Membrane Solubilization by Detergent: Use of Brominated Phospholipids To Evaluate the Detergent-Induced Changes in Ca^{2+}-ATPase/Lipid Interaction†

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ABSTRACT: The solubilization and delipidation of sarcoplasmic reticulum Ca^{2+}-ATPase by different nonionic detergents were measured from changes in turbidity and recovery of intrinsic fluorescence of reconstituted ATPase in which tryptophan residues had been quenched by replacement of endogenous phospholipids with brominated phospholipids. It was found that incorporation of $C_{12}E_8$ or dodecyl maltoside (DM) at low concentrations in the membrane, resulting in membrane "perturbation" without solubilization, displaced a few of the phospholipids in contact with the protein; perturbation was evidenced by a parallel drop in ATPase activity. As a result of further detergent addition leading to solubilization, the tendency toward delipidation of the immediate environment of the protein was stopped, and recovery of enzyme activity was observed, suggesting reorganization of phospholipid and detergent molecules in the solubilized ternary complex, as compared to the perturbed membrane. After further additions of $C_{12}E_8$ or DM to the already solubilized membrane, the protein again experienced progressive delipidation which was only completed at a detergent concentration about 100-fold higher than that necessary for solubilization. Delipidation was correlated with a decrease in enzyme activity toward a level similar to that observed during perturbation. On the other hand, Tween 80, Tween 20, and Lubrol WX failed to solubilize SR membranes and to induce further ATPase delipidation when added after preliminary SR solubilization by $C_{12}E_8$ or dodecyl maltoside. For Tween 80, this can be related to an inability to solubilize pure lipid membrane; in contrast, Tween 20 and Lubrol WX were able to solubilize liposomes but not efficiently to solubilize SR membranes. In all three cases, insertion of the detergent in SR membranes is, however, demonstrated by perturbation of enzyme activity. Correlation between detergent structure and ability to solubilize and delipidate the ATPase suggests that one parameter impeding ATPase solubilization might be the presence of a bulky detergent polar headgroup, which could not fit close to the protein surface. We also conclude that in the active protein/detergent/lipid ternary complexes, solubilized by $C_{12}E_8$ or dodecyl maltoside, most phospholipids remain closely associated with the ATPase hydrophobic surface as in the membranous form. Binding of only a few detergent molecules on this hydrophobic surface may be sufficient for inhibition of ATPase activity observed at high ATP concentration, both during perturbation and in the completely delipidated, solubilized protein. Detergent molecules might also be able to bind at protein/protein interfaces, or even between the ATPase hydrophobic helices in the monomeric polypeptide chain, rendering the bundle of helices looser.

For many membrane-associated enzymes, solubilization by surfactants results in changes in activity, which are generally thought to reflect the loss of membrane lipid or membrane-dependent constraints such as possible breakdown of preexisting oligomers. Detergents are considered to replace phospholipids around the membrane-embedded portion of the protein, but in many instances delipidation is either incomplete or a gradual process. Procedures involving continued exposure to detergent by column chromatography or sedimentation through detergent-free sucrose layers have been used both to produce and to assess delipidation [e.g., Helenius and Simons (1975)]. When it has been given consideration, the extent of delipidation of solubilized protein has been suggested to be critical for enzyme stability and/or activity (e.g., for transport ATPases;[1] le Maire et al., 1976; McIntosh & Ross, 1985; Vilsen & Andersen, 1987; Esmann & Skou, 1984). More specifically, the relevant parameter is likely to be the amount and/or nature of the lipids in the immediate vicinity of the protein rather than the average lipid/detergent/protein stoichiometry in the ternary complex.

Proximity relationships between proteins and lipids can be conveniently evaluated through a study of the interaction of brominated or spin-labeled phospholipids with tryptophan-containing proteins, since such interaction may result in quenching of the protein intrinsic fluorescence provided that tryptophan residues come in close contact with the bromine atoms or the nitroxide groups on the associated lipids. These

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[1] Abbreviations: SR, sarcoplasmic reticulum; ATPase, adenosinetriphosphatase; $C_{12}E_8$, octa(ethylene glycol) dodecyl monoether; DM, dodecyl maltoside ($C_{12}G_2$); Tween 20, C_{12}-sorbitan $E_{(20)}$; Tween 80, $C_{18:1}$-sorbitan $E_{(20)}$; Lubrol WX, $C_{16\&18}E_{(17)}$; IAF, 6-(iodoacetamido)-fluorescein; IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)-ethylenediamine; PC, phosphatidylcholine; PA, phosphatidic acid; DOPC or $C_{18:1}$-PC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; diBrPC or Br-C_{18}-PC, 1,2-bis(9,10-dibromooleoyl)-*sn*-glycero-3-phosphocholine; cmc, critical micellar concentration.

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approaches have already been used to evaluate the specificity of protein/lipid interactions in reconstituted membranes (London & Feigenson, 1981; East & Lee, 1982) and the interaction of various amphiphiles, either long-chain alkyl derivatives or cholesterol derivatives, with a membrane protein (Simmonds et al., 1982; Lee et al., 1982; Froud et al., 1986). The protein used for the above studies was the well-characterized Ca^{2+}-dependent ATPase responsible for active calcium transport in sarcoplasmic reticulum of skeletal muscle. A three-dimensional model derived from the primary sequence has been proposed for this enzyme, in which 11 out of the 13 tryptophans are located in the intramembranous, hydrophobic portion of the protein (Brandl et al., 1986). We show here that a similar approach can be successfully used to study the ability of detergents of various structures to delipidate the ATPase, and we correlate this ability with the effect of detergent on enzyme activity.

Materials and Methods

Methods for preparation of SR vesicles (Champeil et al., 1985), purified leaky ATPase membranes [according to method 2 of Meissner et al. (1973), which gives membrane fragments unable to accumulate calcium], or large unilamellar lipid vesicles [egg PC–egg PA, 9:1 w/w; see le Maire et al. (1987)] and for synthesis of brominated phospholipids (East & Lee, 1982) have already been described. Reconstitution of the Ca^{2+}-ATPase into brominated phospholipid bilayers was performed by cholate dilution, essentially as described by East and Lee (1982) (see Figure 1). Lipids in native SR vesicles were first exchanged for exogenous lipids in a medium containing 1 M KCl, 250 mM sucrose, 50 mM phosphate buffer (pH 8), 2.5 mg/mL SR protein (and 1.25 mg/mL endogenous lipids), 10 mg/mL cholate, and 20 mM exogenous phospholipids, either DOPC or 9,10-diBrPC ($C_{18:1}$-PC or Br-C_{18}-PC), i.e., about 16 mg/mL for nonbrominated and 20 mg/mL for brominated phospholipids. After 0.5–1-h incubation in the cold (6–8 °C) followed by an additional incubation on ice, 5 μL of this mixture was diluted 500-fold by injection into 2.5 mL of buffer [0.1 M KCl, 10 mM Tes–Tris buffer (pH 7.5, 20 °C), 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$]. Small volumes of detergent stock solutions of 10 or 100 mg/mL were sequentially added to this diluted sample.

