The Mechanism of Detergent Solubilization of Liposomes and Protein-Containing Membranes

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ABSTRACT The present study explores intermediate stages in detergent solubilization of liposomes and Ca^{2+}-ATPase membranes by sodium dodecyl sulfate (SDS) and medium-sized ($\sim C_{12}$) nonionic detergents. In all cases detergent partitioning in the membranes precedes cooperative binding and solubilization, which is facilitated by exposure to detergent micelles. Nonionic detergents predominantly interact with the lipid component of Ca^{2+}-ATPase membranes below the CMC (critical micellar concentration), whereas SDS extracts Ca^{2+}-ATPase before solubilization of lipid. At the transition to cooperative binding, *n*-dodecyl octaethylene glycol monoether ($C_{12}E_8$), Triton X-100, and dodecyldimethylamine oxide induce fusion of small unilamellar liposomes to larger vesicles before solubilization. Solubilization of Ca^{2+}-ATPase membranes is accompanied by membrane fragmentation and aggregation rather than vesicle fusion. Detergents with strongly hydrophilic heads (SDS and β-D-dodecylmaltoside) only very slowly solubilize liposomal membranes and do not cause liposome fusion. These properties are correlated with a slow bilayer flip-flop. Our data suggest that detergent solubilization proceeds by a combination of 1) a transbilayer attack, following flip-flop of detergent molecules across the lipid bilayer, and 2) extraction of membrane components directly by detergent micelles. The present study should help in the design of efficient solubilization protocols, accomplishing the often delicate balance between preserving functional properties of detergent sensitive membrane proteins and minimizing secondary aggregation and lipid content.

INTRODUCTION

Detergents are indispensable as solubilizing agents in the isolation, purification, and reconstitution or crystallization of membrane proteins. Furthermore, at lower nonsolubilizing concentrations, these compounds serve useful purposes as agents with which to permeabilize or to perturb membrane structure (Andersen et al., 1983; McIntosh and Davidson 1984; Kragh-Hansen et al., 1993; de Foresta et al., 1994). For the correct use of detergents it is important to have a detailed knowledge of how and in which amounts they interact with integral membrane proteins and membrane lipid, under both solubilizing and nonsolubilizing conditions. This information is also important with respect to functional properties, which are often best preserved when lipid is present together with detergent on the membrane proteins in the transitional states of the solubilization process (Lund et al., 1989; Breyton et al., 1997). According to the three-stage hypothesis, membrane solubilization is an all-or-none process with the characteristics of a phase transition (Jackson et al., 1982; Lichtenberg et al., 1983; Møller et al., 1986; Paternostre et al., 1988; Levy et al., 1990; Lichtenberg, 1995). In stage I detergent is taken up, without solubilization, by the membraneous phase, whereas in stage III the membrane components are fully solubilized by incorporation into mixed micelles. In stage II detergent-saturated membranes are considered to coexist with mixed micelles at a thermodynamic activity close to the critical micellar concentration (CMC).

Although the three-stage hypothesis in a simple and didactic way relates solubilization to fundamental detergent properties, several observations raise questions concerning the sharpness of the transition between the nonsolubilized and solubilized state. A gradual transition is suggested from both the detailed analysis of binding isotherms (Lasch 1995; Kragh-Hansen et al., 1993) and ultrastructural investigations, particularly those obtained with cryotransmission electron microscopy (CTEM). Thus, during solubilization of egg yolk lecithin by octylglucoside (Vinson et al., 1989), *n*-dodecyl octaethylene glycol monoether ($C_{12}E_8$) (Edwards and Almgren, 1991) and Triton X-100 (Edwards et al., 1989), bilayer sheets and extended, threadlike structures have been observed with this noninvasive technique. The formation of nonclosed bilayer sheets has been attributed to hydrophobic shielding of the edges of the bilayer with a rim of detergent (Fromherz et al., 1986; Lasic 1982, 1988), whereas extended, threadlike structures are assumed to reflect the formation of cylindrical micelles with a large proportion of lipid (Vinson et al., 1989; Edwards and Almgren 1991; Silvander et al., 1996).

Another aspect of the use of detergents relates to the nature of the solubilizing species: Is it detergent micelles or monomers? In a recent study Needham et al. (1997) reported that only exposure of liposomes to micelles of a fatty acid detergent (monooleoylphosphatidylcholine) resulted in vesicular rupture, whereas less uptake and no rupture was observed by exposure of the liposomes to detergent at the

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CMC. Furthermore, polyoxyethylene groups incorporated into liposomes sterically protected these against rupture by detergent micelles. In relation to this question, we have previously studied the mechanism of solubilization of Ca^{2+}-ATPase membranes with dodecylmaltoside (Kragh-Hansen et al., 1993). Here binding isotherms were obtained by equilibrium dialysis and combined with a number of other techniques to monitor vesicle fragmentation and solubilization. Our results implied that to obtain rapid solubilization and cooperative interaction, the addition of detergent micelles was required. During solubilization a number of intermediate states were observed. If instead of micelles monomeric detergent was added gradually to the biological membrane suspension, maintaining the free detergent concentration below the CMC, detergent was bound to the membranes by a saturable, noncooperative process that only very slowly (over a period of more than 1 week) was followed by cooperative binding and membrane solubilization. Observations such as these raise questions concerning the existence of different pathways for membrane solubilization with kinetic barriers, depending on the state and nature of the detergent and of the membrane.

As a further step in the characterization of detergent-membrane interaction, we have now compared and studied the solubilizing properties of a variety of detergents. Our data base includes two polyoxyethyleneglycol detergents, $C_{12}E_8$ and Triton X-100 (TX-100), as well as two other nonionic detergents, *N*,*N*-dimethyldodecylamine *N*-oxide (DDAO) and dodecylmaltoside (DM). In addition we present data on one anionic detergent, sodium dodecyl sulfate (SDS). With these compounds we studied general features in the interaction of detergents with Ca^{2+}-ATPase and liposomal membranes. In addition to providing essential data on detergent binding and intermediate states arising during solubilization, our findings indicate important roles of detergent micelles and flip-flop rates across lipid bilayers, to account for the ways in which these detergents act as efficient solubilizers of biological membranes.

MATERIALS AND METHODS

Materials

Sarcoplasmic reticulum (SR) vesicles, isolated from rabbit skeletal muscle according to the procedure of de Meis and Hasselbach (1971), were used for preparation of purified and leaky Ca^{2+}-ATPase membranes by extraction with a low concentration of deoxycholate according to the method of Meissner et al. (1973). SR lipid was prepared from SR vesicles by chloroform/methanol extraction, according to the method of Folch et al. (1957), and purified by removal of proteinaceous (sarcolipin) material by precipitation with diethylether (MacLennan, 1974). Unilamellar liposomes of dioleoylphosphatidylcholine (DOPC) (Avanti Polar Lipids, Highbluff, AL) and SR lipid were prepared by cholate dialysis of the lipid by procedures previously described (Heegaard et al., 1990; Cornelius and Møller 1996). Multilayered vesicles were prepared from thin lipid films, formed by evaporation of chloroform-solubilized dioleoylphosphatidylcholine, which subsequently were dispersed by vigorous vortexing in aqueous media containing 10 mM *N*-tris-(hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES) (pH 7.4), 100 mM NaCl, and 0.1 mM $CaCl_2$.

For the measurement of bound detergent we used freshly prepared batches of the following labeled materials: octaethyleneglycol [1-^{14}C]dodecyl monoether, [1-^{14}C]dodecyldimethyldiamineoxide, and [1-^{14}C]dodecyl-β-D-maltoside, all of which were custom synthesized by Centre d'Etudes Nucleaires de Saclay (Gif-sur-Yvette, France); (Among these, small amounts of labeled dodecylmaltoside are available by request with the necessary authorizations from Marc le Maire free of charge, except for freight.) Triton[*phenyl*-^{3}H(N)] X-100 that was purchased from New England Nuclear (Boston, MA); and dodecyl [^{35}S]sulfate from the Radiochemical Centre (Amersham, England). Unlabeled detergents were obtained from the following sources: $C_{12}E_8$, Nikko Chemicals (Tokyo, Japan); Triton X-100 and dodecyldimethyldiamineoxide, Serva (Heidelberg, Germany); SDS, Merck (Darmstadt, Germany); *n*-dodecyl-β-D-maltoside, Biochemica Boehringer (Mannheim, Germany). The purity of the unlabeled detergents used was analyzed by electrospray ionization mass spectrometry, operated in either the positive ($C_{12}E_8$, DDAO) or negative (SDS, dodecyl-maltoside) mode. In the former case 10-μl samples of 20–50 μM detergent solubilized (by volume) in 50% acetonitrile/50% H_2O plus 0.1% formic acid were injected in an electrospray spectrometer as previously described (le Maire et al., 1993). In the negative mode formic acid was replaced by 0.3% NH_4OH. In all cases, including SDS, the chemical identity of the detergents was confirmed, including the homogeneity of the alkyl chains (cf. Popot et al., 1987). The purity of radiolabeled detergent was regularly checked by gel chromatography as previously described (le Maire et al., 1983), and this method was also used to establish identical behavior (same specific radioactivity) of labeled and unlabeled Triton X-100, eluting from the column in micellar and nonmicellar form.

