have studied in detail the reconstitution of BR, a prototypic membrane protein which, after incorporation into closed proteoliposomes, is able to generate a light-induced transmembrane pH gradient (Oesterhelt et al., 1992).

The reconstitution procedure was derived from the general method previously described for different membrane proteins using other detergents (Rigaud et al., 1995; Cladera et al., 1997). The procedure was carried out in three steps.

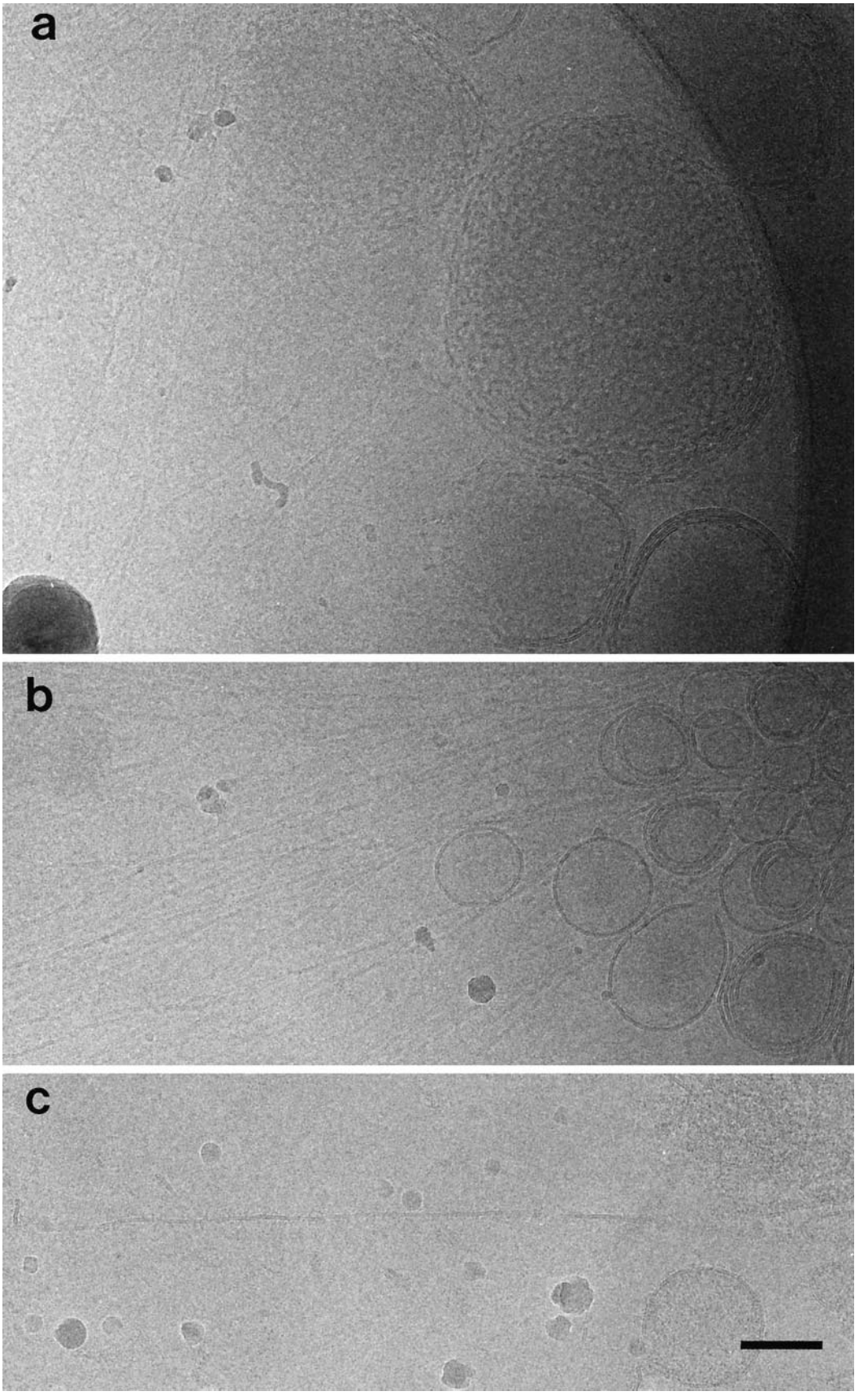

FIGURE 4 Cryo-TEM micrographs of the "gel-like phase" from different preparations. Representative pictures of viscous gel-like phases obtained after the addition of DOM (DOM/lipid = 1.5) to multilamellar liposomes (*a*) and sonicated liposomes (*b*). Representative filamentous structures observed during the solubilization of SR vesicles (*c*). SR vesicles (4 mg protein/ml) and DOM (3.5 mg/ml). Bar = 100 nm.

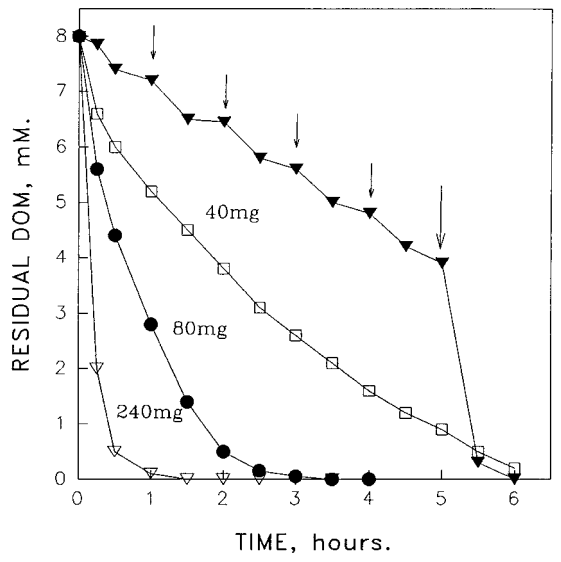

FIGURE 5 Dodecylmaltoside removal by SM2-BioBeads. Kinetics of DOM absorption onto SM2 Bio-Beads. At time zero, Bio-Beads were added to 1-ml micellar solutions containing 5 mM phospholipids and 8 mM DOM supplemented with radioactive detergent. The beads were continuously stirred at 20°C, and 10-μl aliquots from the supernatant were pipetted at various times for determination of DOM concentration. Detergent removal was accomplished by one addition of 240 mg beads (▽), 80 mg beads (●), or 40 mg beads (□), or by five successive additions of 10 mg beads followed by a final addition of 100 mg beads (▼) (successive additions are indicated by *arrows*).

Liposomes were first treated with different amounts of detergent to reach the desired stage in the solubilization process. After 3 h of incubation, previously solubilized membrane proteins were added and incorporation was studied at each step of the lamellar-to-micellar transition. The third step in the reconstitution is to remove the detergent by using SM2 Bio-Beads.

In view of the results reported above, we have studied the effects of the rate of detergent removal upon the efficiency of the reconstitution of BR. Starting from totally solubilized samples, much higher (about twofold) light-induced pH gradients were measured after fast detergent removal than after slow detergent removal (*traces a* and *b* in Fig. 7 *A*). These data can be explained simply on the basis of the electron micrographs reported in Fig. 6. Indeed, as for the reconstitution of pure liposomes, slow detergent removal leads to the formation of multilamellar BR-containing proteoliposomes (Fig. 6 *c*), which are expected to be much less efficient than the homogeneous unilamellar, 110–120-nm-diameter proteoliposomes produced upon fast detergent removal (Figs. 6 *b* and Fig. 3 *B*).