Due to the high cmc of cholate and its corresponding low partition coefficient between water and liposomes, residual cholate in the diluted sample (20 μg/mL) is essentially unbound. The partition coefficient, measured under conditions similar to ours, ranges from 0.05 to 0.1 mM^{-1} (Almog et al., 1986; Almog & Lichtenberg, 1988; Paternostre et al., 1988), corresponding to only 0.0025–0.005 mol of cholate/mol of lipid bound to the lipid phase in the reconstituted membrane [for comparison, $C_{12}E_8$ has a 100–200-fold higher partition coefficient, about 10 mM^{-1} (le Maire et al., 1987), corresponding to a lower cmc]. Moreover, the ATPase environment is not significantly perturbed by residual cholate in the reconstituted sample, on the basis of previously reported activity measurements (Warren et al., 1974). Further, it has been shown that residual cholate has no effect on the fluorescence of the ATPase reconstituted with brominated phospholipids (East & Lee, 1982). Therefore, residual cholate should not interfere with the effects of the other detergents.

Fluorescence spectra were recorded at 20 °C, during continuous stirring, with an SLM 4000S fluorometer. This equipment was also used for measurements of scattering at 90° of incident light.

Ca^{2+}-ATPase activities were measured with a coupled enzyme assay (Møller et al., 1980), as follows: 5 mM MgATP and a regenerating system (1 mM phosphoenolpyruvate, 0.15–0.3 mM NADH, 0.1 mg/mL lactate dehydrogenase, and 0.1 mg/mL pyruvate kinase) were added to the standard medium (0.1 M KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM Tes–Tris buffer, pH 7.5) at 20 °C (Figure 9) or 23–24 °C (Figure 8). The reaction was generally initiated by addition of SR vesicles in the presence or absence of 3 μg/mL A23187, and activity was monitored after sequential additions of specified amounts of detergent. Detergent-induced ATPase denaturation during turnover was negligible during the period required for activity measurement, except when Lubrol WX was used: in this case, only initial ATPase activity was plotted. In some cases, the activity of leaky membranes of purified ATPase was also tested, in the absence of ionophore.

We have plotted our data as a function of total detergent concentration. In fact, the significant parameter is the amount of detergent actually associated with the lipids or proteins, which is in equilibrium with free detergent in solution. Using the formalism of Lichtenberg et al. (1983)

$$D_T = D_W + D_B$$

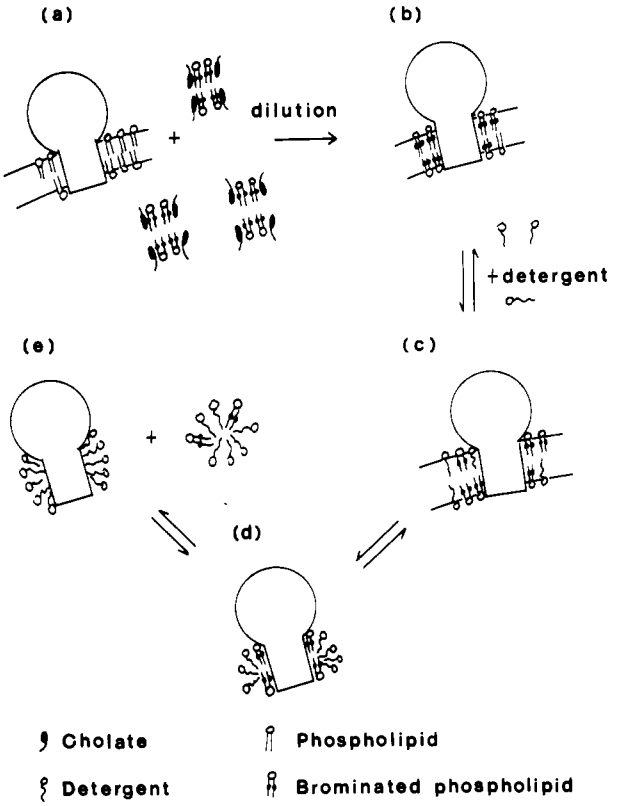

FIGURE 1: Schematic drawing illustrating the various steps of the experimental protocol. (a) The endogenous lipids of sarcoplasmic reticulum membranes are exchanged for brominated phospholipids. The Ca^{2+}-ATPase is represented with a bulky cytoplasmic part; brominated phospholipids, in 13-fold excess over endogenous lipids, are initially in mixed micelles, together with cholate. (b) Dilution of the sample (500-fold) leads to reconstitution of membranes mainly containing brominated phospholipids and the Ca^{2+}-ATPase. Residual cholate will not interfere with the subsequent steps (see Materials and Methods). (c) Addition of low amounts of detergent does not solubilize the reconstituted membrane, but detergent is inserted in the bilayer. (d) At higher concentration some of the detergents are able to break up the membrane (solubilization). This leads to the formation of ternary complexes in which there are still lipids bound to the protein. (e) At very high detergent concentration, complete delipidation and formation of two types of binary complexes occurs. This drawing does not take into account the possible aggregated state of the ATPase. It represents the protein/lipid or detergent interface as a flat, well-defined surface, but this is also an oversimplification (see Discussion).

Table I: Properties of the Various Detergents Mentioned in This Work[a]

	monomer MW (daltons)	cmc at 20 °C (μg/mL)	pure micelle size, MW (daltons) [(no. of monomers per micelle)]
$C_{12}E_8$ (pure compound)	538	50	40 000–65 000 (75–120)[c]
dodecyl maltoside (DM, pure compound)	528	90	60 000–74 000 (114–140)[d]
Tween 80 ($C_{18:1}$-sorbitan $E_{(20)}$)	1320	9–16[b]	80 000 (60)
Tween 20 (C_{12}-sorbitan $E_{(20)}$)	1240	75	
Lubrol WX ($C_{16\&18}E_{(17)}$)	1000	4[b]	92 000 (92)

[a] Data collected in Møller et al. (1986), unless otherwise stated. [b] le Maire et al. (1976). [c] le Maire et al. (1989). [d] le Maire and Møller, unpublished results.

where D_T, D_W, and D_B respectively refer to the total, free in solution, and bound detergent concentrations, or

$$D_T = D_W + R_{eff}[\text{lipid}]$$

with $R_{eff} = D_B/[\text{lipid}]$. R_{eff} is the effective ratio of detergent to lipid, which determines the effect of detergent. The onset of solubilization occurs when the amount of detergent in the membrane, i.e., R_{eff}, reaches a certain value, R_{sat}; under these conditions, the free detergent concentration D_W is close to but slightly lower than the detergent cmc in water (Andersen et al., 1983; Helenius & Simons, 1975). After further detergent addition, the membranous phase is completely converted into mixed micelles when R_{eff} reaches another particular value, R_{sol}.

$C_{12}E_8$ was from Nikko Chemicals, DM was from Calbiochem, Lubrol WX and Tween 80 were from Sigma, and Tween 20 was from Bio-Rad. Table I lists a few known properties of these detergents (Møller et al., 1986).