Equilibrium dialysis

To measure detergent binding we used a thermostatted two-chamber (2 × 250 μl) dialysis system made from Teflon half-alls (Dianorm Geräte, München, Germany), with sample volumes of 225 μl, separated by cellophane tubing (ϕ = 1.8 cm, Visking 18/32 or 20/32, with molecular mass cut-offs of 8 kDa and 10–12 kDa, respectively). Visking 18/32 was used in the experiments with nonionic detergents, whereas the more porous type (20/32) was required to obtain comparable transfer rates in experiments with the more slowly dialyzing SDS. Dialysis was usually carried out at 20°C and pH 7.4 in media, containing 10 mM TES, 100 mM KCl (or NaCl in the presence of SDS), 0.1 mM $CaCl_2$, with 1 mM NaN_3, and gentamycin (20 μg/ml) added to prevent bacterial growth. In *cis* experiments detergent was added at various concentrations to the vesicle preparations (containing 0.5 mg protein or 0.25 mg phospholipid/ml) at the start of the experiment, and dialysis proceeded against detergent-free buffer medium in the other compartment for a minimum of 48 h to attain equilibrium. In *trans* experiments detergent was added to the opposite (membrane-free) compartment, and dialysis was performed for a minimum of 72 h. Concentrations on the *trans* side, indicating the level of unbound detergent, were obtained from duplicate samples in different experiments with an accuracy (SEM) of ±4 (3–6)%. The levels of bound detergent, calculated from the *cis* data after subtraction of radioactive counts from the *trans* side as previously described (Kragh-Hansen et al., 1993), were obtained with an accuracy of ±3 (2–4)%.

Light scattering experiments

The effect of detergent on Rayleigh light scattering of membranous preparations was measured on a Perkin-Elmer M-PF 44A spectrofluorometer with both monochromators set at 600 nm. In ordinary titration experiments small aliquots of stock detergent solutions (50–100 mg/ml) were added stepwise to 2.5 ml of membrane suspensions (0.5 mg protein/ml of Ca^{2+}-ATPase membranes or 0.25–0.5 mg phospholipid/ml) in a well-stirred cuvette in media containing 10 mM TES (pH 7.4), 100 mM KCl (or 100 mM NaCl in the case of experiments involving SDS), and 0.1 mM $CaCl_2$. Sufficient time was allowed for light scattering to stabilize after each addition. In the case of SDS this required several minutes for Ca^{2+}-ATPase

membranes at the critical solubilization levels, and even longer periods ($\frac{1}{2}$–1 h) for liposomal membranes.

For both Triton X-100 and DDAO the decrease in light absorption occurring close to the onset of solubilization was followed by slower, secondary increases arising from membrane aggregation and/or fusion of vesicles. To evaluate the immediate effect of detergent on light scattering, we therefore, instead of performing a whole titration experiment, added the required amount of detergent in one shot and then read the light scattering curve immediately after the addition and again 2–3 min later. This procedure was repeated with fresh membrane samples for each of the detergent concentrations tested. Thus in these one-shot experiments we could obtain under defined conditions the scattering curve arising immediately from the detergent addition and monitor the following slow secondary changes in light scattering that was observed, especially with Triton X-100 and DDAO.

Quasielastic light scattering of detergent treated membranes was studied with a particle sizer analyzer (Nicomp Particle Sizing Systems, Santa Barbara, CA), equipped with a 633-nm high-power He/Ne Laser light source. Data were collected at a right angle over a period of 1 h. Particle sizes were calculated from the diffusion coefficient obtained from a computer analysis with the aid of software provided by the manufacturer. This involved in appropriate cases (DOPC) liposomes) conventional analysis of the data on the assumption of a Gaussian distribution of particle sizes ($\chi^2 < 2$) or, alternatively, analysis in terms of different classes of distributions. The latter procedure (referred to as Nicomp analysis) was performed on an inverted Laplace transform of the data to resolve the autocorrelation time function into its constituent exponential decay components. By this procedure up to three different size distributions could be deduced from the data, provided the use of long analysis times (1 h), but sometimes the presence of the third (large) component was only indicated by a nondecaying residual (persistence of a positive baseline after analysis) in the autocorrelation function.

Flip-flop experiments

Permeation rates of selected detergents ($C_{12}E_8$, SDS, and dodecylmaltoside) across liposomal membranes were examined by a modification of a method previously described (le Maire et al., 1987), but in which we used Biobeads to remove free detergent from the samples instead of agarose columns. To study release of intraliposomal detergent, multilayered vesicles of DOPC (5 mg/ml) were prepared in the presence of a medium containing 0.4 mg/ml detergent with radioactive tracer, 10 mM TES (pH 7.4), 100 mM NaCl, and 0.1 mM $CaCl_2$. At the start of the experiment the preparation was first diluted 100-fold with suspension buffer without detergent. Immediately after the dilution and at various times thereafter, 2.5-ml aliquots were applied to a 1×5 cm BioBeads column, which was rapidly eluted with detergent-free buffer. Measurement of detergent retention by the vesicles was based on determination of the radioactive detergent remaining in the first 8×1-ml fractions collected from the column.

Uptake of detergent by liposomes was studied by the addition of 0.4 mg detergent/ml plus radiolabeled detergent to unilamellar DOPC vesicles at 5 mg phospholipid/ml, prepared by cholate reconstitution. At appropriate times thereafter, uptake of detergent was stopped by 100-fold dilution with detergent free buffer and 2.5 ml of the diluted samples applied to a 1×5 cm BioBeads column and rapidly eluted. The peak of radioactivity associated with phospholipid eluting at 8×1 ml after the application was used for calculation of detergent retained inside the vesicles, with correction for radioactivity eluting in corresponding fractions in control samples that did not contain phospholipid.

These methods were well suited for quantitative measurements of slowly diffusing detergents with half-times for uptake and release of the order of several minutes or hours. To calculate detergent uptake, we applied a zero time subtraction to all data to correct for detergent that remained bound on the outer leaflet at zero incubation time after passage through the column (2–7%, depending on elution rates (1–3 ml/min) and the nature of the detergent (SDS requiring longer contact times than the nonionic detergents)).

RESULTS

Cis and *trans* binding of detergent

To establish conditions for the attainment of thermodynamic equilibrium, the binding of $C_{12}E_8$ to Ca^{2+}-ATPase membranes was measured after dialysis for various times in *cis* experiments, with $C_{12}E_8$ added together with Ca^{2+}-ATPase to the same compartment of the dialysis cell, and in *trans* experiments, with $C_{12}E_8$ added to the opposite compartment. Fig. 1 shows representative data obtained after 3 days of equilibration. It is seen that at low $C_{12}E_8$ concentrations there is no difference between *cis* and *trans* data, but at unbound concentrations of $C_{12}E_8$ above 60 μM the binding curves diverge. In the *cis* situation binding rises steeply and is strongly cooperative, whereas in the *trans* situation binding only rises gradually and remains noncooperative, reaching an upper value at the CMC. As can be seen from Fig. 1, binding data similar to those found here in the *trans* situation were previously obtained in gel chromatographic experiments (Andersen et al., 1983) and in short-term binding experiments on SR vesicles by the application of a rapid filtration method in which large volumes of nonmicellar detergent were flushed through glass filters

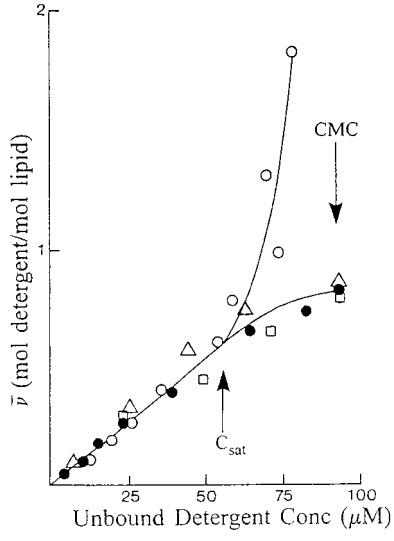

FIGURE 1 *Cis* and *trans* binding of $C_{12}E_8$ by Ca^{2+}-ATPase membranes. Binding of $C_{12}E_8$ by Ca^{2+}-ATPase membranes at 20°C was measured in a Dianorm experiment with an equilibration period of 3 days. The experiment was performed by addition of Ca^{2+}-ATPase membranes at 0.5 mg protein/ml in standard medium (Materials and Methods) to one side of the cell compartment and the addition of 0.09–0.9 mg $C_{12}E_8$/ml to the same (*cis*) side (○) or 0.045–0.6 mg $C_{12}E_8$/ml to the other (*trans*) side of the cell compartments (●). The figure includes previous data obtained by gel chromatography (□) on binding of $C_{12}E_8$ to membrane Ca^{2+}-ATPase (Andersen et al., 1983) and by filtration (△) through GF/F membranes (le Maire et al., 1987). The arrows labeled CMC and C_{sat} indicate the critical micellar concentration and transition concentration between noncooperative and cooperative binding of $C_{12}E_8$. Binding $\bar{\nu}$ is given in terms of moles detergent bound per mole phospholipid. Note that this corresponds to the effective detergent/phospholipid ratio (R_e) often used in experiments employing indirect measurements of detergent binding by the use of different phospholipid concentrations (Lichtenberg, 1985).

with deposited Ca^{2+}-ATPase membranes (le Maire et al., 1987). In these experiments binding also reached a maximum level at $C_{12}E_8$ concentrations close to the CMC, without evidence of cooperative interaction.