In the following, we have thus optimized the incorporation of BR after fast detergent removal from DOM-BR-lipid mixtures equilibrated at the desired lipid/detergent ratio. Fig. 7 *B* depicts the resulting biological activities of BR-proteoliposomes reconstituted from different DOM/lipid ratios. The highest activities were obtained for proteoliposomes formed from a detergent/lipid ratio corresponding to the onset of solubilization (R_{sat}). Starting from liposomes that were partially solubilized, the resulting activities were reduced, and starting from totally solubilized samples (R_{sol}), the activity was less than half that measured at the onset of solubilization (see also *traces a* and *c* in Fig. 7 *A*). This indicates that the optimal DOM-mediated reconstitution occurs by direct incorporation of the protein into preformed liposomes, provided these liposomes are destabilized by a saturating amount of dodecyl maltoside.

Another important aspect in reconstitution is the residual permeability of proteoliposomes, generally related to residual detergent. In this context, the use of Bio-Beads as a detergent-removing agent is an efficient strategy, because, as shown in Fig. 5, provided the amount of beads is above the adsorptive capacity of the beads, almost all of the detergent can be removed. The resulting impermeability of the reconstituted proteoliposomes was confirmed through the large light-induced 9-amino-acridine fluorescence changes similar to those previously reported in other detergent-mediated reconstitutions of bacteriorhodopsin and shown to correspond to pH gradients of ~2 pH units (Rigaud et al., 1988; Pitard et al., 1996). Another piece of evidence for impermeability relies on the effects of valinomycin on the kinetics of the light-induced pH gradients across reconstituted BR-proteoliposomes. As illustrated in Fig. 7 *A*, the rates of light-induced internal acidification by BR-proteoliposomes are strongly accelerated in the presence of this ionophore. Such an acceleration can be related to the collapse of a light-induced electrical transmembrane potential, generated in the absence of valinomycin, and reported to retroinhibit the H^+ pumping by BR (Seigneuret and Rigaud, 1986). In the presence of valinomycin, this electrical potential is overcome by compensatory K^+ movements, and a large pH gradient can develop rapidly because of the high, uninhibited rate of proton pumping.

DISCUSSION

The main goal of this study was a detailed investigation of the phase behavior of mixed phospholipid-detergent systems to increase our understanding of solubilization and reconstitution processes. We have extended the scope of previous investigations on different classes of detergents by including dodecyl maltoside, a glycosylated nonionic detergent with a low critical micellar concentration, which has gained widespread interest in membrane protein biochemistry during the last decade.

Solubilization process

The interaction of dodecyl maltoside with EPC/EPA vesicles was studied at different lipid/detergent ratios by correlating the macroscopic changes observed by bulk sample turbidity measurements with structures observed using cryo-TEM. From turbidity data, the solubilization process