Results

Detergent-Induced Dequenching of ATPase, Reconstituted with Brominated Phospholipid. Figure 2 shows a typical experiment illustrating how delipidation induced by addition of detergent ($C_{12}E_8$ in this example) can be followed after reconstitution of Ca^{2+}-ATPase with brominated phospholipids. The right part of panel A shows the fluorescence emission spectrum (full line) of native SR vesicles. At the low protein concentration used (5 μg/mL), intrinsic fluorescence of the Ca^{2+}-ATPase (around 335 nm) was only a few fold larger than the peak due to Raman scattering (around 310 nm) and the background fluorescence of the buffer (dashed line). On the left part of panel A, the Rayleigh peak, indicative of light scattering by SR vesicles (80–200-nm diameter), was recorded at a 100-fold lower electronic gain. Panel C shows that ATPase reconstituted with nonbrominated $C_{18:1}$-PC displayed a similar tryptophan fluorescence level and a higher Rayleigh peak than native SR vesicles [due to the presence of a relatively high amount (32 μg/mL) of exogenous lipids]. Addition of $C_{12}E_8$ to this sample reduced the Rayleigh peak, due to solubilization, but the tryptophan fluorescence level remained virtually unaltered. Panel D shows that ATPase reconstituted with brominated lipids had a much lower fluorescence level (60% quenching). When $C_{12}E_8$ was added to this sample, the Rayleigh peak again dropped upon solubilization, but the tryptophan fluorescence was gradually dequenched and, at a concentration of 10 mg of $C_{12}E_8$/mL, rose to a level very similar to that of the unquenched sample. Panel B is a control where detergent was added to buffer alone.

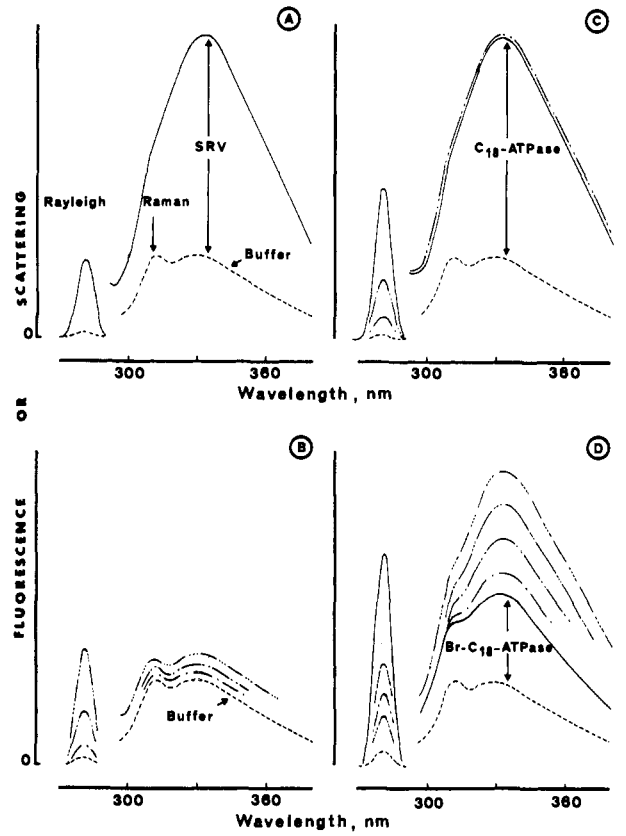

FIGURE 2: Typical fluorescence spectra and Rayleigh peaks demonstrating the use of brominated phospholipids to monitor Ca^{2+}-ATPase delipidation and membrane solubilization with $C_{12}E_8$. For each panel: (left part) Rayleigh scattering; (right part) fluorescence intensity. (—) sample; (– –) buffer. Excitation was set at 280 nm, with 4- and 8-nm bandwidths at the excitation and emission wavelengths, respectively. For Rayleigh intensity recordings, the photomultiplier gain was 0.01-fold that for fluorescence intensity recordings. (Panel A) Native sarcoplasmic reticulum membranes at a protein concentration of 5 μg/mL (and 2.5 μg of endogenous lipid/mL). (Panel B) Buffer alone (– –), to which various amounts of $C_{12}E_8$ were added, as a control: 1 (–·–), 4 (–··–), and 10 mg/mL (–···–). Note that for very high detergent concentrations light scattering by the micelles becomes significant. This is exemplified in Figures 3 and 4, open symbols. (Panel C) ATPase sample, reconstituted with DOPC. The final sample contained 5 μg of protein/mL, 2.5 μg of endogenous lipid/mL, 32 μg of exogenous nonbrominated or 40 μg of brominated lipid/mL, and 20 μg of cholate/mL, in 100 mM KCl, 10 mM Tes, pH 7.5, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.5 mM sucrose, 0.1 mM phosphate, and various concentrations of $C_{12}E_8$: 0, (—), 0.2 (–·–), or 4 mg/mL (–··–). (Panel D) ATPase sample reconstituted with Br-C_{18}-PC, as above, without (—) or with different additions of $C_{12}E_8$: 0.2 (–·–), 0.4 (–··–), 1 (–···–), and 10 mg/mL (–····–). Temperature was 20 °C.

Figure 3A depicts the quantitative relationship between tryptophan fluorescence recovery, Rayleigh scattering, and detergent addition. From the drop in scattering intensities (open symbols), it is apparent that the reconstituted samples have been almost completely solubilized by addition of 0.2 mg/mL $C_{12}E_8$. At this point, the tryptophan fluorescence in the sample reconstituted with brominated phospholipids (closed triangles) is still quenched to an appreciable degree, compared to the situation before addition of detergent. The detergent-solubilized complex therefore contains a large number of phospholipids in direct contact with the protein. Tryptophan fluorescence does not recover the value characteristic of the unquenched state until after further addition of a large excess of detergent (10 mg/mL). To attain half-maximal de-

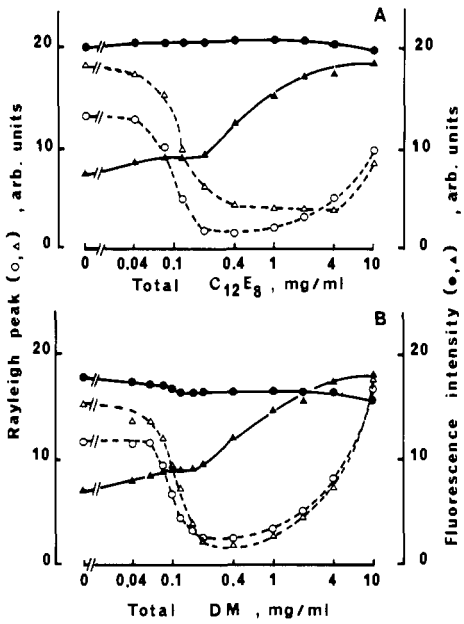

FIGURE 3: Intrinsic fluorescence and turbidity of reconstituted ATPase samples, in the presence of various concentrations of $C_{12}E_8$ (panel A) or DM (panel B). The samples were reconstituted with DOPC (circles) or Br-C_{18}-PC (triangles) as in Figure 2. (Closed symbols) Fluorescence intensity (λ_{exc} = 280 nm and λ_{em} = 335 nm with respective bandwidths of 4 and 8 nm), corrected for the "blank" fluorescence of buffer plus detergent (see Figure 2B). (Open symbols) Rayleigh scattering determined at $\lambda_{exc} = \lambda_{em}$ = 280 nm; same bandwidths as above. The detergents were sequentially added from concentrated solutions (10 or 100 mg/mL). Fluorescence data were corrected for dilution. Temperature was 20 °C.