Similar characteristics of cooperative and noncooperative interaction in the *cis* and *trans* modes, respectively, were also observed with the other detergents used in this investigation and in our previous study of dodecylmaltoside, where it was demonstrated that only binding in the *cis* mode is correlated with membrane solubilization (Kragh-Hansen et al., 1993). Common for all detergents, equilibration in the *cis* mode can be obtained within a relatively short period (40–48 h), as when the Dianorm apparatus is used for ligand binding measurements by water-soluble proteins like serum albumin (Kragh-Hansen et al., 1990), whereas cooperative binding of detergent is obtained much more slowly (1–2 weeks) in the *trans* situation. The reason(s) for the sluggish binding of detergent in the *trans* situation as well as in other procedures (gel chromatography, filtration) are not entirely clear, but probably involves slow kinetic features in the cooperative interaction of the membranes with detergent at concentrations below the CMC (cf. the end of Discussion concerning micellar versus nonmicellar detergent interactions with Ca^{2+}-ATPase membranes). Therefore, in the experiments reported below, we routinely measured only detergent binding in the *cis* mode after an equilibration period of 48–72 h.

Detergent binding to Ca^{2+}-ATPase

Fig. 2 and Table 1 summarize salient features of the interaction of $C_{12}E_8$, Triton X-100, DDAO, and SDS with Ca^{2+}-ATPase membranes and protein-free liposomes. It should be noted that as in Fig. 1, detergent binding is expressed on a lipid basis, i.e., in terms of the number of moles bound detergent per mole lipid ($\bar{\nu}$). This is done regardless of the kind of detergent-binding species, whether it is the amount of detergent taken up by unsolubilized membranes or that which is present as detergent-lipid micelles and solubilized protein-lipid-detergent complexes. Fig. 2 shows that when the data are expressed in this way, there is no detectable difference between binding of any of the three nonionic detergents to Ca^{2+}-ATPase membranes and binding to liposomes made from SR lipids. This can be taken as an indication that the predominant part of nonionic detergent interacts with the lipid component of the Ca^{2+}-ATPase membranes, in both the noncooperative and cooperative binding modes, rather than with the protein part. During the solubilization of Ca^{2+}-ATPase and SR lipid membranes, the concentration of free detergent approaches the CMC for pure detergent. On the other hand, we find that interaction of detergent with DOPC is somewhat different from that of SR lipid, at least for $C_{12}E_8$ and Triton X-100, because DOPC is solubilized over a very narrow concentration range below the CMC.

In all cases the transition between noncooperative and cooperative detergent binding gives rise to a break point in the binding curves below the CMC. The concentration of free detergent at which this break point occurs is termed C_{sat}. As shown in Table 1, C_{sat} corresponds to 47–72% of the CMC of the detergents under study. Furthermore, we find that there is hardly any difference between SR lipid and DOPC with respect to their C_{sat} values and partition coef-

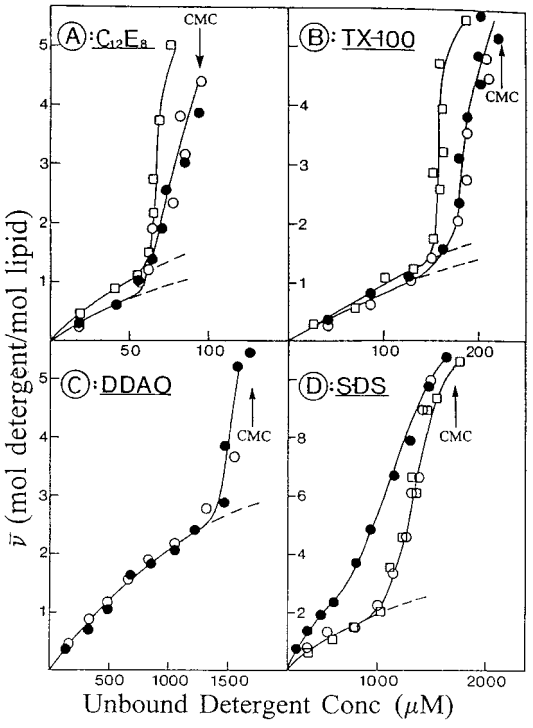

FIGURE 2 Binding of detergents by Ca^{2+}-ATPase, SR lipid, and pure dioleoylphosphatidylcholine membranes. The experiments were performed as Dianorm *cis* experiments over a period of 48–72 h with Ca^{2+}-ATPase membranes (●, 0.5 mg protein/ml), SR lipid (○, 0.25 mg lipid/ml), and dioleoylphosphatidyl unilamellar liposomes (□, 0.25 mg lipid/ml), to which were added various amounts of $C_{12}E_8$ (*A*), Triton X-100 (*B*), DDAO (*C*), and SDS (*D*) in standard medium. Data (mean values of duplicate samples from 3–5 experiments) are plotted as the molar ratio of bound detergent and phospholipid as a function of the free concentration of detergent measured as the concentration of the detergent on the *trans* side. Numerical values for the CMC are given in Table 1.

TABLE 1 Noncooperative interaction of detergents with lipid

Detergent	CMC (mM)	Lipid	K_p* (M/M)	C_{sat} (mM)	$\bar{\nu}$ (C_{sat})#	C_{sat}/CMC
$C_{12}E_8$	0.094	SR	24,000	0.055	0.80	0.59
—	—	DOPC	28,000	0.055	1.12	0.59
DM§	0.18	SR	27,000	0.085	0.97	0.47
—	—	DOPC	28,000	0.095	0.70	0.53
TX-100	0.23	SR	11,000	0.128	1.00	0.56
—	—	DOPC	12,000	0.144	1.37	0.63
DDAO	1.8	SR	4,000	1.3	2.5	0.72
SDS	1.7	SR	6,000	0.9	1.8	0.53
—	—	DOPC	6,000	0.9	1.8	0.53

* Moles detergent per liter lipid phase divided by moles detergent per liter aqueous phase.
# Moles detergent taken up per mole phospholipid at C_{sat}.
§ dodecyl-maltoside.

ficents (calculated from the linear uptake at low detergent concentrations). With respect to the uptake of the different detergents, the partition coefficients tend to be inversely related to CMC, a circumstance that probably reflects a correlation between an affinity for lipid and a tendency toward micelle formation. However, the correlation is not perfect, a finding that is in accord with a number of NMR and other spectroscopic studies, showing that there are significant differences in the way that various detergents perturb the hydrocarbon and headgroups of phospholipids (Jackson et al., 1982; Ollivon et al., 1988; Goñi et al., 1986; Otten et al., 1995; Wenk et al., 1997; Lasch, 1995). The partition coefficients obtained in the present study with radiolabeled detergent seem to be somewhat (two- to threefold) higher than can be estimated from previous data obtained by indirect procedures for $C_{12}E_8$ (Heerklotz et al., 1994) and dodecylmaltoside (de Foresta et al., 1992).

Concerning the interaction of SDS with the membranes (Fig. 2 *D*), a number of special features are observed: binding of this detergent to the Ca^{2+}-ATPase membranes is clearly in excess of that of SR lipid, being characterized by a cooperative transition that starts at a lower concentration (~0.4 mM) than for the liposomal preparations (~0.9 mM). In supplementary experiments we found that cooperative binding of SDS to Ca^{2+}-ATPase is accompanied by complete and irreversible inactivation of ATP hydrolysis (not shown). Thus for SDS there is evidence of a cooperative and separate interaction with protein at a lower concentration than with lipid. As shown below, this difference plays an important role in the mechanism by which SDS solubilizes Ca^{2+}-ATPase membranes. In contrast to the nonionic detergents, there is during solubilization no difference between binding of detergent by SR lipid and DOPC liposomes, which for both lipids takes place over a certain concentration range, i.e., the solubilization process does not proceed at a constant concentration, even for the homogeneous DOPC liposomes.

Steps in Ca^{2+}-ATPase solubilization

Fig. 3 shows the result of experiments in which the effect of detergent on the physical state of the Ca^{2+}-ATPase membranes was monitored by both Rayleigh light scattering and sedimentation experiments. For $C_{12}E_8$ (Fig. 3 *A*) we could establish, by these procedures, that during the solubilization process the membranes pass through three different phases (I, IIA, and IIB) before reaching the state of full solubilization (phase III). Phase I is characterized by a slight decrease in light scattering, but with virtually complete