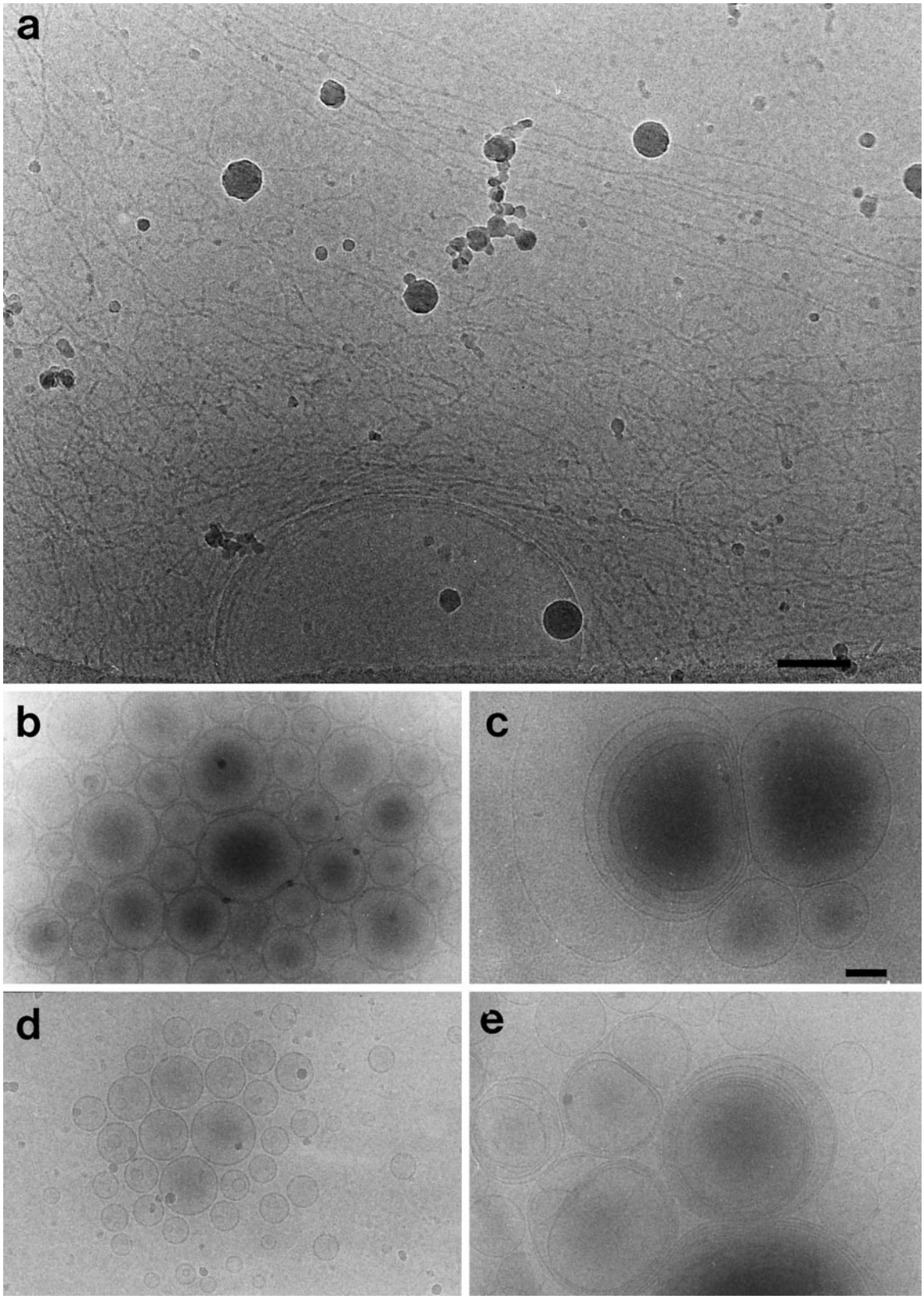

FIGURE 6 Cryo-TEM micrographs of reconstituted liposomes and proteoliposomes. Liposomes and bacteriorhodopsin-containing proteoliposomes were reconstituted by detergent removal from DOM-lipid and DOM-BR-lipid micellar solutions, respectively. Slow detergent removal corresponds to successive additions of 10 mg Bio-Beads (▼ in Fig. 5), whereas fast detergent removal corresponds to one addition of 240 mg Bio-Beads (▽ in Fig. 5). (*a*) Representative picture of a "gel-like phase" obtained upon slow detergent removal from DOM-BR-lipid micellar solutions. (*b* and *c*) BR containing proteoliposomes produced upon fast (*b*) or slow (*c*) detergent removal. (*d* and *e*) pure liposomes produced upon fast (*d*) and slow (*e*) detergent removal. Bar = 100 nm.