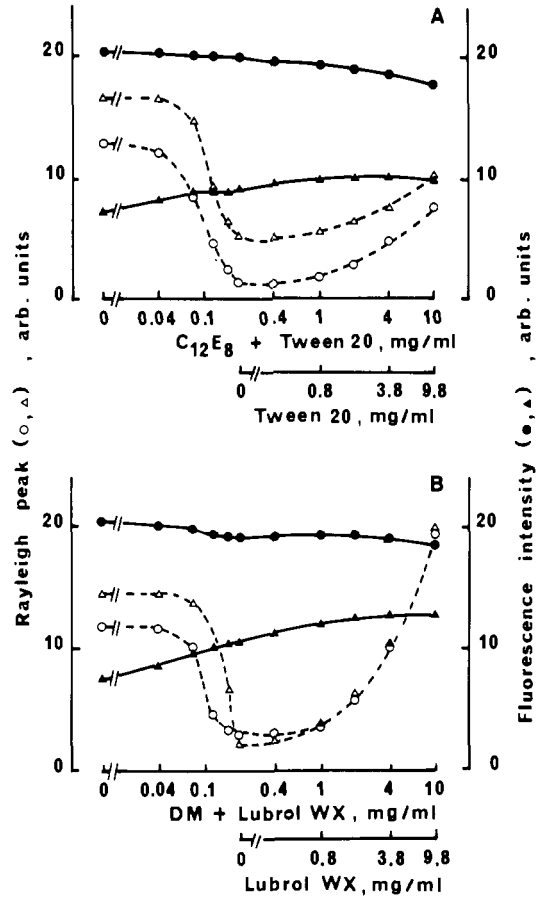

FIGURE 4: Intrinsic fluorescence and turbidity of reconstituted ATPase samples, in the presence of various concentrations of either $C_{12}E_8$ and Tween 20 (panel A) or DM and Lubrol WX (panel B). The experimental procedure was similar to the one of Figure 3 except that in panel A $C_{12}E_8$ was first added, up to 0.2 mg/mL (solubilization), and then Tween 20 was added; in panel B, dodecyl maltoside was first added, up to 0.2 mg/mL (solubilization), and then Lubrol WX was added. Symbols are as in Figure 3.

quenching, about 1 mg/mL $C_{12}E_8$ is required, i.e., a 20–30-fold (w/w) excess of $C_{12}E_8$ over exogenous PC. It should be stressed that even at 0.2 mg/mL detergent, the molar ratio of nonmonomeric detergent to brominated lipid is high, close to 8:1 (0.16 mg/mL nonmonomeric $C_{12}E_8$ and 40 μM lipid). The significant quenching observed under these conditions shows that lipids bind to the protein hydrophobic surface much more strongly than detergent molecules. Similarly, on going from 0.2 mg/mL detergent to 0.4 mg/mL for instance, the amount of nonmonomeric detergent increases by more than a factor of 2 (from 0.16 to 0.36 mg/mL), whereas the tryptophan quenching is only reduced by approximately one-third, again suggesting preferential retention of phospholipid at the protein surface.

Using a different experimental protocol in the absence of cholate, we also observed progressive ATPase delipidation only in the presence of $C_{12}E_8$ concentrations much higher than those required for solubilization (Lund et al., 1989).

Note that there is not much difference in tryptophan quenching in nonsolubilized membranes almost saturated with detergent [added $C_{12}E_8$ about 0.04 mg/mL; see Andersen et al. (1983) and le Maire et al. (1987)] and in the initially solubilized detergent/lipid/protein ternary complexes ($C_{12}E_8$ $\approx$ 0.2 mg/mL). This plateau contrasts with the drastic changes observed upon solubilization when energy transfer is monitored between ATPase chains labeled with fluorescent groups in reconstituted membranes (Champeil et al., 1982) or between labeled phospholipids in pure liposomes (Ollivon et al., 1988).

Addition of dodecyl maltoside (DM) instead of $C_{12}E_8$ to the lipid-substituted ATPase leads to similar results (Figure 3, panel B); i.e., the ATPase is only delipidated in the presence of a large excess of DM. Careful inspection of the data shows that perturbation by nonsolubilizing concentrations of DM resulted in a slightly larger recovery of fluorescence than perturbation with $C_{12}E_8$, but the higher final fluorescence level for Br-C_{18}-PC/ATPase, compared to $C_{18:1}$-PC/ATPase, is probably not significant (Figure 3B).

Further experiments performed with phospholipids with shorter acyl chains ($C_{14:1}$-PC and Br-C_{14}-PC) gave comparable results: when endogenous lipids were substituted for Br-C_{14}-PC, tryptophan fluorescence was even more efficiently quenched—70% compared to 60%—than in the presence of Br-C_{18}-PC. Perturbation and initial solubilization again resulted in only small increases of the fluorescence level, while the presence of excess $C_{12}E_8$ or DM allowed progressive recovery of tryptophan fluorescence (data not shown).

Figure 4 shows opposite results obtained with other detergents, which have been used for preparation of active oligomeric transport ATPases after initial solubilization with $C_{12}E_8$ (le Maire et al., 1976; Hastings & Reynolds, 1979). In the experiment illustrated in panel A, the lipid-substituted ATPase was first solubilized with 0.2 mg/mL $C_{12}E_8$, and Tween 20 (C_{12}-sorbitan $E_{(20)}$) was then added. Panel A shows that Tween 20 was unable to strip most of the Br-C_{18}-PC from the ATPase, even at a total detergent concentration of 10 mg/mL. The same type of experiment was repeated with Tween 80 ($C_{18:1}$-sorbitan $E_{(20)}$) and Lubrol WX ($C_{16\&18}E_{(17)}$), with similar results, both after initial solubilization with 0.2 mg/mL $C_{12}E_8$ (data not shown) and after initial solubilization with 0.2 mg/mL DM. Thus panel B in Figure 4 shows that under the latter conditions Lubrol WX again only slightly relieved the Br-induced quenching of ATPase tryptophans.

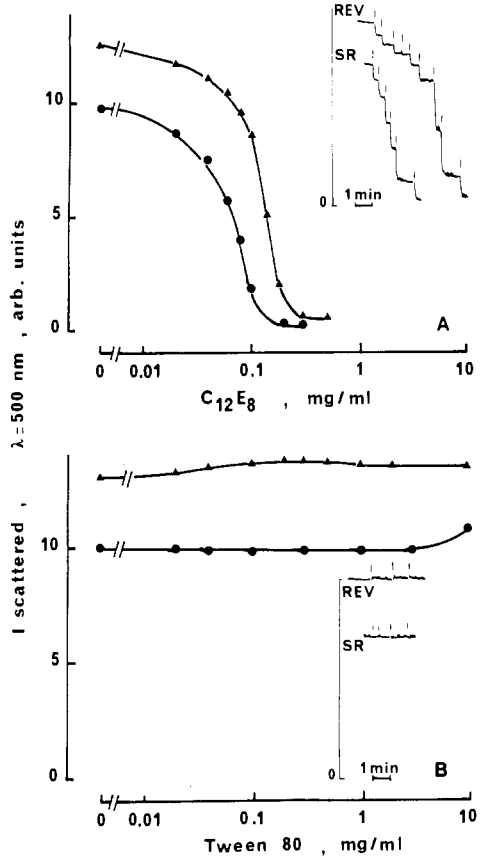

FIGURE 5: Detergent solubilization assay of sarcoplasmic reticulum vesicles (circles) or liposomes (triangles), with $C_{12}E_8$ (panel A) or Tween 80 (panel B). The solubilization index was the intensity of Rayleigh scattered light at 500 nm. Sarcoplasmic reticulum vesicles (25 μg of protein/mL and 12.5 μg of endogenous lipid/mL) or liposomes (125 μg of lipid/mL) were treated with increasing concentrations of detergent in the same cuvette under continuous stirring, in a medium containing 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 10 mM Tes–Tris, pH 7.5, at 20 °C. (Panel A) Clarification produced by $C_{12}E_8$. (Panel B) Absence of clarification in the presence of Tween 80. (Insets) Kinetics of the drops in intensity.