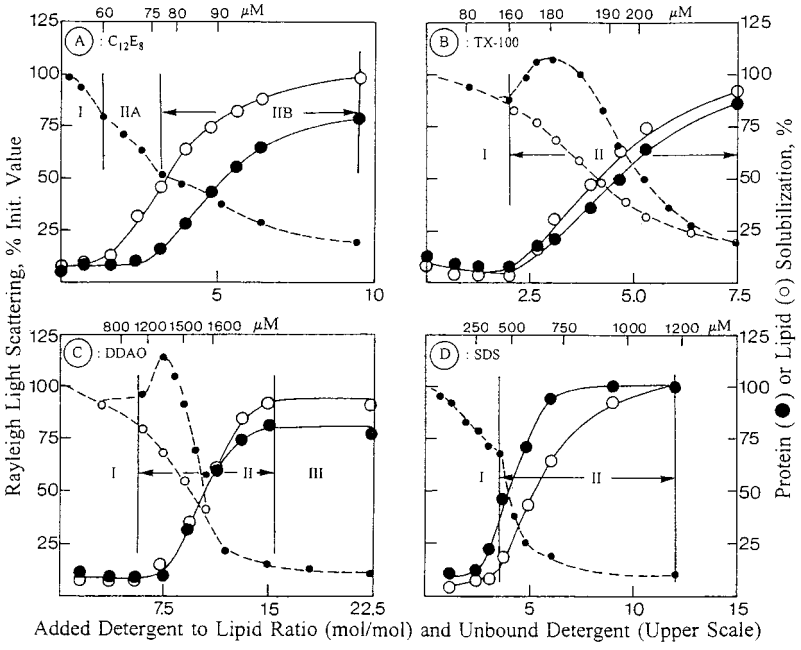

FIGURE 3 Solubilization and Rayleigh light scattering of Ca^{2+}-ATPase membranes during detergent solubilization. Aliquots of Ca^{2+}-ATPase membranes (0.5 mg protein/ml), suspended in standard medium with various concentrations of detergent, were centrifuged for 1 h at 100,000 × *g* in the Beckman Airfuge. The figure shows the concentration of protein (●) and phospholipid (○) in the supernatant (measured by the method of Bartlett, 1959), relative to that of an uncentrifuged specimen. Rayleigh light scattering was measured at 600 nm in titration experiments after allowing sufficient time to approach equilibrium (●– – –●) or recorded in one-shot experiments, immediately after the addition of detergent to the required concentration (o– – –o) in *B* and *C*. The data are mean values of three or four experiments with a SEM of ±2–8%. Note that to provide clearer visualization of the different phases, data are plotted as a function of molar ratio of added detergent to phospholipid, without correction for unbound detergent. The level of unbound detergent, corresponding to the different additions, as deduced from Fig. 2, is indicated at the top of the frames. The vertical bars show the transition between noncooperative and cooperative binding (I-II boundary), also based on the binding data of Fig. 2, and maximum solubilization (II-III boundary), based on the light scattering and lipid sedimentation data.

sedimentation of membrane material after centrifugation for 1 h at 100,000 × *g*. Phase I corresponds to noncooperative $C_{12}E_8$ binding, a treatment that according to a previous freeze-fracture analysis only gives rise to slight changes in vesicle size and morphology (Andersen et al., 1983). In the present study we confirmed the lack of an effect of nonsolubilizing $C_{12}E_8$ concentrations on vesicular size by quasielastic light scattering (QELS) analysis on SR vesicles that had been size fractionated on agarose (Fig. 4 *A*) and examined before (Fig. 4 *B*) and after the addition of $C_{12}E_8$ in nonsolubilizing amounts (Fig. 4 *C*). Although as previously shown this level of detergent clearly perturbs ATPase activity (Andersen et al. 1983), it did not result in any detectable change in vesicular diameter, as can be seen by a comparison of Fig. 4, *B* and *C*. However, the addition of further $C_{12}E_8$, corresponding to the start of cooperative detergent binding, was accompanied by marked changes in particle size (Fig. 4 *D*), as will be further discussed below.

The sedimentation data of Fig. 3 *A* indicate that after phase I there is preferential solubilization of lipid, which appears in the supernatant before protein (indicated as phase IIA in Fig. 3 *A*). This is followed in phase IIB by solubilization of the major part of the protein and all remaining lipid, processes that are accompanied by a further decrease in Rayleigh light scattering. In the transition between phases IIA and IIB there is evidence of a slight but reproducible break point in the Rayleigh light scattering curve. QELS analysis of various phase IIA preparations indicates pronounced size heterogeneity with the presence of both small (~8–10 nm), intermediate (60–110 nm), and large (200–400 nm) diameter particles (Fig. 4 *D*). The small particles represent mixed lipid-detergent micelles that have weakly light scattering properties and therefore only give rise to a small peak. Probably, the peak of intermediate size in Fig. 4 *D* reflects unsolubilized membrane vesicles or fragments, and the large particles aggregation of membrane fragments and protein. In accord with the appreciable Rayleigh scattering persisting in phase III, QELS analysis indicates the presence of large particles in phase III (200–400 nm) under conditions where the lipid is fully solubilized (data not shown). According to the sedimentation data, these aggregates represent 20–25% of the total protein. This material is devoid of enzyme activity and remains unsolubilized, even after the addition of very large amounts of nonionic detergent. We conclude that the aggregates represent denatured and delipidated protein, which is either present in a preformed state in the preparation, and/or are formed during solubilization with $C_{12}E_8$.

Somewhat different results were obtained by addition of Triton X-100 and DDAO (Figs. 3 B and 3 C). For these detergents the sedimentation experiments indicated practically concomitant solubilization of lipid and protein, and a subdivision of phase II in two subphases based on this criterion is blurred, if at all present. Instead, at the onset of solubilization we observe in the ordinary titration experiments an immediate decrease in Rayleigh scattering, followed by slow time-dependent increases, as shown for Triton X-100 in Fig. 5 A. The data obtained from such experiments are shown as the upper Rayleigh light scattering curves for Triton X-100 and DDAO (●– – –●) in Fig. 3 B and 3 C. (Also in the case of $C_{12}E_8$ there is a slight time dependent upwards trend in Rayleigh light scattering, corresponding to phase IIA, which results in the discontinuity which can be observed between phase IIA and IIB in Fig. 3 A.) For Triton X-100 and DDAO, QELS analysis of phase II preparations indicates extreme size heterogeneity with the presence of both small (~10 nm), intermediate (50–100 nm), and very large (500–1000 nm) particles (not shown).

To be able to characterize the instantaneous changes in Rayleigh scattering during solubilization, we obtained an

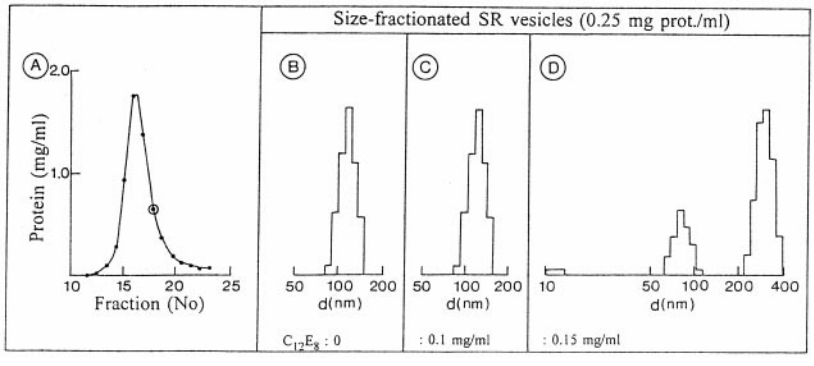

FIGURE 4 Dynamic light scattering analysis of the effect of $C_{12}E_8$ on SR particle size parameters. (*A*) Representative experiment, in which SR vesicles (8 mg protein) were size fractionated on a Sepharose 2B (1.5 × 80 cm) column, equilibrated with 10 mM TES (pH 7.4), 100 mM KCl, and 0.1 mM $CaCl_2$. Fraction 18 (the *circled point*) from the trailing edge of the chromatographed vesicle (with a protein concentration of 0.50 mg/ml) was used for the QELS experiments, after dilution with an equal volume of chromatography buffer (*B*) or after dilution with an equal volume of buffer, containing 0.2 mg $C_{12}E_8$/ml (*C*); this detergent addition corresponds to saturation of the membranes with detergent ($\bar{\nu}$ = 0.8) but does not lead to solubilization or change in particle size. *D* shows the QELS spectrum obtained after partial solubilization of lipid by addition of 0.3 mg $C_{12}E_8$/ml; this is seen as a small peak of weakly light scattering mixed micelles at 9 nm, which is present together with 60–110 nm vesicles or membrane fragments and larger (200–400 nm), probably aggregated forms. The spectra obtained are the result of a long-term (1 h) Nicomp multiexponential analysis of the autocorrelation time curve of the intensity fluctuations in scattered light.

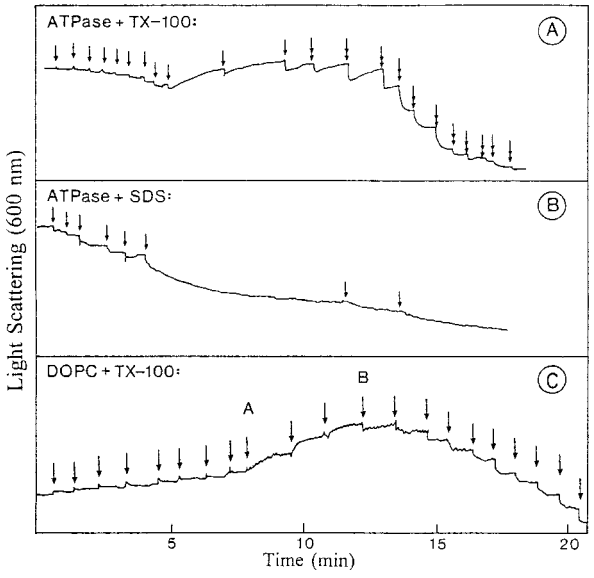

FIGURE 5 Time dependence of Rayleigh light scattering changes occurring during detergent solubilization of Ca^{2+}-ATPase and DOPC membranes. The curves are from representative experiments that were performed with Ca^{2+}-ATPase membranes (0.5 mg protein/ml) and Triton X-100 or SDS in the upper two panels (*A* and *B*), and with DOPC unilamellar liposomes (0.25 mg phospholipid/ml) and Triton X-100 in *C*. The membranes were suspended in 2.5 ml standard medium in a cuvette with continuous stirring in a Perkin-Elmer 44A spectrofluorometer. Detergent was added from a stock solution (50 mg/ml) in 2-μl (*single arrows*) or 4-μl (*double arrows*) aliquots. In the experiment with DOPC and Triton X-100 (*C*), the positions of break points A and B are indicated to illustrate that slow time-dependent increases only occur after break point A.