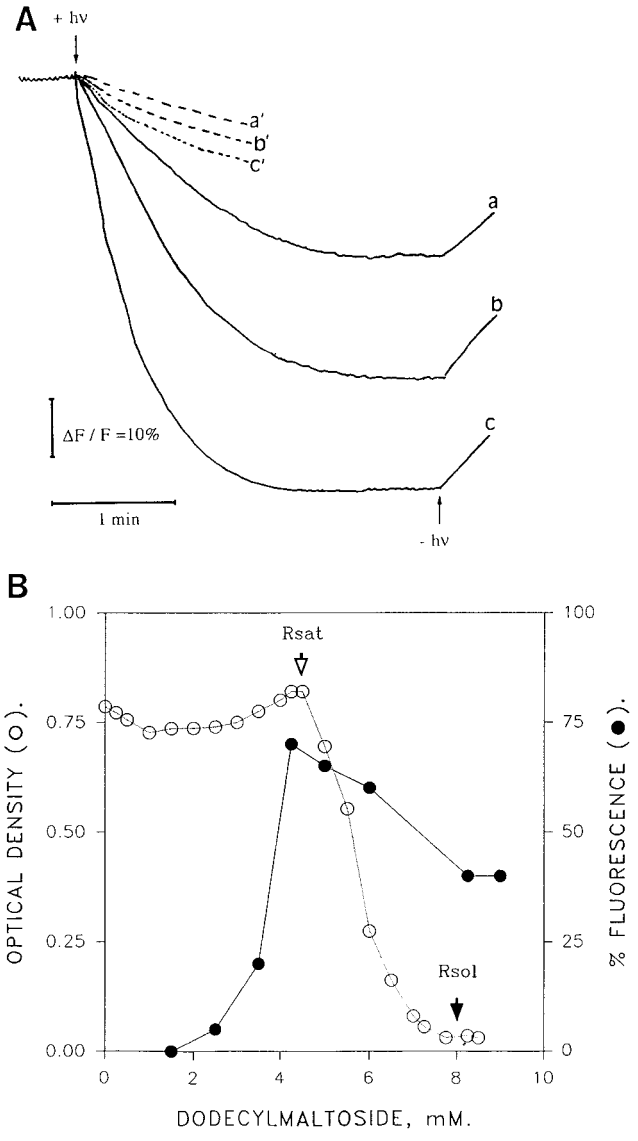

FIGURE 7 Dodecyl maltoside-mediated reconstitution of bacteriorhodopsin. Liposomes were first treated with variable amounts of DOM and allowed to equilibrate for 3 h. Then solubilized BR was added and equilibrated for an additional hour at room temperature. The final DOM concentrations take into account the small amount of detergent added with the protein. Final lipid concentration, 5 mM (i.e., 4 mg/ml); final BR concentration, 100 μg/ml. DOM was removed from the lipid-DOM-BR mixtures by one addition of 240 mg Bio-Beads (fast detergent removal) or by successive addition of 10 mg beads (slow detergent removal), as depicted in the legends of Figs. 5 and 6. After total detergent removal, light-induced pH gradients were measured at 20°C, as changes in the fluorescence intensity of 9-amino-acridine. (*A*) Light-induced pH gradients across BR-proteoliposomes. Samples were reconstituted from total solubilization ($R_{eff} = R_{sol} = 1.6$) by slow (*traces a, a′*) or fast (*traces b, b′*) detergent removal; samples reconstituted from onset of solubilization by fast detergent removal (*traces c, c′*). Traces *a, b,* and *c*: in the presence of 0.1 μM valinomycin; traces *a′, b′,* and *c′*: without valinomycin. (*B*) Bacteriorhodopsin incorporation measured by steady-state light-induced changes in 9-aminoacridine fluorescence (●) as a function of initial DOM concentration. ○, Absorbance of the lipid-BR-DOM mixtures before detergent removal. White and black arrows correspond to onset and total solubilization, respectively.