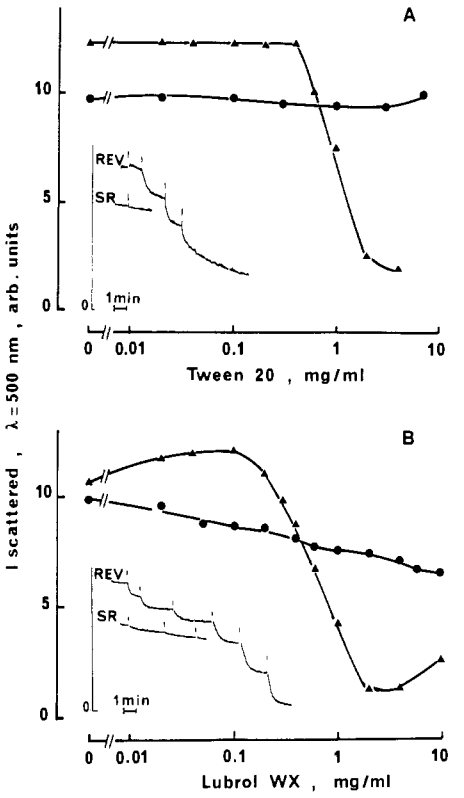

FIGURE 6: Detergent solubilization assay of SR vesicles and liposomes using Tween 20 (panel A) or Lubrol WX (panel B). Symbols and experimental procedures as in Figure 5. In both cases only the liposomes (triangles) were completely clarified.

Similarly, for Tween 80, we obtained no evidence for ATPase delipidation, but the experiment was complicated by the light absorption properties and high fluorescence level of the commercial detergent used.

Solubilization of SR and Pure Lipid Vesicles by Detergent. As shown above, some surfactants do not succeed in delipidating the ATPase, even if the membrane has been previously solubilized. A trivial explanation for such behavior would be that the micelles of the specified surfactant are unable to accept any lipid. To evaluate this possibility, we measured the ability of the various detergents to solubilize either sarcoplasmic reticulum membranes (i.e., protein and endogenous lipids, 0.5 g of lipid/g of protein) or pure lipidic vesicles of similar size, obtained by reverse-phase evaporation (REV) (egg PC–egg PA, 9:1 w/w). SR at 25 μg of protein/mL (and 12.5 μg of endogenous lipid/mL) and REV at 125 μg of lipid/mL scattered incident light at 500 nm with efficiencies of the same order of magnitude. For both samples, we measured the detergent-induced changes in scattered light, again taking clarification as an approximate, but convenient assay for solubilization [see Watanabe and Inesi (1982), Champeil et al. (1982), and Paternostre et al. (1988)].

Figure 5 shows the effect of $C_{12}E_8$ and Tween 80 on light scattering. $C_{12}E_8$ (panel A) clarified both SR membranes (circles) and liposomes (triangles), at total concentrations consistent with what is known to be necessary for total solubilization of SR membranes—total $C_{12}E_8$ required ≈ cmc + (1–1.5 g of $C_{12}E_8$/g of protein)[protein] (le Maire et al., 1978)—or pure lipids—total $C_{12}E_8$ required ≈ cmc + (1.8–2.7 g of $C_{12}E_8$/g of lipid)[lipid]; the latter value is the R_{sol} defined under Materials and Methods, generally expressed in mol/mol units, which gives 2.6–4 mol of detergent/mol of lipid (Møller et al., 1986; Paternostre et al., 1988). The inset to panel A shows that stepwise clarification took place within seconds in both cases. On the other hand, panel B confirms that Tween 80 is a nonsolubilizing surfactant: neither SR vesicles nor lipid vesicles were clarified by addition of up to 10 mg/mL Tween 80.

Compared to these two extreme cases, Figure 6 shows an intermediate behavior for Tween 20 (panel A) and Lubrol WX (panel B). Despite their inefficiency in displacing phospholipids from solubilized ATPase, these two detergents did solubilize pure lipids, although solubilization took place rather slowly (within minutes, see insets) and apparently was not completed until a high detergent concentration, about 2 mg/mL, was reached. Thus, micelles of these detergents are able to accommodate a small amount of phospholipids, corresponding to uptake of a few phospholipid molecules per detergent micelle. On the other hand, the data indirectly indicate that a significant number of detergent molecules can be incorporated into the lipidic membrane without solubilization, since the decrease in light scattering occurs at a detergent concentration much higher than the cmc (see Table I). Assuming that the point at which light scattering drops is a reliable index of the onset of solubilization, about 0.4 mol of Tween 20 is bound per mole of phospholipid (R_{sat}), as deduced from a systematic study of the dependence of this point on the lipid concentration, in which results were evaluated as in Paternostre et al. (1988; data not shown). In contrast, Tween 20 was unable to solubilize SR membranes within 15

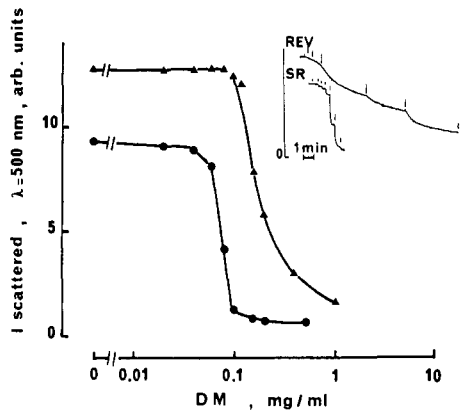

FIGURE 7: Detergent solubilization assay of SR vesicles and liposomes using dodecyl maltoside. Symbols and experimental procedures as in Figure 5.

min (see circles and inset in panel A). In other experiments involving longer incubation periods, a very slow clarification of SR vesicles could be observed after several hours in the presence of Tween 20: this might indicate slow solubilization of bulk SR lipids, whose properties are somewhat different from those of lipids present in a liposome [see, for instance, Lentz et al. (1985)]. In the presence of Lubrol WX (panel B), solubilization of pure lipids occurred and was preceded by a slight rise in turbidity, possibly arising from time-dependent fusion of vesicles or from an increase in vesicle size due to detergent incorporation. However, for SR vesicles, only a slight and gradual decrease in turbidity was observed within 15 min (circles), up to the high Lubrol WX concentrations examined: this decrease can be assigned both to vesicle perturbation, which changes light scattering properties (Watanabe & Inesi, 1982; Champeil et al., 1982), and, again, to very slow partial solubilization.

Dodecyl maltoside, like $C_{12}E_8$, was found to clarify both lipidic vesicles and SR membranes (Figure 7). Clarification of SR membranes started at a DM concentration lower than the reported cmc in water (90 μg/mL; Møller et al., 1986). This is in line with previous findings and theoretical predictions that the free detergent at solubilization is somewhat lower than the cmc in the absence of lipids (Andersen et al., 1983). One unexpected feature was that clarification of pure lipidic vesicles took place slowly while solubilization of SR membranes was instantaneous (see inset).