additional set of light scattering data immediately after detergent addition (i.e., without waiting for the slow rise in light scattering, as shown in Fig. 5 *A*). These data were obtained as one-shot experiments in which we made only one detergent addition per sample and read the ensuing light scattering from the curves immediately after the addition. Under these conditions we observed for Triton X-100 and DDAO, as shown by the lower set of light scattering curves (○-----○) in Fig. 3, *B* and *C*, a monotonous decrease in the light scattering of the same type as observed with $C_{12}E_8$. Note that the upper set of light scattering curves (●------●), which includes the contribution of secondary changes, shows that light scattering reaches a maximum at detergent concentrations close to those permitting solubilization, but merges with the lower curve at the end of the solubilization process in phase II. Both the sedimentation and QELS experiments indicate that at the start of phase II there is immediate solubilization of part of the membranous material by Triton X-100 and DDAO, followed by aggregation of membranous or solubilized material, most of which deaggregates at the end of phase II. As in the case of $C_{12}E_8$, a small fraction of Ca^{2+}-ATPase remains insoluble in the presence of excess Triton X-100 and DDAO. We have obtained indications that the processes of solubilization and aggregation at the start of phase II are, in fact, independent phenomena. Thus we observed that the increase in light scattering is strongly dependent on solubilization conditions (buffer composition, protein concentration). Furthermore, aggregation is completely absent if the cuvette temperature is lowered to 4°C; under these conditions light scattering curves similar to those obtained in one-shot experiments were obtained in ordinary titration experiments (not shown).

With SDS we found that, in contrast to the nonionic detergents, solubilization of Ca^{2+}-ATPase protein precedes solubilization of membrane lipid, i.e., Ca^{2+}-ATPase is preferentially extracted from the membranes, leaving lipid behind in the sediment (Fig. 3 *D*). This takes place at a free concentration of SDS $\approx$ 0.4 mM, corresponding to the onset of cooperative transition for the Ca^{2+}-ATPase vesicles. For SDS no increase in light scattering was observed in phase II, as can be seen from Fig. 5 *B*, but the extraction of protein from the membranes, taking place at the phase I to II boundary, is a slow process, requiring 5–10 min for completion. However, at high SDS concentrations, solubilization was more complete, i.e., SDS treatment led to solubilization also of the fraction of inactive, aggregated Ca^{2+}-ATPase, in accordance with the utility of this detergent for gel electrophoretic analysis.

Solubilization of DOPC liposomes

To further investigate the solubilization process and avoid some of the complexities attending the use of protein-containing membranes, we performed a number of experiments on unilamellar DOPC liposomes, reconstituted by cholate dialysis. By QELS analysis these preparations had narrow unimodal vesicle size distributions with average dimensions ranging from 38 to 40 nm among various preparations and with a size dispersion of $\pm$8–15 (SD) nm. Rayleigh light scattering data of these preparations as a function of addition of $C_{12}E_8$, Triton X-100, and DDAO are shown in the upper part of Fig. 6. In all of these cases stepwise detergent addition resulted in a biphasic increase in light scattering intensity, followed by a gradual decline to a very low level after complete solubilization. The curves are characterized by three break points, indicated as A, B, and C on Fig. 6. According to the binding data of Fig. 2, the first increase in light scattering (up to point A) corresponds to phase I, and the following steeper increase to the first part of phase II. During the noncooperative binding of detergent in phase I, the increase in light scattering occurs rapidly, and according to QELS analysis, it is not accompanied by any detectable change in vesicle size (see *A**, *lower part* of Fig. 6). The second increase (from break point A to B) is time dependent (as demonstrated with Triton X-100 in Fig. 5 *C*). According to QELS analysis (see *B***, *lower part* of Fig. 6) this step is associated with a considerable increase in vesicle size (from 39 to 71 $\pm$ 9 nm for $C_{12}E_8$ and similar increases for the other detergents; see legend to Fig. 6). But note that this level of detergent addition, which corresponds to phase IIA for Ca^{2+}-ATPase membranes (Fig. 3 *A*), does not result in the same heterogeneous particle size distribution as ob-

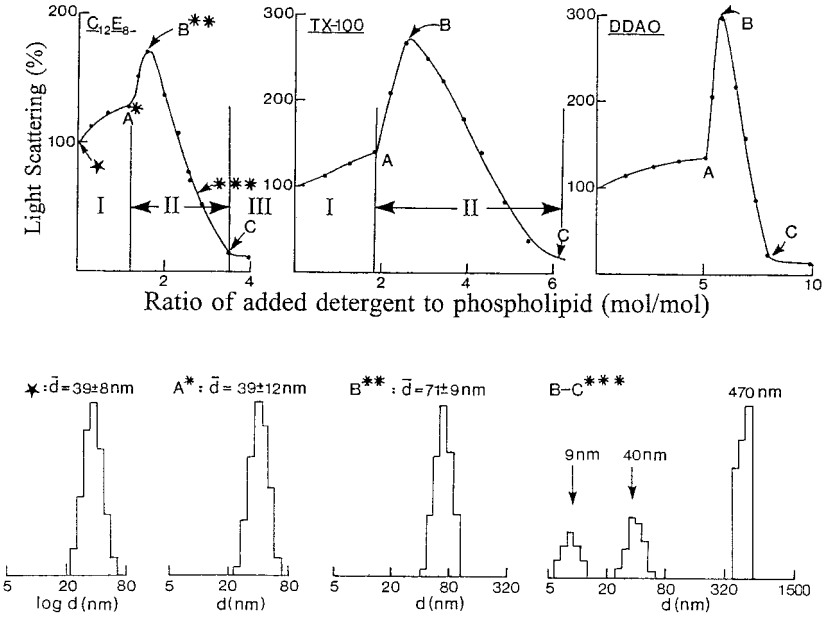

FIGURE 6 Effects of detergent addition on Rayleigh and dynamic light scattering changes of unilamellar DOPC vesicles during detergent solubilization. DOPC unilamellar liposomes, prepared by cholate dialysis and suspended at a phospholipid concentration of 0.25 mg/ml in 2.5 ml standard medium, were titrated with detergent stock solutions (50 mg/ml), added in 2-μl aliquots. The upper part of the figure shows the changes in Rayleigh light scattering at 600 nm caused by detergent addition, obtained in representative experiments. The lower part of the figure shows selected QELS data on samples prepared by the addition of $C_{12}E_8$ to DOPC samples, corresponding to break point A (*), break point B (**), and the lower third of the solubilization curve (***). For comparison, QELS analysis of vesicle size in the absence of detergent is also shown (⋆). The addition of detergent up to break point A does not change the vesicle diameter (d = 39 nm), whereas detergent addition to reach break point B results in an increase in vesicle diameter to 71 nm. In similar experiments with Triton X-100 and DDAO an increase in vesicle size from 40 nm to 72 and 78 nm, respectively, was obtained between break points A and B. After break point B, an additional peak of small particles (d = 9 nm), representing detergent/phospholipid micelles, is present, and this is accompanied at the end of the solubilization phase by particles of intermediary and very large sizes (***), as revealed by Nicomp multiexponential analysis of the autocorrelation function. The same pattern of size heterogeneity at the end of the solubilization phase is observed with Triton X-100 and DDAO.

served with Ca^{2+}-ATPase membranes (Fig. 4 *D*). The increase in DOPC size observed probably represents vesicle fusion (Lasch 1995), and at break point B we have from the QELS data no evidence that solubilization of lipid as mixed micelles has yet occurred: first, by addition of detergent beyond break point B, we can detect the emergence of small particles ($d \approx 8$–10 nm) in the QELS spectrum, together with unsolubilized vesicles. In the initial part of the solubilization phase the unsolubilized vesicular material retains the same size as measured at break point B (data not shown). However, toward the end of phase II (shown as *B-C* *** in Fig. 6), the 71-nm-diameter vesicles are replaced by two new peaks with particle sizes of 40 and 470 nm, in addition to a small peak of mixed micelles (9 nm). This recalls the CTEM description of the presence of various threadlike structures and large bilayer sheets during detergent solubilization (Vinson et al., 1989; Edwards et al., 1989; Edwards and Almgren, 1991). Finally, as expected from the decrease in Rayleigh scattering, these particles disappear from the QELS analysis at the end of the solubilization phase after break point C, as the result of complete solubilization of the lipid as mixed micelles (not shown).