by DOM was described in line with the three-stage model: detergent incorporation, lamellar-to-micellar transition, and total solubilization (Lichtenberg, 1985; Rigaud et al., 1995). Turbidity measurements as a function of lipid concentration allowed quantitative determination of the critical effective DOM-to-lipid ratios at which the lamellar-to-micellar transition started ($R_{sat} = 1$ mol/mol) and finished ($R_{sol} = 1.6$ mol/mol). These values are similar to those reported by De La Maza and Para (1997) and can be compared with those measured under strictly similar conditions using other detergents. For example, Triton X-100, $C_{12}E_8$, octylglucoside, and cholate saturated liposomes at molar ratios of 0.64, 0.7, 1.3, and 0.3, respectively, whereas total solubilization occurred at respective ratios of 2.5, 2.2, 3, and 0.9 (Paternostre et al., 1988; Rigaud et al., 1995). Such a comparison indicates that 1) The lamellar-to-micellar transition is much shorter with DOM as compared to other detergents. There is a factor of only 1.6 between R_{sol} and R_{sat} for DOM, compared to a factor of ~3 for other detergents. 2) At subsolubilizing levels, DOM has a higher capacity for saturating EPC/EPA liposomes than other low-cmc detergents, and up to 1 mol DOM/mol lipid can be incorporated at saturation. 3) At solubilizing levels, DOM has a slightly higher ability to solubilize bilayers than the other low-cmc nonionic detergents and a much higher (about twofold) solubilization efficiency than octylglucoside. It is difficult, however, to rationalize all of these comparative data, which have to take into account interfering physicochemical properties of detergents such as shape, size, geometry, amphiphilicity, cmc, packing defects, and fusogenic ability.

A significant feature of the present data was the slowness (2–3 h) of DOM equilibration times when mixed with EPC/EPA unilamellar liposomes, as compared to previously reported equilibration times (a few minutes) observed for other detergents (Paternostre et al., 1988; Ollivon et al., 1988; Almog et al., 1990). This observation, made on pure liposomes, corroborates that made during DOM solubilization of biological membranes (Kragh-Hansen et al., 1993). A possible interpretation would be to consider the bulky glycosylated headgroup of DOM, which could hinder the binding of the detergent to the interface region of the liposomes, and its integration into the hydrocarbon region by rearrangement of detergent and lipid molecules (see discussion below on protein insertion). Other factors, such as viscosity and detergent flip-flop, are probably involved, in addition to detergent-detergent, lipid-detergent interactions and geometrical constraints. Whatever the final interpretation, one must include time among the important variables examined in optimizing the solubilization conditions by DOM of liposomes and biological membranes.

Intermediate structures in the vesicle-micelle transition

To address directly the microstructures of the aggregates formed during the solubilization process, we have per-

formed cryo-transmission electron microscopic studies. Cryo-TEM has several advantages for the present study. First, cryo-TEM, by analyzing vitrified samples, traps the potentially labile structures associated with intermediates in membrane solubilization. Second, entire hydrated aggregates of liposomes can be imaged, as opposed to the single fracture planes through the specimen that are observed with freeze-fracture electron microscopy. Third, no stains or cryoprotectants are used, reducing the number of artifacts.

Between the apparent upper and lower phase boundaries of the lamellar-to-micellar transition, different structures were observed: open vesicles, large bilayer sheets, and long threadlike micelles. Although relatively similar structures have already been reported for other detergent-lipid systems, despite the vast difference in R_{eff}'s at each transition, our data on DOM relate an unexpected new microstructure, corresponding to a macroscopic "gel-like" phase. Near the end of the lamellar-to-micellar transition, the DOM-lipid suspensions became very viscous, forming a gel-like phase. This new phase is composed of filamentous structures spanning the field of view. When one compares these threadlike micelles to the cylindrical, wormlike or rodlike micelles seen in other surfactant systems or in phospholipid-detergent mixtures (Vinson et al., 1989; Walter et al., 1991; Edwards et al., 1993; Silvander et al., 1996), their unusual feature is their long persistence length, over microns in length. Such long and numerous entangled structures, which are observed whatever the mode of preparation of liposomes, may cause the high viscosity and the gel consistency of the DOM-lipid mixtures. They can also explain the flow behavior of the phase upon stirring, namely an increase in the gel consistency and a clear Weissenberg effect. Solubilization of SR vesicles, i.e., vesicles with a high protein content, demonstrate that long threadlike micelles are still present, but the presence of protein and/or the lipid heterogeneity in SR (see Kragh-Hansen et al., 1993) prevents the large increase in number and length observed for lipid-DOM mixtures, possibly explaining the absence of the gel-like consistency of the phase.