Dependence of Ca^{2+}-ATPase Activity on Detergent Concentration. To gain further insight into the delipidation process and its implications, we also measured the ATPase activity of SR membranes at high ATP concentration as a function of detergent concentration for the above-mentioned detergents. For $C_{12}E_8$ (panel A in Figure 8) and DM (panel B in Figure 8), three stages could be distinguished when ATPase activity was measured in the presence of ionophore, which prevented ATPase inhibition by accumulated calcium. At nonsolubilizing concentrations of detergent, ATPase activity was reduced. This is what has been characterized earlier as "perturbation" of the nonsolubilized SR ATPase (Lüdi et al., 1982; Andersen et al., 1983; McIntosh & Davidson, 1984; Champeil et al., 1986). At slightly higher detergent concentrations (about 0.1 mg/mL), ATPase activity reverted to a high value. This rise occurs more or less parallel to solubilization [ATPase turnover might influence the precise extent of solubilization, as has been documented for $C_{12}E_8$ by Watanabe and Inesi (1982)]. At total solubilization, both for $C_{12}E_8$ and for DM (0.1–0.15 mg/mL), activity has reached its maximal value (compare Figures 5A and 8A or Figures 7 and 8B).

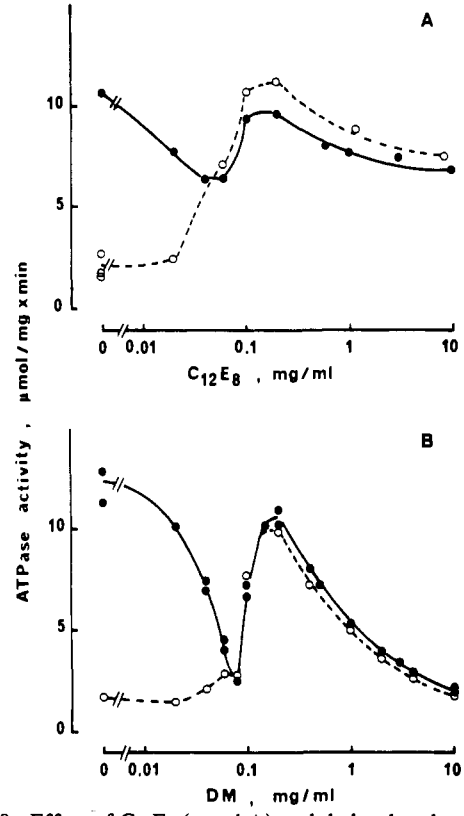

FIGURE 8: Effect of $C_{12}E_8$ (panel A) and dodecyl maltoside (panel B) on Ca^{2+}-ATPase activity, in the presence of Ca^{2+} ionophore A23187 (closed circles) or in its absence (open circles). Ca^{2+}-ATPase activity was measured by a spectrophotometric coupled enzyme assay on sarcoplasmic reticulum vesicles at a final concentration of 25 μg of protein/mL, i.e., as in Figures 5–7, in the following medium: 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM Tes–Tris, pH 7.5, 5 mM MgATP, 0.1 mg/mL pyruvate kinase, 0.1 mg/mL lactate dehydrogenase, 1 mM phosphoenolpyruvate, 0.3 mM NADH, and ±3 μg/mL A23187. Temperature was 23–24 °C in these experiments. At 20 °C, the activity of both our SR preparation in the presence of ionophore and purified ATPase in this medium was 5–6 μmol mg^{-1} min^{-1} (see Figure 9), and the pattern of ATPase activity as a function of $C_{12}E_8$ or DM concentration was similar. The effect of sequential addition of detergent in the same stirred cuvette (2.5 mL) was followed by continuous recording of optical density at 340 nm. NADH and phosphoenolpyruvate were added when exhausted.

At very high detergent concentrations, up to 10 mg/mL, activity dropped again toward the level previously observed in the perturbed state [this was not recognized in our earlier experiments with $C_{12}E_8$; see Andersen et al. (1983)]. This drop is clearly concomitant with what we know from Figure 3 to be ATPase delipidation, and it is particularly prominent in the presence of dodecyl maltoside. It is not caused by detergent-induced irreversible inactivation: when phospholipids were added to ATPase solubilized with a large excess of DM, activity distinctly reverted to a high value (data not shown). The ratio of activities measured here for SR ATPase in the presence of high amounts of $C_{12}E_8$ or DM agrees with that obtained for ATPase which had been previously delipidated through a HPLC procedure and further incubated in the same detergents (Lund et al., 1989); this is consistent with the idea that 10 mg of either detergent/mL efficiently delipidates the ATPase.

When similar experiments were repeated in the absence of ionophore (open symbols in Figure 8), the initial drop in activity was no longer observed, since it was counterbalanced by the increased leakiness of SR toward calcium (Andersen et al., 1983). The activity curves in the absence or presence of ionophore only became coincident, within experimental

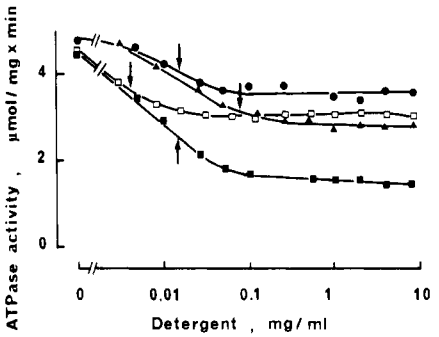

FIGURE 9: Effect of Tween 80 (circles), Tween 20 (closed triangles), Lubrol WX (open squares), and Brij 99 (closed squares) on ATPase activity of leaky, purified Ca^{2+}-ATPase membranes. Activity was measured at 5 µg of protein/mL by the spectrophotometric method as described in the legend of Figure 8, but in the absence of Ca^{2+} ionophore. Temperature was 20 °C. Arrows indicate the cmc of the detergents.

error, near solubilization. Experiments performed on leaky purified ATPase in the absence of ionophore gave results similar to those obtained with SR vesicles to which ionophore had been added (data not shown).

Figure 9 shows the effect of Lubrol WX, Tween 80, and Tween 20, as well as another nonsolubilizing detergent, Brij 99 ($C_{18:1}E_{\langle 20\rangle}$), on the activity of purified ATPase membranes, which are completely leaky to calcium. It is seen that activity was reduced to various extents upon addition of purified ATPase to the detergent-containing assay medium and that the inhibitory effects extended only slightly beyond the cmc for these detergents. In contrast to $C_{12}E_8$ and DM, concentrations in excess of cmc did not induce any further change in ATPase activity, consistent with the lack of solubilization and delipidation in this case. When similar experiments were performed with SR vesicles in the presence of ionophore, perturbation of ATPase activity was again observed at detergent concentrations below cmc. However, for very high concentrations of these nonsolubilizing detergents, the ionophore partitioned predominantly into the detergent micelles rather than into the membrane: therefore, SR vesicles were no longer leaky and ATPase activity dropped, precluding observation of a plateau similar to the one observed with purified ATPase (data not shown).

Discussion

Different Stages of Detergent Solubilization and Delipidation. Our experiments concerning quenching of tryptophan fluorescence by brominated lipids in the presence of increasing amounts of detergents like $C_{12}E_8$ or DM give some insight into the possible modes of interaction of efficiently solubilizing detergents with SR ATPase and the surrounding lipids. Several stages in this interaction can be distinguished.