An interesting aspect of the experiments with DOPC membranes is the correlation between morphological changes and the detergent binding isotherms (Fig. 2). As mentioned above, break point A corresponds to the transition from noncooperative to cooperative binding, whereas the formation of mixed micelles, accompanying the decline in the light scattering curve from break points B to C, mainly corresponds to the vertical part of the DOPC binding curve. That is, the formation of larger vesicles of defined size observed after break point A corresponds to the start of the cooperative interaction of detergent with the lipid. At this point solubilization has not yet started, but significant reorganization, including membrane fusion, must take place, resulting in the formation of the increased vesicle size between A and B. Solubilization of the lipid as mixed micelles requires a slightly higher concentration of free detergent than C_{sat}, corresponding to the steep slope seen in Fig. 2, *A* and *B*, and gradually also results in the formation of various mesomorphic phases of remaining unsolubilized lipid.

Solubilization and flip-flop rates of detergents

When experiments similar to those described above were repeated with SDS, much to our surprise we observed that solubilization of DOPC bilayers is an extremely slow pro-

cess, requiring more than 1 h to go to completion under usual conditions. The slow solubilization of lipid bilayers by SDS is reminiscent of the rather slow solubilization of lipid membranes by dodecylmaltoside (de Foresta et al., 1989). The kinetic aspects of solubilization of DOPC liposomes with dodecylmaltoside and SDS are shown in Fig. 7. It is seen that for both detergents there is a lag phase of several minutes before the onset of solubilization, which then proceeds slowly to completion like a cooperative process. The duration of the lag phase and rate of solubilization is somewhat dependent on the concentration of added detergent but approaches a limiting value after the addition of detergent in amounts sufficient to produce full solubilization in the final state. A distinctive feature of the solubilization process is that it is extremely temperature sensitive, requiring strict thermostatting to obtain comparable curves. To complete the experiments shown in Fig. 7 within a reasonable time period, they were conducted, as indicated on the figure, at slightly elevated temperatures: 27°C for dodecylmaltoside and 30°C for SDS.

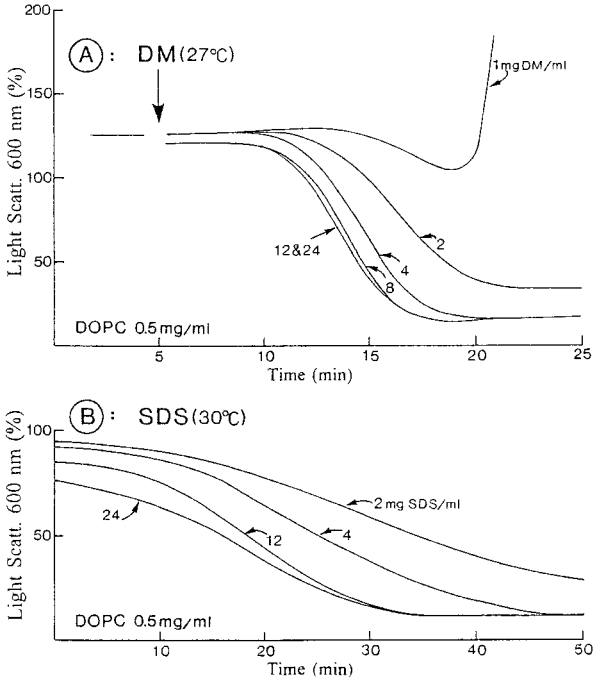

FIGURE 7 Delay and slow solubilization of DOPC vesicles by dodecylmaltoside and SDS. (*A*) Samples of a unilamellar liposome preparation were suspended in standard medium and placed in a stirred cuvette and thermostatted at 27°C in the cuvette compartment of a Perkin-Elmer 44A spectrofluorometer. To this was added, as indicated by the arrow, aliquots of a stock solution of dodecylmaltoside (100 mg/ml), also thermostatted at 27°C, to give the following final concentrations of dodecylmaltoside: 1, 2, 4, 8, 12, or 24 mg/ml, and 0.5 mg DOPC/ml. Changes in Rayleigh light scattering were recorded at 600 nm. Note that a lag period is required before a decrease in light scattering occurs. Furthermore, note that at the lowest detergent concentration (1 mg/ml) solubilization is followed by reaggregation, probably because of slow exposure of the inner bilayer leaflet to detergent. (*B*) To a unilamellar vesicle preparation (0.5 mg DOPC/ml), suspended in standard medium and thermostatted at 30°C, were added aliquots of a stock solution of SDS to final concentrations of 2, 4, 12, or 24 mg SDS/ml. Changes in Rayleigh scattering after SDS addition were recorded at 600 nm. Note that the incorporation of SDS into the liposomes (without solubilization) results in a decrease in light scattering. By contrast, incorporation of dodecylmaltoside results in an increase in light scattering above 100% (*A*), but in contrast to the other nonionic detergents (Fig. 6) the increase is monophasic, because the A-to-B transition is absent, resulting in a plateau value already at 1 mg/ml.

In the search for a plausible explanation for the marked differences with regard to kinetic properties of the various detergents in producing solubilization, we first note that efficient and quick membrane solubilizers like $C_{12}E_8$, Triton X-100, and DDAO are characterized by a higher degree of lipophilicity, e.g., they are easily dissolved in a variety of organic solvents (le Maire et al., 1987). On the other hand, both dodecylmaltoside and, in particular, SDS have headgroups with marked hydrophilic properties. As a consequence, it can therefore be anticipated that the latter detergents are slowly transported across the bilayer leaflet of closed lipid vesicles. This might impede solubilization, if this process is dependent on the presence of appreciable amounts of detergent on both sides of the membrane for concomitant interaction with the outer and inner bilayer leaflets. To evaluate the relevance of these considerations to the detergents studied here, we have measured flip-flop rates of SDS and dodecylmaltoside across lipid bilayers and compared them with that of a polyethylenglycol detergent ($C_{12}E_8$). The outcome of these studies is shown in experiments that were performed at detergent concentrations sufficiently low to ensure intact bilayer vesicles (Fig. 8). Release of detergent from DOPC multilayers was first measured at timed intervals with the aid of Bio-Beads columns, which were used to remove detergent present in the medium and presumably from the outer leaflet of the bilayer. As can be seen from Fig. 8, *B* and *C*, there is, over several hours, barely any detectable escape of dodecylmaltoside and SDS from multilayered vesicles with encapsulated detergent. Uptake of these detergents into unilamellar DOPC vesicles was then measured. The data obtained by this approach also indicate that lipid bilayer permeation of dodecylmaltoside and SDS is a slow process (Fig. 8, *D* and *E*). The uptake proceeds with half-times of hours and reaches a final level after 24 h, when about a third of the detergent present in the sample is taken up. This is a reasonable equilibrium level, because the Bio-Beads extract both unbound detergent (~10%) and detergent present on the outer bilayer leaflet, which has a slightly larger surface than the inner leaflet. Similar experiments with intact SR vesicles indicate that dodecylmaltoside and SDS also slowly permeate through these membranes (data not shown). These findings are markedly different from those observed in the case of $C_{12}E_8$. With this detergent there is, as shown in Fig. 8, *A* and *D*, no restriction in detergent transport across the membrane in either direction, i.e., all detergent is removed from both multilamellar and unilamellar DOPC preparations by quick passage (1 min) through the Bio-Beads column, whether the lipid preparations are pre-equilibrated

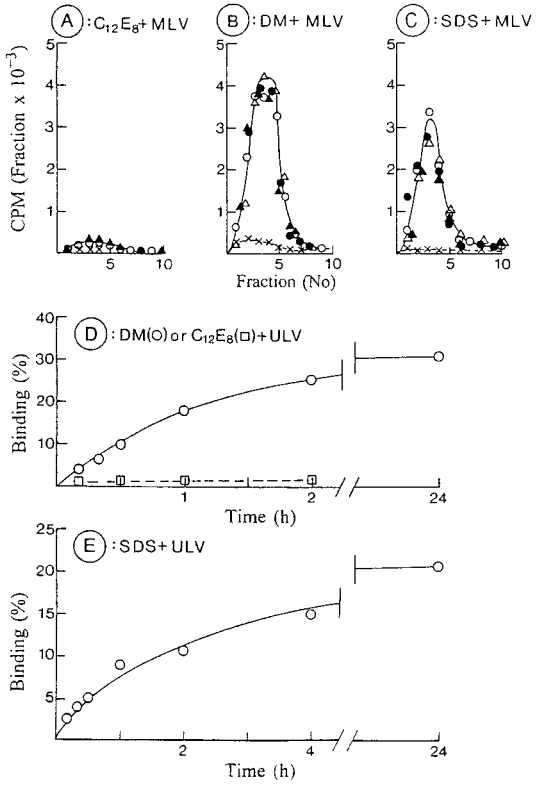

FIGURE 8 Detergent release from DOPC multilayers and uptake by DOPC unilamellar liposomes at 20°C. (*Upper figures, A, B,* and *C*) To examine detergent release $C_{12}E_8$ (*A*), dodecylmaltoside (*B*) or SDS (*C*) was encapsulated in a DOPC liposome preparation (at 5 mg/ml) during the formation of multilayered vesicles (MLVs) by vortexing thin DOPC films in 10 mM TES (pH 7.4), 100 mM NaCl, and 0.1 mM $CaCl_2$, containing 0.4 mg/ml of detergent with radioactive tracer. At the start of the experiment (zero time), a sample was diluted 100 times with detergent-free buffer, and 2.5-ml aliquots of this dilution (containing 12,000–13,000 cpm) were applied to a Biobeads column (1 × 5 cm) immediately after dilution (○) and at different times thereafter: ●, 15 min; △, 30 min; and ▲, 5 h. x——x, shows the elution from control samples, without phospholipid, treated the same way to indicate the completeness of detergent extraction from the buffer by passage through the column. (*Middle and lower figures, D and E*) To examine detergent uptake by liposomal membranes, radiolabeled and unlabeled detergent (0.4 mg/ml) was added at time 0 to unilamellar vesicles (ULVs) of DOPC liposomes (5 mg/ml), prepared by cholate dialysis, and suspended in 10 mM TES (pH 7.4), 100 mM NaCl, and 0.1 mM $CaCl_2$ at time 0. At various times thereafter, aliquots of the samples were diluted 100 times with detergent-free buffer, and 2.5 ml of these dilutions (containing 12,000–13,000 cpm) was applied to the Biobeads column. Intravesicular uptake of dodecylmaltoside and SDS (○) was calculated from the radioactivity in the first eight fractions, with a subtraction of the zero time elution value (2–7%) to correct for the presence of detergent remaining in the medium and in the outer bilayer leaflet after passage through the column. Note that in the case of $C_{12}E_8$ (□), the apparent lack of uptake reflects the fact that the release is too fast to allow retention by the liposomes of detergent taken up during the incubation period within the time scale (2 min) of the subsequent chromatography (cf. le Maire et al., 1987).

with or exposed to the detergent at various times. This is in agreement with the results of a previous study, which indicated rapid flip-flop (within fractions of a second) of $C_{12}E_8$ (le Maire et al., 1987).