It is of interest to relate these observations to those reported in the studies of the liposome-micelle transition by octylglucoside, another glycosylated detergent. Near the micellar phase boundary, the appearance of oil-like droplets, accompanied by a dramatic increase in the turbidity, was observed in octylglucoside-lipid mixtures (Paternostre et al., 1988; Ollivon et al., 1988; Almog et al., 1990). Such turbid mixtures separated into two bulk phases; indeed, after a few hours of incubation at room temperature, a spontaneous macroscopic phase separation occurred, resulting in a clear, viscous lower phase and a turbid upper phase (Ollivon et al., 1988; Almog et al., 1990). On the basis of lipid and detergent composition, the upper phase was suggested to be composed of lamellar structures, whereas the structure of the lower phase, which was clear, viscous, and enriched in lipid and OG has not been determined. Although there is no a priori reason to expect OG and DOM interactions with phospholipid to be identical (there are large differences in the hydrophobic chain length and in the structure of the polar headgroup, leading to large differences in cmc's and in the R_{eff}'s for the transitions), it is tempting to correlate the macroscopic gel-like phase observed in the presence of DOM with the viscous phase observed after phase separation in the presence of OG. Furthermore, because long threadlike structures similar to those reported here for DOM have been observed for phosphoglycolipid micelles (Danino et al;, 1997), it can be proposed that surfactants with a glycosylated polar headgroup have the tendency to give this very viscous phase the viscosity or gel-like consistency, depending on the structure of the glycosylated headgroup, the amphiphilicity of the detergent molecule, and/or the lipid and protein composition of the micelles.

Liposome formation upon detergent removal

Another important part of this work is related to the study of liposome reconstitution upon detergent removal from DOM-lipid mixed micelles. The protocol that was employed, namely adsorption of DOM onto Bio-Beads, was well suited to investigating the role of kinetics factors in the vesiculation process. In this context, our cryo-TEM studies demonstrated that the rate of detergent removal critically affected the morphology of reconstituted liposomes. Upon slow detergent removal, a high proportion of multilamellar structures were formed, whereas upon rapid removal, almost unilamellar liposomes were produced. Such an observation appears to be specific to DOM-mediated reconstitutions, because our previous studies with OG, Triton X-100, $C_{12}E_8$, and different ionic detergents have shown that, when the same strategy of detergent removal was used, whatever the rate of removal, only unilamellar liposomes were produced. The only significant effect of the rate of detergent removal was a propensity to form small liposomes upon rapid detergent removal (Levy et al., 1990a,b, 1992; Cladera et al., 1997).

This specific property of DOM-mediated reconstitutions led us to tentatively correlate the multilamellar tendency to a slow transition through the specific gel-like phase. In the light of the models proposed for vesicle formation by detergent depletion techniques (Lasic, 1988; Wrigglesworth et al., 1987; Schurtenberger et al., 1984), it has been proposed that three steps may occur during the overall process: micellar equilibration (micellar growth), bilayer closure, and liposome growth (due to residual detergent in the formed liposomes). Thus a possible interpretation of multilamellar formation upon slow DOM removal from mixed micelles would be that in the early stage of detergent removal, mixed micelles would fuse, leading to the formation of very long threadlike micelles. Upon further detergent removal, these entangled filamentous micelles would bend and coalesce, leading to multilamellar bilayer formation.

In this context, we would like also to mention that recent 2D crystallization trials, using SM2 Bio-Beads to remove DOM, have shown that the rate of detergent removal dras-

tically influenced the morphology and shape of melibiose permease crystals (Rigaud et al., 1997), confirming the importance of kinetic factors in the DOM micellar-to-lamellar transition.