At concentrations of $C_{12}E_8$ below the cmc detergent molecules partition between the aqueous phase, where they exist in a monomeric form, and the membrane phase. Up to 0.25 g of $C_{12}E_8$/g of protein, or 0.5 g of $C_{12}E_8$/g of endogenous lipid, may be accommodated in the perturbed membrane before saturation (Andersen et al., 1983; le Maire et al., 1987). This is a large amount, corresponding to an R_{sat} value of about 0.7 mol of $C_{12}E_8$/mol of phospholipid, which is present on both leaflets of the bilayer (le Maire et al., 1987). Quenching of tryptophan fluorescence by the brominated phospholipids is only slightly affected under these conditions (added $C_{12}E_8$ or DM between 0.04 and 0.06 mg/mL in Figure 3). This shows (i) that a few phospholipid molecules in the first lipid shell around the protein have been exchanged for detergent, correlating with perturbation of ATPase activity (Figure 8), and (ii) that the protein nevertheless remains in close contact with many phospholipids under these conditions, most detergent molecules being dissolved in the bulk lipid phase (Andersen et al., 1983; le Maire et al., 1987).

The next stage during stepwise detergent addition corresponds to progressive solubilization, a point at which detergent cooperatively interacts with the membranes. For pure lipid systems, this has been described as a transition between membranes saturated with detergent and detergent micelles saturated with lipid (Lichtenberg et al., 1983; Møller et al., 1986). For SR vesicles at this stage, proteins and some of the associated lipids are cosolubilized in the same complexes. Moreover, as judged from the similar level of tryptophan quenching in Figure 3 between 0.06 and 0.2 mg/mL added $C_{12}E_8$ or DM, our data show that average protein/lipid proximity relationships do not differ much in detergent-saturated membranes and in these ternary complexes. In terms of models for solubilized membrane proteins [le Maire et al. (1983) and Figure 1d), this implies that phospholipids are still maintained close to the protein surface in the solubilized ternary complexes.

Only when a large excess of $C_{12}E_8$ or DM is added does the solubilized protein experience complete delipidation: protein and lipids, at the end stage of this process, are now segregated into different binary complexes, viz., detergent/protein and detergent/lipid complexes. At this point, it might be useful to recall that previous attempts to partially delipidate the ATPase have not demonstrated selective enrichment of any particular type of phospholipid, so that it is reasonable to assume that brominated phospholipids in our experiments (Figures 2–4) reflect the behavior of the main endogenous phospholipids [see, for instance, le Maire et al. (1978)].

Progressive delipidation of solubilized ATPase was only observed with $C_{12}E_8$ and DM. Among the detergents tested here, Tween 80, Tween 20, and Lubrol WX were virtually ineffective in delipidating the solubilized protein, even after initial solubilization with DM or $C_{12}E_8$ (Figure 4). In the case of Tween 80, this correlates with the previous finding that $C_{12}E_8$-solubilized ATPase, layered on a column equilibrated with Tween 80, was eluted from this column in a form which still had phospholipids bound to it (le Maire et al., 1976, 1978). Considering Tween 20 and Lubrol WX, Figure 6 conclusively shows that the reason why these two detergents do not delipidate the ATPase surface is NOT that lipids are unable to be inserted into micelles formed by these detergents, since pure lipidic vesicles were solubilized by these detergents. Thus phospholipids prefer to bind to the surface of the ATPase rather than to detergent micelles, while detergent binds only very weakly to the surface of the ATPase and is thus not able to displace phospholipids. In other words, the partition equilibrium of phospholipids between the protein surface and the detergent micelles is very unfavorable for Tween 80, Tween 20, and Lubrol WX and more favorable for detergents like $C_{12}E_8$ and DM. Even the affinity of these latter two detergents for the ATPase surface is still relatively low, in view of the large excess of $C_{12}E_8$ or DM which is required to obtain half-delipidation of SR ATPase. However, in the experiments with Tween 20 and Lubrol WX illustrated in Figure 4, the intrinsic fluorescence of ATPase tryptophan residues was only monitored within a short period of time after detergent addition; therefore, we cannot completely exclude the possibility that the preferential binding of phospholipids to the protein surface rather than to micelles of these detergents is due to extremely slow exchange kinetics rather than to a truly un-

favorable partition equilibrium. This requires further experiments.

In an attempt to rationalize the observation that some detergents delipidate and others do not, one correlation found in the limited series of detergents tested here concerns the polar head group: only those detergents with a relatively small head group ($C_{12}E_8$, DM) were able to delipidate the ATPase. By contrast, Tween 20, with the same dodecyl hydrocarbon chain and a similar cmc but with a bulkier headgroup, is not an efficient solubilizer. Taking into consideration the three-dimensional structure predicted for ATPase, it might be speculated that detergents with large headgroups cannot fit all around the stalk below the bulky ATPase hydrophilic portion (Taylor et al., 1986; Castellani et al., 1985). This does not exclude the possibility that at least a few of the sites close to the ATPase are accessible to detergents with large headgroups, as evidenced by ATPase perturbation by these detergents, too. Charge also seems to be an important parameter in determining binding at the lipid/protein interface. Froud et al. (1986a) studied the ability of a variety of oleyl derivatives to displace phospholipids from the ATPase and found that oleylamine and oleic acid bound relatively strongly to the ATPase but that neutral molecules like oleyl alcohol and methyl oleate bound weakly, consistent with the weak binding observed here for neutral detergent molecules.

The role suggested here for the size of the detergent headgroup is worth a wider scan among surfactants. Indeed in a more systematic parallel study, all detergents with a large number (more than 16) of oxyethylene units tested proved inefficient solubilizers (Lund et al., 1989). It is remarkable that the four detergents considered in a recent report to be the best for maintenance of activity and for crystallization of the ATPase had a polar headgroup with only 8 or 10 oxyethylene groups (Pikula et al., 1988). On the other hand, a relatively small headgroup is probably not the unique requirement for efficient SR solubilization and ATPase delipidation; the acyl chain must also be taken into consideration, since a detergent like Brij 96, which also has a relatively small headgroup (Brij 96 is $C_{18:1}E_{\langle 10\rangle}$; i.e., it comprises an oleoyl chain and an average number of 10 oxyethylene groups), did not efficiently solubilize SR membranes in our hands (data not shown).

For DM, we found that solubilization of SR membranes was very fast compared to liposome solubilization (Figure 7). Possibly, the endogenous SR lipids might be solubilized by DM more efficiently than the egg PC and egg PA used for liposome preparation. Alternately, a tentative explanation would be that DM induces a preferential disruption of protein/protein or protein/lipid interactions versus lipid/lipid interaction. This would be consistent with the fact that fluorescence recovery in the presence of perturbing amounts of DM was slightly larger than in the presence of equivalent amounts of $C_{12}E_8$ (Figures 3 and 4). Concerning the slow solubilization of liposomes by DM, the rate at which one detergent solubilizes pure lipid liposomes might be related to the degree of membrane destabilization resulting, for instance, from changes in membrane fluidity or from geometrical constraints (the "wedge effect"). $C_{12}E_8$ has been shown to enhance the fluidity of liposomes below its cmc (Andersen et al., 1983), but the insertion of other detergents (e.g., DM) might not give rise to such a destabilization of the membrane.