DISCUSSION

Thermodynamic aspects of nonionic detergent binding

Previous studies on the mechanism of membrane solubilization by detergents have mainly been concerned with binary detergent/lipid mixtures. In the present study we included studies on the effect of a third component (integral membrane protein) to address biologically relevant questions concerning the solubilization of biomembranes. For this we chose to use Ca^{2+}-ATPase membranes, prepared from SR vesicles and mainly (~90%) containing the 110-kDa SERCA 1a ATPase. The interaction of these membranes with a number of efficiently solubilizing detergents was compared with those of SR lipid and pure lipid (DOPC) membranes.

For pure DOPC membranes we found, in agreement with the three-stage hypothesis, that solubilization by nonionic detergent takes place at a constant concentration of unbound detergent below the CMC, indicating coexistence in stage II of DOPC/detergent micelles at the same thermodynamic activity as the detergent-saturated membranes. However, we also found that this critical concentration was slightly above C_{sat}, the concentration at which detergent starts to bind in a cooperative manner. This implies the existence of an intermediate state where detergent is taken up by membrane lipid by both noncooperative and cooperative binding, before solubilization takes place. In agreement with this conclusion, López et al. (1998) recently published CTEM pictures suggesting the presence of micellar structures of Triton X-100 within liposomal membranes immediately before solubilization.

In the case of SR lipid, we found that solubilization proceeds over a range of concentrations, starting at the C_{sat} and ending close to the CMC. It seems probable that it is the marked variations in phospholipid hydrocarbon chain length and degree of unsaturation of SR lipid species (C_{12-24}, with up to six double bonds; cf. Warren et al., 1974; Bick et al., 1987; Infante 1987), rather than differences in phospholipid headgroups, that are the most important factors involved in this heterogeneous response, because previous data on SR solubilization, as monitored by ^{31}P-NMR spectroscopy, indicated similar solubilities of the various neutral and acidic phospholipids (Roux and Champeil 1984).

We conclude from the identity of the binding of nonionic detergent by Ca^{2+}-ATPase membranes and that by SR lipid (Fig. 2) that the interaction and solubilization of the membranes by these compounds are processes that are primarily governed by the lipid component. This does not exclude binding of small amounts of detergents at specific protein sites, for which there is both functional (Simmonds et al., 1982; Andersen et al., 1983; de Foresta et al., 1989, 1992) and kinetic (le Maire et al., 1987) evidence. Nonetheless, the lack of an effect of the protein component on bulk detergent binding is surprising, in view of the organizing effect that the protein exerts on the lipid phase. Thus it has

been estimated that ~20–25 molecules of phospholipid, corresponding to about one-quarter to one-third of the lipid content, are localized close to the membranous sector per Ca^{2+}-ATPase monomer, where because of diffusion restrictions they are bound in a relatively immobilized state (Marsh 1995). In agreement with a particular status of annular lipid, previous studies with brominated phospholipid suggested that detergents are unable to displace phospholipid in contact with Ca^{2+}-ATPase (de Foresta et al., 1989). However, it was subsequently found that bromosubstituted detergents are not only taken up by ordinary bilayer phospholipids, but are also in close contact with the protein already at concentrations below the onset of solubilization (de Foresta et al., 1996). This latter finding confirms the conclusion from the present study that annular lipid participates in all phases of detergent binding.

SDS interaction with membranes

In contrast to nonionic detergents, we found that SDS specifically interacts with the protein component of Ca^{2+}-ATPase membranes, leading to cooperative unfolding and release of Ca^{2+}-ATPase before solubilization of SR lipid. This aggressive behavior, which probably is the result of a combination of ionic and hydrophobic interactions, occurs in the same concentration range and is very similar to the attack of SDS on water-soluble proteins. As in the case of serum albumin, interaction of SDS with Ca^{2+}-ATPase probably comprises different phases, starting with binding of SDS at discrete binding sites at low detergent concentrations and cooperative unfolding of protein at higher concentrations (Reynolds et al., 1967; Tanford 1972; Jones 1992). On the other hand, predominantly ionic interactions are presumably the basis for the efficient purification of some natural membrane proteins by treatment with low amounts of SDS, which leads to the removal of a number of loosely bound membrane-associated proteins, e.g., of Na^+,K^+-ATPase containing microsomes (Jørgensen, 1988).

Compared to the effect on proteins, the interaction of SDS with lipid is weaker, requiring higher concentrations of unbound SDS, close to the CMC. A solubilization process different from that of the three-stage hypothesis, involving a gradual transition between the vesicular and mixed micellar states, is suggested by the fact that solubilization of DOPC vesicles proceeds over a relatively large range of concentrations of unbound SDS (Fig. 2 *D*).

Membrane morphology during solubilization

It is a common observation that detergent solubilization of small liposomes is preceded by vesicle fusion (Edwards et al., 1989; Edwards and Almgren 1991, Lasch 1995; Paternostre et al., 1995). In our experiments this phenomenon takes place in the intermediate state mentioned above, where detergent is bound cooperatively to the membranes without solubilization. It can be envisioned that under these conditions the membranes are in a fluctuating and dynamic state, characterized by rapid exchange of phospholipid between vesicles. In comparison to this we find for Ca^{2+}-ATPase no evidence of intermediate fusion of the vesicles before vesicle solubilization. Presumably the protein component, via protein-protein and protein-lipid interactions, serves as a scaffold that imparts greater mechanical stability to the membrane fragments, preventing them from fusion into larger vesicles, as is the case for pure lipid membranes. However, the detergent-modified membrane fragments have a pronounced tendency toward secondary, temperature-dependent aggregation, a feature especially prominent in the case of Triton-X100 and DDAO (Fig. 3, *B* and *C*). During this transitional state we may assume that, because of incomplete covering of hydrophobic surfaces, the membrane fragments formed at the start of the solubilization process have "sticky" properties, leading to their aggregation into larger complexes. Consonant with this view, we observed that membrane aggregation is markedly increased by a rise in temperature, i.e., it is entropy driven, consistent with a predominantly hydrophobic origin of the aggregation process (Kauzmann, 1959; Tanford, 1980).

In the case of liposomal membranes there is also evidence for secondary aggregation processes, leading to an increased turbidity at the end of the solubilization process, a phenomenon previously observed with β-octylglucoside at high lipid concentrations (Paternostre et al., 1988; Ollivon et al., 1988). In our experiments such large particles were only detectable by QELS analysis at the end of the solubilization phase (Fig. 6). In agreement with López et al. (1998), we did not by this approach obtain evidence for a variegated morphology, as has otherwise been reported to exist by CTEM already at the start of the solubilization phase of egg yolk phosphatidylcholine liposomes with $C_{12}E_8$ and Triton X-100 (Edwards et al., 1989; Edwards and Almgren, 1991; Silvander et al., 1996) and β-octylglucoside (Vinson et al., 1989). One reason for these differences could be that, in contrast to earlier investigations, we conducted our solubilization experiments at low concentrations of phospholipid (0.3–0.7 mM).

Protein solubilization procedures

The present study results in some practical suggestions for the use of detergents in solubilizing detergent-sensitive membrane proteins in an active form. For this a protocol should be worked out to approach a solubilized state close to, but not crossing, the phase II-III boundary (Fig. 3). In this way it will be possible to effect solubilization of a major part of the membrane protein(s), without the risk of taking delipidation of the solubilized protein to a state that may cause inactivation and dissociation of functional complexes (cf., e.g., Breyton et al., 1997). (Notwithstanding that some membrane proteins, that have been obtained in a 3D crystalline state, e.g., reaction center (Deisenhofer et al., 1985), porin (Cowan et al., 1992), cytochrome oxidase (Iwata et

al., 1995), and *N. crassa* H^+-ATPase (Cyrklaff et al., 1995), can be essentially delipidated without loss of native structure.) Solubilization can be conveniently monitored by following the evolution of light scattering changes after the stepwise addition of detergent. At the appropriate point, nonsolubilized (very often inactive and aggregated) protein can then be removed simply by centrifugation at medium speed (e.g., for 60 min at 105,000 × *g*, a usual criterion for solubilization). This criterion is, of course, operational and should be used with caution. More stringent criteria are related to size measurements by methods such as CTEM or QELS analysis, sedimentation velocity, and size exclusion chromatography with columns equilibrated with detergent near the CMC or with lipid-containing micelles. Furthermore, solubilization should be performed at a low temperature (0–4°C), not only to better preserve activity, but also to minimize aggregation, which was a particularly prominent feature in the case of Triton X-100 and DDAO (Fig. 3, *B* and *C*).