Membrane protein reconstitution

Using the same strategy of reconstitution with other detergents and other proteins (Rigaud et al., 1995; Pitard et al., 1996; Cladera et al., 1996, 1997), we have identified three mechanisms by which membrane proteins can associate with lipids to give functional proteoliposomes. Depending on the nature of the detergent, proteins can be either directly incorporated into detergent-saturated liposomes (OG-mediated reconstitutions), transferred from mixed micelles to detergent-saturated liposomes (Triton X-100-mediated reconstitutions), or participate in proteoliposome formation during the micellar-to-lamellar transition (ionic detergents). Our data on DOM-mediated reconstitution of bacteriorhodopsin are in agreement with the mechanisms described for OG-mediated reconstitutions of different classes of membrane proteins, i.e., by direct incorporation of proteins into detergent-saturated liposomes. In this context, recent studies, using our step-by-step protocol for reconstitution of the bovine heart mitochondrial ATP synthase (Groth and Walker, 1996) and of the lactose transport protein of *Streptococcus thermophilus* (Knol et al., 1996), demonstrated that the highest transport activities were obtained when the liposomes were titrated with saturating amounts of DOM. Although not analyzed in this paper, an ultimate consequence of this mechanism of direct protein incorporation into preformed liposomes is related to the final orientation of the protein: indeed, upon direct incorporation, protein orientation has been shown to be asymmetrical, leading to much more efficient biological activities than reconstitution from micellar solutions in which protein orientation is generally more random (Rigaud et al., 1995).

Common mechanisms described for OG- and DOM-mediated reconstitution may be related to the only common property of these two detergents, i.e., a glycosylated polar headgroup. In this connection, recent NMR studies on the interactions of OG with lipid bilayers suggested an alignment of detergent and lipid molecules where the sugar moiety of the detergent penetrated approximately up to the level of the glycerol backbone, with the headgroup region tightly packed and the conformation of the $P\text{-}N^+$ dipole of the lipid almost unchanged (Wenk et al., 1997). The large cross-sectional area of the OG headgroup, as well as the short C_8 chain of the detergent produced packing defects in the central part of the membrane. Such specific defects induced by glycosylated detergents on lipid bilayers may be clues in driving the direct incorporation of membrane proteins into detergent-saturated liposomes.

As a last remark, this work establishes the potential of SM2 Bio-Beads for removing DOM. Besides providing a convenient way to control the rate of detergent removal and thus the homogeneity and unilamellarity of liposome or proteoliposome preparations, another advantage of Bio-Beads is to allow almost complete detergent removal, leading to relatively impermeant proteoliposomes. Dialysis would have been as efficient in removing all of the detergent, but would have required a very long time for total removal, which might be drastic when dealing with unstable membrane proteins. Furthermore, related to our results on the effects of detergent removal rate, dialysis would result in the formation of multilamellar structures. Thus the most important benefit in using Bio-Beads is to produce unilamellar liposomes and proteoliposomes with a low ionic permeability, which is obviously crucial to the study of transport membrane proteins.

CONCLUSION

The solubilization of biological membranes by detergents as well as the formation of proteoliposomes from lipid-protein-detergent mixtures have found wide application in membrane research. Examples are the preparation of lipid vesicles of defined size and composition, the reconstitution of membrane proteins in functional forms, and more recently, 2D crystallization of membrane proteins. However, it is clear that a basic understanding of the lipid-detergent interactions is an indispensable prerequisite for efficiently seeking the optimal conditions for solubilization and reconstitution of a given membrane protein. The present paper on the use of dodecylmaltoside is a piece of this wide systematic work needed to optimize the incorporation of membrane proteins for which the use of this detergent is convenient. Studies on 2D crystallization mediated by this detergent are currently in progress in our laboratory (Rigaud et al., 1997).

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