Effect of Detergent on Ca^{2+}-ATPase Function. In terms of models for protein/lipid/detergent interaction, it is a puzzling observation that for both $C_{12}E_8$ and DM ATPase activity was initially reduced to a low level by addition of a perturbing, nonsolubilizing amount of detergent, then recovered a high value upon solubilization, and finally dropped to a low value again upon ATPase delipidation (Figure 8). A very similar activity profile has been observed on addition of oleyl alcohol to the ATPase reconstituted with a particular lipid, di-13-*cis*-docosenoylphosphatidylcholine, although the effects of oleyl alcohol on activity are very different for the ATPase reconstituted with other phospholipids (Froud et al., 1986b). The effect of detergents on overall activity is primarily the result of effects on rate-controlling (slow) steps of the reaction sequence. A previous analysis suggested that the inhibitory effect of perturbing concentrations of $C_{12}E_8$ on turnover at high ATP concentration is due to an inhibition of dephosphorylation, the intermediate step in the cycle which mainly contributes to rate limitation under these conditions (Champeil et al., 1986). Inhibition in this study was attributed to $C_{12}E_8$ molecules in close contact with the ATPase, rather than to a general modification by $C_{12}E_8$ of the "bulk" lipid phase (Andersen et al., 1983). The experiments reported here (Figure 3) further show that this inhibition occurs when only a few phospholipid molecules have been displaced from the ATPase [Figure 1c; see also le Maire et al. (1987)]. At this stage, the experiments do not say whether dephosphorylation is particularly sensitive to the displacement of a few phospholipids from particular regions of the ATPase–lipid interface or whether a few detergent molecules bound anywhere on this surface cause inhibition.

Since dephosphorylation is likely to contribute even more markedly to rate limitation in the presence of perturbing amounts of detergent than in the absence of detergent, one has to assume that the slowing down of this particular step is reversed upon membrane solubilization to account for the observed recovery of ATPase activity upon solubilization. Reversal of the inhibition of dephosphorylation has been unambiguously demonstrated through oxygen exchange experiments after solubilization with another nonionic detergent, Triton X-100 (McIntosh & Davidson, 1984). The molecular basis for this reversal has never been determined. We here tentatively suggest that it might be due to reorganization of phospholipid during the initial solubilization stage, in the following way: the tendency for lipids to be displaced from the protein surface could be reversed in the solubilized ternary complex, resulting in reoccupancy of critical regions. When a membrane already saturated with detergent faces additional detergent, ATPase polypeptides and associated lipids are solubilized, but at this point, the number of detergent molecules in the resulting ternary complex is just sufficient to keep the complex solubilized. One may then imagine that these detergent molecules now mainly concentrate at locations where they are most needed for covering the hydrophobic surface of the lipids, and that lipids cannot exchange with them because of curvature constraints (Figure 1d). In this situation there would be a "pull" on $C_{12}E_8$ away from the protein surface, and the vacant site might again become occupied by phospholipid. As a consequence of this reorganization, ATPase activity would revert to the high value characteristic of the membranous form. Reversal of ATPase delipidation on a small fraction of the protein surface is indirectly suggested by the lack of further recovery of average tryptophan fluorescence upon initial solubilization, since the increase in detergent/phospholipid ratio during the transition from saturated vesicles to solubilized complexes is expected to result at least in some dequenching effect. On these premises it is easy to explain the final decline of ATPase activity observed after addition of a large excess of $C_{12}E_8$ or DM, since such detergent concentrations induce

progressive delipidation of the ATPase (Figure 3): under these conditions, detergent molecules again come into close contact with the protein surface, resulting in a drop in activity to a level similar to that observed in the perturbed state (Figure 8; model in Figure 1e).

The above description of the binding of detergent to the ATPase hydrophobic surface is not intended to account for all aspects of detergent effects on ATPase function. There is ample evidence that detergent affects other steps in the catalytic cycle, and some of these effects are observed both in the perturbed membrane and in the solubilized ATPase [e.g., McIntosh and Ross (1985)]. Under conditions where isomerization between phosphorylated ATPase forms contributes to rate limitation of the catalytic cycle, e.g., for intermediate or low ATP concentrations, increases in ATPase activity to levels higher than those of native ATPase have been observed upon solubilization [data not shown, but see, for instance, Møller et al. (1980), Kosk-Kosicka et al. (1983) and Lund and Møller (1988)]. It has been suggested that solubilization increases the rate of phosphoenzyme isomerization and that ATP accelerates this step with a higher affinity in the solubilized ATPase than in the membranous ATPase (Gould et al., 1986). Previously, similar increases in ATPase activity observed in the presence of a variety of hydrophobic molecules (long-chain alkyl derivatives) have been attributed to modulation of the same step caused by binding of these molecules to nonannular sites on the ATPase, i.e., sites not available for phospholipid binding (Froud et al., 1986b). We therefore suggest that, in addition to the reversal of inhibition of the dephosphorylation rate, the increase in ATPase activity caused by intermediate concentrations of detergent ($C_{12}E_8$ or DM) could also be concomitant with binding of detergent at nonannular sites. This would also be consistent with the observed lack of dequenching of tryptophan fluorescence during solubilization in the experiments illustrated in Figure 3.

The location of these nonannular sites, occupied by detergent at solubilization, is then the next question. Protein/protein interfaces could provide nonannular binding sites for amphiphiles [Simmonds et al., 1982; Lee et al., 1982; see also Andersen et al. (1981)]. The functional significance of these protein/protein contacts in the membrane and the relative contribution of the hydrophobic portion of the ATPase to such interaction are not yet ascertained, but it is clear that such contacts do exist within the membrane in the absence of detergent. Alternately, as also proposed originally for other amphiphiles (Simmonds et al., 1982; Lee et al., 1982), detergent aliphatic chains might be able to become inserted between the protein transmembrane helices predicted by Brandl et al. (1986). Detergent molecules have indeed already been found trapped in the hydrophobic interior of proteins (Yonath et al., 1977). This would obviously result in alterations in the catalytic cycle. More specifically, insertion of detergent aliphatic chains could misalign the negative charges on the helices which form the binding sites for calcium in the ATPase postulated channel (Inesi, 1987; Khananshvili & Jencks, 1988). This would cause changes in both the affinity and the cooperativity of calcium binding to the ATPase, which have aroused much controversy [unpublished observations; see, for instance, Verjovski-Almeida and Silva (1981), Lüdi and Hasselbach (1982), Andersen et al. (1982), Kosk-Kosicka et al. (1983), McIntosh and Davidson (1984), and Lund and Møller (1988)]. In addition, the suggestion that detergent binds to the delipidated protein as a monolayer (le Maire et al., 1983) obviously offers the possibility of such interpenetration of the detergent chains and the protein moiety. This could be a basis for the instability observed in the soluble delipidated ATPase.

Concluding Remarks. In this paper, we attempted to clear up a few aspects of the interaction of SR membranes with detergents. The methodology described here, using brominated phospholipid for studies of detergent solubilization, might be easily extended to other membrane proteins. It might also be extended to a related question, the kinetics of brominated phospholipid exchange when mixed micelles (and not pure detergent micelles) are added to the reconstituted ATPase (Warren et al., 1984). Finally, using ATPase labeled with IAF or IAEDANS (Vanderkooi & Martonosi, 1977; Champeil et al., 1982; Watanabe & Inesi, 1982; Ross, 1987), it might also be possible from parallel measurements of fluorescence transfer to study the aggregation state of the solubilized ATPase and therefore to describe precisely how delipidation and oligomer formation are related to each other and how both events correlate with ATPase activity [see previous discussions in le Maire et al. (1978) and McIntosh and Ross (1985)].

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