Kinetic and molecular aspects of detergent solubilization

It remains to consider questions concerning the detailed mechanism of detergent solubilization. As illustrated in Fig. 9 *A*, single noncooperatively interacting detergent molecules are likely to be inserted in the membrane with a main orientation normal to the membrane plane, whereas micellar assemblies of cooperatively bound detergent are likely to form curved structures that disrupt the bilayer. Depending on the extent of cooperatively bound detergent, this will result either in the formation of holes in the membrane structure (Kragh-Hansen et al., 1993) or in the formation of bilayer discs sealed at the edges by detergent molecules (Lasic, 1982; Fromherz et al., 1986).

This picture of the initial states in the solubilization process helps to explain certain kinetic features of the present study, viz., the slow solubilization of DOPC vesicles by SDS and dodecylmaltoside. For detergents with a rapid flip-flop, the formation of micellar structures in the membrane will be aided by the concerted attack of monomeric detergent from both the outside and inside aspects of the vesicles (see Fig. 9 *A* and the accompanying text to the figure), whereas solubilization will be impeded for detergents like SDS and dodecylmaltoside, which mainly interact with the outer layer of the lipid bilayer leaflet (Fig. 9 *B*). The absence of bilayer discontinuities as intermediate states during solubilization with SDS and dodecylmaltoside is further suggested by the lack of vesicular fusion observed with these detergents in the presolubilization region, corresponding to the A-to-B transition of $C_{12}E_8$, Triton X-100, and DDAO (cf. Fig. 7). It is probable that slow penetration is responsible for the paucity of structural changes in liposomes observed during solubilization with SDS and dodecylmaltoside and their slow solubilization.

As an alternative to a transbilayer mechanism with detergent in nonmicellar form, membrane lipid as shown in Fig. 9 *B* can be assumed to be extracted from the outer part of the membrane by interaction with detergent micelles on the outside of vesicles. During this process the outer bilayer will be partially depleted of phospholipid, resulting in vesicle reorganization and redistribution of phospholipid molecules from the inner to the outer bilayer leaflet. As a consequence of these changes, vesicles slowly open up, leading to fragmentation and finally to solubilization by the detergent micelles. This chain of proposed events we may

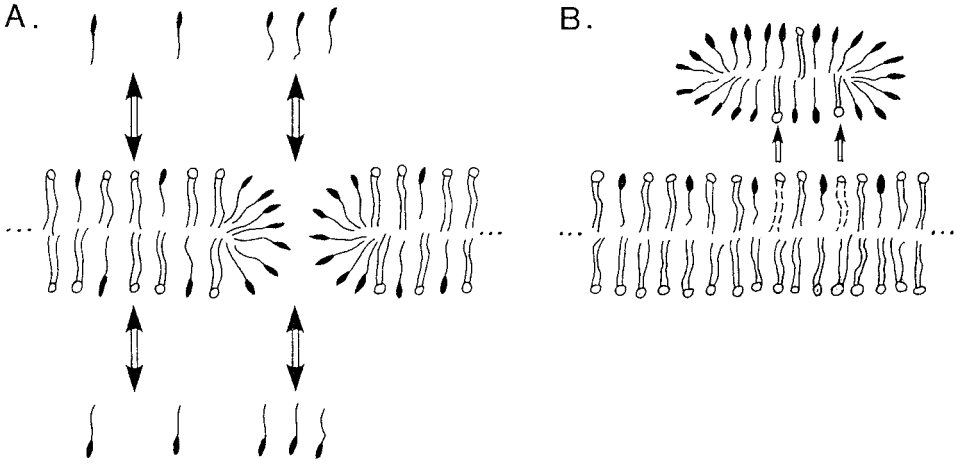

FIGURE 9 Fragmentation and solubilization of lipid membranes by detergent. (*A*) Illustration of the formation of cooperatively bound assemblies of detergent, spanning the membrane. The scheme assumes that monomeric detergent equilibrates by rapid flip-flop across the membrane (*left-hand part of A*). Above C_{sat} this is followed by the formation of a membrane-spanning assembly of detergent monolayer, resulting from the concerted attack of detergent molecules from both sides of the membrane (*right-hand part of A*). Note that by contrast noncooperatively bound detergent molecules have their main orientation normal to the membrane plane. (*B*) Extraction of phospholipid from the outer bilayer leaflet into detergent micelles at the membrane/water interface. This is suggested to account for the slow solubilization of phospholipid in liposomes by dodecylmaltoside and SDS, i.e., for solubilization by detergents with a slow flip-flop rate. ═○, phospholipid; —●, detergent; – – –○, phospholipid extracted from bilayer into a micelle, as indicated by the arrows in *B*.

term the **micellar mechanism** of solubilization, because it depends, directly or indirectly, on the interaction of membranes with detergent micelles, in contrast to the **transbilayer mechanism** depicted in Fig. 9 *A*. Micellar solubilization generally can be expected to be a slow process, because of a requirement for higher activation energies in the interaction with membranes than for nonmicellar detergent. This will account for the delay observed in the solubilization of liposome membranes by SDS (Fig. 7 *B*) and dodecylmaltoside (Fig. 7 *A*). For protein containing membranes the situation is somewhat different, because dodecylmaltoside quite rapidly solubilizes both Ca^{2+}-ATPase and SR membranes. In the case of SDS, solubilization of SR membranes at room temperature is also a slow process (see Fig. 5 *B*) but quicker than flip-flop, which at room temperature requires a time scale of hours. This difference can be accounted for on the basis of the fact that SDS first extracts proteins from the Ca^{2+}-ATPase containing membranes, a process that can be considered to open the way for a subsequent detergent attack on the membrane lipid. However, for the quick solubilization of SR membranes by a detergent like dodecylmaltoside, which predominantly interacts with membrane lipid (Kragh-Hansen et al., 1993), a different mechanism must exist; in this case we propose that liposomal vesicles with a smooth surface and orderly disposition of lipids are less accessible to solubilization by the micellar mechanism than phospholipid in the protein-containing membranes with a more irregular disposition and hence easier extractability of phospholipid.

In support of the idea that detergent micelles are involved in the solubilization of membranes, Needham et al. (1997), in a study of a fatty acid detergent (monooleoylphosphatidylcholine), recently obtained direct evidence for the interaction of micelles with unilamellar liposomes. This evidence was based on a higher detergent uptake by normal liposomes than by sterically protected liposomes, containing polyethyleneglycol-substituted phospholipid. With respect to the present data, it is likely that the cytosolic projections of Ca^{2+}-ATPase, like the polyoxyethylene groups in the study by Needham et al. (1997), also will provide an efficient shield against fusion of detergent micelles with intact membranes. The SR surface is very crowded (Toyoshima et al., 1993) and is unlikely to offer open patches of protein-free lipid surface of a diameter sufficient to allow direct contact with detergent micelles (diameters 50–100 Å; cf. Møller and le Maire, 1993). However, this will not matter, if the concentration of nonmicellar detergent near the vesicle surface (which is very close to the CMC) suffices to cause defects in the membrane structure, as suggested by ultrastructural data (Kragh-Hansen et al., 1993), thereby exposing membrane lipid at the edges directly to micellar attack (cf. Fig. 9 *A*). Furthermore, the presence of detergent micelles in the vicinity of the membranes provides these with a detergent reservoir to replenish newly bound detergent and to counteract the effect of unstirred layers during the solubilization process. Finally, a special role could be played by vicinal detergent micelles in providing a source of premicellar detergent aggregates that are likely to be present in the transition zone between the nonmicellar and micellar states (Mukerjee et al., 1958; Kale et al., 1980) and that would have dimensions sufficiently small to approach the lipid bilayer. A combination of these effects on a relatively vulnerable protein-containing membrane could account for a rapid solubilization process in the presence of detergents with slow flip-flop rates.

CONCLUDING COMMENTS

In our study we have demonstrated both individual and common features in the way in which detergents interact with lipid and protein-containing membranes. For solubilization to occur, cooperative binding is required, and for nonionic detergents, in contrast to SDS, this is achieved predominantly by interaction with the membrane lipid component of protein-containing membranes. There also appears to be differences with respect to the way detergents interact with liposomes versus protein-containing membranes, especially concerning the importance of transbilayer and micellar attack mechanisms. The importance of a transbilayer mechanism, especially to liposome solubilization, is demonstrated by correlation with detergent flip-flop. There are also differences between protein-containing and liposomal membranes with respect to the morphology of intermediate forms arising during solubilization; in the case of liposomes, detergent-loaded membrane fragments have a pronounced tendency to fuse to reform vesicles with defined size, from which lipid can be solubilized by the further addition of detergent. On the other hand, the less flexible Ca^{2+}-ATPase membrane fragments produced by interaction with detergent have a pronounced tendency to aggregate rather than to fuse, before being fully solubilized by further addition of detergent.

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